Original Article

Titanium dioxide nanoparticles induce COX-2 expression through ROS generation in human periodontal ligament cells

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ABSTRACT — Titanium dioxide nanoparticles (TiO₂-NPs) are used to improve the aesthetic of toothpaste. While TiO₂-NPs have been used safely in toothpaste products for a long time, there haven't been studies to determine whether absorption of TiO₂-NPs by the mucous membranes in the mouth induces pathogenic conditions. Here, we assessed whether TiO₂-NPs induce cyclooxygenase-2 (COX-2) and investigated the molecular mechanisms underlying the pro-inflammatory effect of TiO₂-NPs on human periodontal ligament (PDL) cells. Treatment of PDL cells with TiO₂-NPs led to induction of both COX-2 mRNA and protein expression. TiO₂-NPs stimulated the nuclear translocation of nuclear factor-kappaB (NF-κB) as well as its DNA binding by inducing phosphorylation and subsequent degradation of the inhibitory protein IκBα in PDL cells. TiO₂-NPs treatment resulted in rapid activation of extracellular signal-regulated kinase (ERK)1/2 and Akt, which could be upstream of NF-κB. Treatment of PDL cells with both the MEK1/2 inhibitor U0126 and the PI3K inhibitor LY294002 strongly attenuated TiO₂-NPs-induced activation of NF-κB, and also the expression of COX-2. PDL cells treated with TiO₂-NPs exhibited increased accumulation of intracellular reactive oxygen species (ROS). Pretreatment of cells with ROS scavenger *N*-acetyl cysteine (NAC) abrogated the stimulatory effect of TiO₂-NPs on p65, p50, and COX-2 expression. In conclusion, ROS, concomitantly overproduced by TiO₂-NPs, induce COX-2 expression through activation of NF-κB signaling, which may contribute to the inflammatory effect of PDL cells.

Key words: Titanium dioxide nanoparticles, Cyclooxygenase-2, Reactive oxygen species, Periodontal ligament cells

INTRODUCTION

Titanium dioxide nanoparticles (TiO₂-NPs) exist in different crystal structures: anatase, rutile and brookite, or a mixture of these; they have unique physicochemical properties including a bright white color, ability to block UV light, and anti-microbial activity (Wiesenthal *et al.*, 2011). TiO₂-NPs are widely used as a pigment in personal care products such as toothpastes and sunscreen

(Rompelberg *et al.*, 2016). Generally, TiO₂-NPs at micro-scale dimensions have been considered biologically inactive and physiologically inert in both human and animals under non-overload conditions (Chen and Fayerweather, 1988). However, when its particle size is reduced to the nanoscale, the physical properties may change differently. TiO₂-NPs have a large surface-area-to-weight ratio and a high redox activity (Oberdörster *et al.*, 2005), which increases their potential to cause adverse

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effects or intrinsic toxicity to human health (Grande and Tucci, 2016). Actually, human exposure to TiO₂-NPs occurs through its use as a pharmaceutical additive, food intake, and toothpaste. In addition, studies dealing with oral exposure of TiO₂-NPs in mice have demonstrated the presence of particles in distant organs such as the liver, spleen, kidney and lung (Jia *et al.*, 2017). These reports suggest that TiO₂-NPs can travel to other tissues and organs following uptake by the gastrointestinal tract, with blood circulation primarily implicated in its bio-distribution. While most studies have concluded that TiO₂-NPs are safe for topical use on skin because these NPs are not absorbed, there haven't been studies to determine if TiO₂-NPs are absorbed by the mucous membranes in the mouth. Although toothpastes contain 1% to 10% TiO₂-NPs, toxicological study of TiO₂-NPs in dentistry is limited. Therefore, understanding the mechanisms of TiO₂-NPs-induced periodontal toxic effects will be helpful for risk assessment and ensuring human health.

The accumulation of reactive oxygen species (ROS) is an important cofactor in pathogenesis of oral and dental diseases including pulpal or mucosal inflammation, as well as inflammatory processes in periodontitis (Waddington *et al.*, 2000). In addition, there are many intervention studies carried out to investigate the effects of antioxidants in periodontal therapy (Chapple *et al.*, 2002).

A variety of transition metals are known to generate ROS through Haber-Weiss and Fenton reaction mechanisms (Knaapen *et al.*, 2004). Multiple lines of evidence suggest that the pro-inflammatory potential of TiO₂-NPs linked to the toxicity of this NP through the production of ROS in various cells (Hu *et al.*, 2016; Meena *et al.*, 2015).

One of the key enzymes in precipitating tissue inflammation is cyclooxygenase-2 (COX-2), which is induced by various endogenous or exogenous inflammatory stimuli (Ricciotti and FitzGerald, 2011). COX-2 acts as one of the bridging molecules in linking accumulation of ROS and chronic inflammation (Kundu and Surh, 2012). Oxidative stress-induced inappropriate activation of intracellular kinases, such as mitogen-activated protein (MAP) kinase and Akt kinase can increase DNA-binding activities of a variety of transcription factors, such as nuclear-factor kappa-B (NF-κB) and activator protein-1 (AP-1), which bind with the consensus sequences located in the *cox-2* gene promoter (Chun and Surh, 2004).

To determine whether TiO₂-NPs induce an inflammatory response in human periodontal ligament (PDL) cells, we investigated the expression and activation of molecules related to inflammatory signaling pathway in TiO₂-NPs-treated PDL cells. Here, we report that TiO₂-NPs induced

COX-2 expression through the activation of NF-κB signaling by two distinct mechanisms in PDL cells: (1) the activation of ERK1/2 kinase and (2) the amplification of the Akt pathway.

MATERIALS AND METHODS

Materials

We purchased powder-form TiO₂-NPs (99% anatase phase, particle size 15 nm, surface area 240 m²/g) from Nanoamor (Los Alamos, NM, USA). We checked the size and shape by a high resolution transmission electron microscopy (TEM) (Leo-912 AB OMEGA, Zeiss, Oberkochen, Germany). We also checked the dynamic light scattering based size and zeta potential by Zetasizer Nano ZS (Malvern Instruments Limited, Malvern, UK). The process was previously reported (Cho *et al.*, 2013; Lee *et al.*, 2017a). *N*-acetyl cysteine (NAC) and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against ERK1/2, p-ERK1/2, Akt, p-Akt and survivin were procured from Cell Signaling Technology Inc. (Beverly, MA, USA). Primary antibodies against p65, p50, p-IκBα, IκBα, Lamin A, and COX-2 as well as horse-radish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 2'-7' dichlorofluorescein diacetate (DCF-DA) was procured from Invitrogen (Carlsbad, CA, USA). LY294002 and U0126 were purchased from Cell Signaling Technology Inc. Hank's balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA, USA).

PDL Cell Culture and treatment

PDLs were obtained from extracted human molars donated by the Department of Oral and Maxillofacial Surgery, Kyung Hee University. All subjects involved in this study were informed about its purpose and procedures, and the study was approved by the Review Board of Kyung Hee University. PDLs were collected from the middle thirds of roots and cultured in minimal essential medium (α-MEM; Invitrogen) containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 g/mL) according to a previously described method (Kim *et al.*, 2013; Lee *et al.*, 2017b). All experiments were carried out with passage 4-7 cells.

Cytotoxicity assay

The cell growth effect was measured by the MTT assay. Cells (2 × 10³) were incubated in triplicate in a 96 well plate in the presence or absence of TiO₂-NPs in a final volume of 100 μL for different time intervals at 37°C. There-

after, 10 μ L of MTT solution (5 mg/mL) was added to each well and incubated for 4 hr. Medium was removed, the formation of formazan was dissolved in DMSO and absorbance at 550 nm was measured by using a microplate reader (Tecan Trading AG, Männedorf, Switzerland). Cell viability was described as the relative percentage of control.

Western blot analysis

Cells were harvested and lysed with radioimmunoprecipitation (RIPA) cell lysis buffer (Thermo Scientific, Waltham, MA, USA) and collected protein samples were quantified by using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis were done according to the protocol described earlier (Chae *et al.*, 2014). Immunoblot membranes were incubated with Super-signal pico-chemiluminescent substrate or dura-luminol substrate (Thermo Scientific) according to manufacturer's instruction and visualized with imagequant™ LAS 4000 (Fujifilm Life Science, Kanagawa, Japan).

Preparation of nuclear extracts

The nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific). Cells were washed with ice cold PBS, collected and centrifuged at 1,600 $\times$ g for 15 min at 4°C. Pellets were suspended in 50 μ L of Cytoplasmic Extraction Reagent (CER) I for 15 min, added CER II for additional 2 min. The mixture was centrifuged for 10 min at 16,000 $\times$ g. The pellets were washed with Nuclear Extraction Reagent and incubated on ice for 1 hr and centrifuged at 16,000 $\times$ g for 15 min. The supernatant containing nuclear proteins was collected and stored at -70°C after determination of protein concentration by using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoretic mobility gel shift assay (EMSA)

The EMSA for NF- κ B DNA binding was performed using a DNA-protein binding detection kit, according to the manufacturer's protocol (GIBCO BRL, Grand Island, NY, USA). The nuclear extract was prepared from cells incubated with or without TiO₂-NPs. The NF- κ B oligonucleotide probe 5'- AGT TGA GGG GAC TTT CCC AGG C-3' (Promega, Madison, WI, USA) was labeled with [γ -³²P] ATP and the EMSA was performed according to the protocol described earlier (Kim *et al.*, 2016).

Real-time Quantitative Polymerase Chain Reaction (Real-time qPCR)

Real-time qPCR was performed on the complementary DNAs (cDNAs) using the selective primers for COX-2 (sense; 5'-TGAGCATCTACGGTTTGCTG-3', antisense; 5'-AACTGCTCATCACCCCATTC-3'), and GAPDH (sense; 5'-TGCACCACCAACTGCTTAGC-3', antisense; 5'-GGCATGGACTGTGGTCATGA-3'). PCRs were performed in a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) using the Light Cycler DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's instruction. The PCR thermal profile was 95°C for 10 min, and 45 cycles of 95°C for 10 sec, 60°C for 40 sec followed by a cooling step at 40°C for 30 sec. For relative quantification, the cycle threshold (CT) value of COX-2 was subtracted from that of GAPDH and comparative method ($2^{-\Delta\Delta C_t}$) values were then normalized using results from untreated cell groups.

Measurement of ROS accumulation

Cells were treated with TiO₂-NPs in the presence or absence of NAC (5 mM) for 1 hr and then loaded with 25 μ M of DCF-DA. After incubation for 30 min at 37°C in a 5% CO₂ incubator, cells were washed twice with HBSS solution, suspended in the complete media and were examined under a fluorescence microscope to detect the intracellular accumulation of ROS. The fluorescence of oxidized DCF was also measured at an excitation wavelength of 480 nm and emission wave length of 525 nm using an Infinite® 200 PRO microplate reader (Tecan Trading AG).

Statistical analysis

When necessary, data were expressed as mean $\pm$ S.D. of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t*-test and a *p* value less than 0.05 was considered as statistically significant.

RESULTS

TiO₂-NPs induce COX-2 expression in PDL cells

We initially examined whether TiO₂-NPs induce any cytotoxicity in PDL cells. Treatment of these cells with TiO₂-NPs at relatively low concentration (10 μ g/mL) for 24 hr did not affect the cell viability. Moreover, incubation with same concentration of TiO₂-NPs for 48 hr maintained percentages of cell viability about 80%. However, incubation with high concentrations (20 ~ 50 μ g/mL) of TiO₂-NPs for 24 or 48 hr resulted in the cytotoxic effect in PDL cells. Incubation of PDL cells with TiO₂-NPs

Fig. 1. Effects of TiO₂-NPs on cell viability and COX-2 expression in PDL cells. (A) PDL cells were treated with the indicated concentrations of TiO₂-NPs for 24 or 48 hr. Cell viability was determined by the MTT assay. The results are presented as means $\pm$ S.D. (n = 3). Significantly different from control group (** $P < 0.005$). (B) PDL cells were treated with of TiO₂-NPs (2.5 and 10 µg/mL) for 8 hr. (C) PDL cells were treated with 10 µg/mL TiO₂-NPs for the indicated times. The protein level of COX-2 was investigated by immunoblot analysis. Actin was used as a loading control. Data shown are a representative of repeated experiment. (D) Expression of *cox-2* mRNA was detected by real-time PCR analysis. The results are presented as means $\pm$ S.D. (n = 3). Significantly different from control group (* $P < 0.01$).

(10 µg/mL) concentration for 24 or 48 hr did not show the cytotoxicity (Fig. 1A). When PDL cells were treated with TiO₂-NPs for various concentrations, COX-2 mRNA and protein expression was evident at 10 µg/mL (Fig. 1B and 1D). TiO₂-NPs (10 µg/mL) increased the COX-2 pro-

tein level in a time-related manner with maximal expression observed at 8 hr (Fig. 1C).

TiO₂-NPs induce NF- κ B activation in PDL cells

Because NF- κ B is known to play a critical role in reg-

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ulating the induction of COX-2, we determined whether TiO₂-NPs could induce activation of this transcription factor in PDL cells. Treatment of cells with TiO₂-NPs induced expression of p65 and p50, which are the functionally active subunits of NF- κ B, and then led to phosphorylation and degradation of I κ B α , an inhibitory subunit of NF- κ B in a time-dependent manner (Fig. 2A). TiO₂-NPs treatment also led to substantial accumulation of these subunits in the nucleus (Fig. 2B), followed by the up-regulation of its DNA binding activity (Fig. 2C). These results indicate that TiO₂-NPs activate NF- κ B signaling through induction of its subunit expression and I κ B α phosphorylation.

TiO₂-NPs induce ERK1/2 and Akt activation in PDL cells

Accumulating evidence indicates that NF- κ B activation is modulated by ERK1/2 as well as Akt (Pires *et*

al., 2018). As shown in Fig. 3A, ERK1/2 and Akt in PDL cells were phosphorylated in response to TiO₂-NPs treatment. Phosphorylation of ERK1/2 (Thr 202/Tyr 204) and Akt (Ser 473) was detectable in as early as 30 min after the TiO₂-NPs treatment with activation sustained up to 3 hr. To determine whether ERK1/2 or Akt is involved in activation of NF- κ B in TiO₂-NPs-stimulated PDL cells, we investigated the inhibitory effect of the pharmacological inhibitors of MEK1/2 and PI3K, upstream kinase of ERK1/2 and Akt, on NF- κ B activation by TiO₂-NPs treatment. We confirmed that both U0126 and LY294002 suppressed the phosphorylation of ERK1/2 and Akt, respectively (Fig. 3B and 3C). Treatment of both U0126 and LY294002 decreased expression of COX-2, p65 and p50 protein in TiO₂-NPs-incubated PDL cells (Fig. 3D and 3F). And then, nuclear translocation of p65 and p50 was also attenuated by incubation of U0126 and LY294002 in TiO₂-NPs-treated PDL cells (Fig. 3E and 3G). These

Fig. 2. TiO₂-NPs induce activation of NF- κ B signaling pathway. (A) PDL cells were treated with TiO₂-NPs of 10 μ g/mL for the indicated times and the expression of p65, p50 and I κ B α as well as its phosphorylation were detected by immunoblotting. (B) The nuclear expression of p65 and p50 were confirmed by Western blot analysis using nuclear fraction extracted from TiO₂-NPs-treated PDL cells for the indicated times. Lamin A was used as a loading control for nuclear extracts. (C) Nuclear extracts prepared from PDL cells treated with TiO₂-NPs were assessed for the DNA-binding activity of NF- κ B. Probe only; labelled probe with no sample, Cold probe; fifty-fold excess of unlabeled NF- κ B oligonucleotide as a competitor to the reaction mixture.

Fig. 3. Role of ERK and Akt in TiO₂-NPs-induced COX-2 expression and NF-κB activation in PDL cells. (A) PDL cells were treated with TiO₂-NPs (10 μg/mL) for the indicated time periods. Cell lysates were subjected to immunoblot analysis for detecting the phosphorylation as well as the total expression of ERK and Akt. (B, C) PDL cells were pretreated with U0126 (10 μM) and LY294002 (10 μM), pharmacological inhibitors of ERK and Akt, respectively, for 1 hr prior to incubation with TiO₂-NPs. And then, cells were treated with the TiO₂-NPs (10 μg/mL) for 0.5 or 1 hr and the activation/expression levels of ERK and Akt were checked by Western blot analysis. Data shown are representative of repeated experiment. (D and F) Under the same experimental condition, cells were treated with the TiO₂-NPs for 3 hr, and then levels of COX-2, p65 and p50 protein expression were determined by Western blot analysis. (E and G) Nuclear extracts prepared from PDL cells treated with TiO₂-NPs for 3 hr were subjected to immunoblot analysis against p65 and p50 antibody. Lamin A was used as a loading control for nuclear extracts.

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results suggest that the activation of NF- κ B occurs via the ERK1/2 and/or Akt-dependent pathway in TiO₂-NPs-stimulated PDL cells.

TiO₂-NPs induce generation of ROS in PDL cells

Since previous studies have reported that the treatment with various NPs, including titanium, resulted in the generation of ROS in multiple cell lines (Gali *et al.*, 2016; Minai *et al.*, 2013; Kundu *et al.*, 2018) and the accumulation of intracellular ROS can induce COX-2 expression (Kundu *et al.*, 2018; Nishanth *et al.*, 2011), we examined whether TiO₂-NPs accumulate intracellular ROS by

measuring the fluorescence intensity of DCF-DA in PDL cells. The accumulation of ROS is induced by treatment of TiO₂-NPs (2.5, 5 or 10 μ g/mL) for 1 hr (Fig. 4A and 4B), and pretreatment with ROS scavenger NAC abrogated the TiO₂-NPs (10 μ g/mL)-induced ROS generation (Fig. 4C and 4D).

The possible involvement of ROS in TiO₂-NPs-induced the expression of COX-2 and the activation of upstream kinases

To further determine direct involvement of ROS accumulation and COX-2 expression in TiO₂-NPs-treated PDL

Fig. 4. Effect of TiO₂-NPs on ROS generation in PDL cells. (A-B) PDL cells were treated with TiO₂-NPs (2.5, 5 or 10 μ g/mL) for 1 hr and then examined for the intracellular accumulation of ROS under the fluorescence microscope (left panel) and a microplate reader (right panel) using DCF-DA fluorescence staining method. Fluorescence data are representative of experiments performed in triplicate. Significantly different from control group (* $P < 0.01$, ** $P < 0.005$). RFU; Relative Fluorescence Unit. (C-D) PDL cells were incubated with NAC (5 mM) for 1 hr before treatment with TiO₂-NPs, and then intracellular ROS generation examined in the same way. ** $P < 0.005$, control versus TiO₂-NPs alone; NAC plus TiO₂-NPs versus TiO₂-NPs alone groups.

cells, we examined the inhibitory effect of NAC pretreatment on COX-2 expression and its regulatory signaling pathways. We observed that NAC pretreatment abolished phosphorylation of ERK1/2 and Akt (Fig. 5A and 5B) as well as expression of COX-2, p65 and p50 (Fig. 5C). Furthermore, NAC pretreatment suppressed the TiO₂-NPs-induced nuclear translocation of p65 and p50 (Fig. 5D). Taken together, above findings suggest that the TiO₂-NPs-induced ROS generation leads to the upregulation of COX-2 expression through the activation of NF- κ B via ERK1/2 and Akt phosphorylation in PDL cells.

DISCUSSION

NPs have attracted much attention due to their application in various fields, including electronics, cosmetics and biotechnology. NPs are beneficial for applications in wide areas due to unique physiochemical properties, such as small size and large specific surface area, and excellent optical properties, etc. Thus, humans are becoming increasingly exposed to NPs through diverse routes, such

as oral ingestion, inhalation, or dermal absorption. Despite the many benefits offered by NPs in advanced biotechnology, the potential of human health hazards induced by NPs remains largely underestimated.

During recent decades, TiO₂-NPs have been used in many applications due to their ability to confer a whiteness to various products. TiO₂-NPs have been classified in humans as biologically inert, which partially contributes to its relatively positive acceptance by the public (Skocaj *et al.*, 2011). TiO₂-NPs are being used in toothpaste, food colorants and nutritional supplements on a large scale. Therefore, dental exposure to TiO₂-NPs may happen through consumption of such products.

Because TiO₂-NPs are used in toothpaste and dental implants, we were interested to examine whether TiO₂-NPs application can incite inflammation when exposed to human PDL cells. We, therefore, attempted to determine the effect of TiO₂-NPs on the expression of COX-2, a marker of tissue inflammation, and investigate its underlying molecular mechanisms in PDL cells. Our findings indicate that TiO₂-NPs induce COX-2 expression at both

Fig. 5. Role of ROS in TiO₂-NPs-induced NF- κ B signaling activation in PDL cells. (A, B) PDL cells were pretreated with NAC for 1 hr before treatment with TiO₂-NPs (10 μ g/mL), and then total and phosphorylated ERK and Akt levels were determined by immunoblot analysis. (C) To confirm levels of COX-2 protein as well as total p65 and p50 protein expression, PDL cells were stimulated TiO₂-NPs (10 μ g/mL) for 3 hr in the presence or absence of NAC. (D) The nuclear levels of p65 and p50 subunit were measured using specific antibodies. Lamin A was used as a loading control for nuclear extracts.

protein and mRNA levels are in agreement with previous studies (Dinesh *et al.*, 2017; Kumar *et al.*, 2016).

NF- κ B is a major transcriptional factor of inflammation-related gene induction, which could be activated by TiO₂-NPs (Hong *et al.*, 2017; Ye *et al.*, 2017). Administration of TiO₂-NPs led to activation of NF- κ B in Wistar rats (Kumar *et al.*, 2016) and induction of NF- κ B subunits in hippocampus of CD-1 mouse (Ze *et al.*, 2014). Our study revealed that treatment of PDL cells with TiO₂-NPs induced phosphorylation and degradation of I κ B α , thus freeing NF- κ B complexes to translocate to the nucleus. They bound to NF- κ B DNA response elements, and induced the DNA binding activity. This finding suggests that TiO₂-NPs upregulate COX-2 expression, at least in part, through activation of NF- κ B in PDL cells.

The NF- κ B family is composed of either hetero- or homodimers of five subunit members. These include p65 (RelA), p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), c-Rel and RelB. NF- κ B presents predominantly as a p65/p50 heterodimer and transactivate a battery of pro-inflammatory genes (Barchowsky *et al.*, 2000). In most cell types, the p65/RelA-p50 heterodimer is sequestered in the cytoplasm by the inhibitor of κ B, because I κ B binding masks the nuclear localization sequence of p65. Generally, it is accepted that nuclear translocation and subsequent DNA binding of NF- κ B are critical events required for the activation of NF- κ B-dependent gene expression (Baldwin, 1996). The efficient transcriptional activation of NF- κ B depends on the phosphorylation of its active subunit p65/RelA, particularly at serine 536 residue. Phosphorylation on serine 335 of p50 increases the DNA binding capacity of NF- κ B subunit (Hou *et al.*, 2003). Several lines of evidence suggest that ERK and Akt kinase may regulate transcriptional activity of NF- κ B (Kim *et al.*, 2014; Madrid *et al.*, 2001). To investigate the underlying mechanisms of NF- κ B activation and COX-2 expression by TiO₂-NPs, we determined the effect of TiO₂-NPs on the activation of ERK1/2 and Akt, known to regulate NF- κ B, and the role of ROS in stimulating these signaling pathways. We found that inhibitors of MEK1/2 and PI3K attenuated COX-2 expression and nuclear translocation of p65 and p50 in TiO₂-NPs-treated PDL cells. We also revealed that TiO₂-NPs-induced ROS generation accounts for phosphorylation of ERK1/2 and Akt, and then subsequent activation of NF- κ B signaling. These findings were verified by pretreatment with NAC in TiO₂-NPs-treated PDL cells. Therefore, we suggest that TiO₂-NPs activate ERK1/2 and Akt phosphorylation, leading to activation of NF- κ B, via ROS accumulation. Our results are consistent with previous publications (Han *et al.*, 2013; Jin *et al.*, 2008; Murphy-Marion and Girard, 2018). Han *et al.* reported

that TiO₂-NPs treatment induced both phosphorylation of ERK1/2 and Akt as well as NF- κ B DNA binding activity in primary vascular endothelial cells (Han *et al.*, 2013). Recently, Murphy-Marion and Girard (Murphy-Marion and Girard, 2018) demonstrated that treatment of human eosinophil cells with TiO₂-NPs activated Akt, while did not affect ERK1/2 phosphorylation. These findings support our findings that phosphorylation of ERK1/2 and/or Akt can induce NF- κ B activation in TiO₂-NPs-treated PDL cells and imply that cells can differently respond to TiO₂-NPs. However, it remains largely unresolved how translocation of p65 and p50 is induced by TiO₂-NPs in PDL cells. We need to investigate further whether TiO₂-NPs stimulate the phosphorylation of p65 and p50.

TiO₂-NPs have been reported to mediate oxidative stress. Petković *et al.* (Petković *et al.*, 2011) demonstrated that anatase TiO₂-NPs induce significantly intracellular ROS, and then leading to DNA damage in human hepatoma HepG2 cells. Gurr *et al.* (Gurr *et al.*, 2005) also found that both anatase and rutile TiO₂-NPs induce oxidative damage in human bronchial epithelial cells. Multiple lines of evidence suggest that oxidative stress stimulates the

Fig. 6. Schematic representation of the proposed mechanism underlying the effects of TiO₂-NPs in inflammation signaling. TiO₂-NPs induces the activation of NF- κ B signaling through ROS-mediated phosphorylation of ERK and Akt, which in turn leads to the COX-2 expression. This may contribute to periodontal inflammation.

pro-inflammatory response in various cell lines (Ventura *et al.*, 2009). Accumulation of ROS triggers the release of pro-inflammatory cytokines and mediators through activation of transcription factors sensitive to oxidative stress (Wu and Tang, 2018). It has recently been reported in cell culture and animal model that TiO₂-NPs show the potential of the pro-inflammatory responses (Hu *et al.*, 2016; Liu *et al.*, 2013; Meena *et al.*, 2015).

The periodontium consists of the alveolar bone, the tooth, and the gingiva as well as the PDL which connects the tooth and the surrounding alveolar bone (Gölz *et al.*, 2014). The PDL cells play an important role in the homeostasis of periodontal tissue by mechanical stress derived from mastication, such as tension and compression (Narayanan and Page, 1983). Sometimes these excess forces may induce local inflammation and oxidative stress, which is associated with the pathogenesis of chronic periodontitis (Chapple *et al.*, 2000). Local ROS accumulation may promote the induction of pro-inflammatory cytokines or enzyme expression with consecutive activation of macrophages, leading to periodontal destruction (Rousset *et al.*, 2013).

In conclusion, as illustrated in Fig. 6, TiO₂-NPs-derived ROS accumulation induce phosphorylation of ERK1/2 and Akt, thereby activating NF-κB signaling and inducing COX-2 expression in PDL cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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