



Research article

Exposure to airborne particulate matter induces renal tubular cell injury *in vitro*: the role of vitamin D signaling and renin-angiotensin systemEungu Kang^a, Hyung Eun Yim^{a,*}, Yoon Jeong Nam^b, Sang Hoon Jeong^b, Joo-Ae Kim^c, Ju-Han Lee^d, Min Hwa Son^a, Kee Hwan Yoo^e^a Department of Pediatrics, Korea University Ansan Hospital, 123, Jeokgeum-ro, Danwon-gu, Ansan-si, Gyeonggi-do 15355, Republic of Korea^b Medical Science Research Center, Korea University Ansan Hospital, 123, Jeokgeum-ro, Danwon-gu, Ansan-si, Gyeonggi-do 15355, Republic of Korea^c Department of Earth and Environmental Sciences, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea^d Department of Pathology, Korea University Ansan Hospital, 123, Jeokgeum-ro, Danwon-gu, Ansan-si, Gyeonggi-do, Gyeonggi 15355, South Korea^e Department of Pediatrics, Korea University Guro Hospital, 148, Gurodong-ro, Guro-gu, Seoul 08308, Republic of Korea

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ABSTRACT

Background: Exposure to air pollution can interfere with the vitamin D endocrine system. This study investigated the effects of airborne particulate matter (PM) on renal tubular cell injury *in vitro* and explored the underlying mechanisms.**Methods:** HK-2 human renal proximal tubule cells were treated with PM with or without 1,25(OH)₂D₃ analog, 19-Nor-1,25(OH)₂D₂ (paricalcitol, 10 nM) for 48 h. The dose- and time-dependent cytotoxicity of PM with or without paricalcitol was determined via cell counting kit-8 assay. Cellular oxidative stress was assessed using commercially available enzyme-linked immunosorbent assay kits. The protein expression of vitamin D receptor (VDR), cytochrome P450(CYP)27B1, CYP24A1, renin, angiotensin converting enzyme (ACE), angiotensin II type 1 receptor (AT1), nuclear factor erythroid 2-related factor 2 (Nrf2), nuclear factor-kB (NF-kB), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 was determined.**Results:** PM exposure decreased HK-2 cell viability in a dose- and time-dependent manner. The activities of superoxide dismutase and malondialdehyde in HK-2 cells increased significantly in the group exposed to PM. PM exposure decreased VDR and Nrf2, while increasing CYP27B1, renin, ACE, AT1, NF-kB, TNF-α, and IL-6. The expression of VDR, CYP27B1, renin, ACE, AT1, and TNF-α was reversed by paricalcitol treatment. Paricalcitol also restored the cell viability of PM-exposed HK-2 cells.**Conclusion:** Our findings indicate that exposure to PM induces renal proximal tubular cell injury, concomitant with alteration of vitamin D endocrine system and renin angiotensin system. Vitamin D could attenuate renal tubular cell damage following PM exposure by suppressing the renin-angiotensin system and by partially inhibiting the inflammatory response.

1. Introduction

Exposure to particulate matter (PM) is an established risk factor for pulmonary and cardiovascular diseases (Brook et al., 2017). Recent evidence indicates that ambient PM contributes to the risk of developing incident chronic kidney disease (CKD), renal function decline and end-stage renal disease (Bowe et al., 2017; Mehta et al., 2016). The inflammatory mediators induced by PM particles translocate from the lung into the circulation, which leads to systemic inflammation, oxidative stress and injury to remote organs including kidneys (Brook et al., 2010).

Experimental studies suggest that exposure to PM causes renal hemodynamic disturbances, acute kidney damage with oxidative stress, inflammation, and DNA damage in renal tissue, and exacerbates chronic renal injury in murine models (Nemmar et al., 2016; Zhang et al., 2018).

PM is a complex mixture of particles consisting of both organic and inorganic compounds, such as carbon, nitrates, sulfates, ammonium, organic chemicals, and metals (Cho et al., 2018). Organic carbon and transition metals are increasingly important because of their ability to induce inflammatory and cytotoxic activities (Bates et al., 2019). Based on aerodynamic diameter, PM is classified into three categories: coarse

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particles or PM₁₀ (ranging from 2.5 to 10 µm), fine particles or PM_{2.5} (less than 2.5 µm), and ultrafine particles or PM_{0.1} (less than 0.1 µm). Fine and ultrafine particles are particularly harmful since they can penetrate distal airways and alveoli, leading to systemic inflammatory response (Chin, 2015). Recent findings suggest that exposure to urban fine particles may interfere with the vitamin D endocrine system, increasing vitamin D deficiency (BaSalamah et al., 2018; Mousavi et al., 2019). By inhibiting ultraviolet B photons or reducing outdoor activities, ambient air pollution contributes to decreased cutaneous synthesis of vitamin D. In addition, heavy metal components of PM may downregulate serum levels of vitamin D by increasing renal tubular dysfunction and decreasing the transcription of mitochondrial cytochrome P450 mixed-function oxidases (CYPs) (Mousavi et al., 2019). While the biologically active vitamin D [1,25(OH)₂D₃] is synthesized mainly in the mitochondria of renal proximal tubular cells by the enzyme 1 α -hydroxylase (CYP27B1), the hormone activities are mostly regulated by its catabolizing enzyme 1, 25(OH)₂D-24-hydroxylase (CYP24A1) (DeLuca, 2004). The coordinated actions of CYP27B1 and CYP24A1 maintain the circulating levels of the active form of vitamin D, and the regulation and maintenance of vitamin D by CYP27B1 and CYP24A1 is disrupted in various kidney diseases (Bland et al., 1999; Helvig et al., 2010). The transcription factor vitamin D receptor (VDR) is the nuclear target of active vitamin D, which mediates most of the known biological actions of vitamin D. VDR binding to 1, 25(OH)₂D₃ is followed by recruitment of a variety of coregulatory complexes and attachment to specific DNA-binding sites for transcriptional regulation (Valdivielso, 2009). VDR activity is impaired by vitamin D deficiency, which contributes to the progression of kidney diseases (Yang et al., 2018). The decrease in VDR is an early event in CKD based on findings from renal biopsies as well as mouse models of renal fibrosis (Xiong et al., 2012). Conversely, VDR activation has been shown to be involved in the protection against renal injury by various mechanisms, such as anti-inflammation, inhibition of renal fibrogenesis, restoration of mitochondrial function and suppression of renin angiotensin system (RAS) (Freundlich et al., 2014; Xiong et al., 2012; Xu et al., 2015). Disruption of vitamin D signaling is known to activate the RAS inappropriately, which is a crucial mechanism associated with the progression of CKD and increasing evidence reveals that VDR activation with vitamin D analogs leads to renoprotective effects by blocking the RAS (Chandel et al., 2017; Tiryaki et al., 2016).

In the present study, we hypothesized that PM particles induce renal tubular cell injury *in vitro* and aberrant RAS activation via impaired vitamin D signaling might be an underlying mechanism associated with the harmful effects of PM exposure. We investigated the cytotoxicity, oxidative stress and inflammatory responses of fine PM with or without D analog in human proximal renal tubular cells. Simultaneously, changes in vitamin D signaling pathway and RAS components after airborne PM exposure were analyzed in HK-2 cell lines. Furthermore, the effects of the vitamin D analog on RAS gene expression were assessed to explore the possible underlying mechanisms and to identify novel strategies for the management of renal injury induced by ambient PM exposure.

2. Materials and methods

2.1. PM collection, analysis and preparation

Airborne fine PM sample, which was collected with quartz fiber filters (QR-100, Sibata, Tokyo, Japan) using a high-volume air sampler (HV-1700RW, Sibata, Tokyo, Japan) operating at 1000 L/min flow on the rooftop of Korea University Ansan Hospital, in Ansan-si, Gyeonggi-province, South Korea, was used. As a single filter was used each day for air sampling, two filters above the WHO guidelines for PM_{2.5} (average of $\geq 25 \mu\text{g}/\text{m}^3$ per day) were selected for the experiment (December 9, 2019 and December 23, 2019). We referred to real-time air quality data (<https://www.airkorea.or.kr/eng>) to establish the concentration of fine PM. To obtain the PM suspension, the filters adhering to atmospheric particles were dried in an auto-desiccator (Sanpla Dry Keeper, Sanplatec

Corp, Osaka, Japan) before extracting PM. Subsequently, the filters were cut into small segment measuring about 2 cm² each, immersed in 100 mL of phosphate-buffered saline (PBS), and sonicated three times each for 30 min. After separating the PM suspension from the quartz fiber filters by shaking for 10 min, the PM suspension was filtered with a 0.2 µm syringe filter to remove larger interfering particles. For analysis of water-soluble organic carbon (WSOC) and ionic PM components, soluble ion components (F⁻, NO₃⁻, SO₄²⁻, PO₄³⁻, NH₄⁺, Ca²⁺, Mg²⁺) were analyzed by ion chromatography (Eco IC, Metrohm AG, Herisau, Switzerland) and WSOC was quantified with a total organic carbon analyzer (TOC-L, Simadzu Co., Kyoto, Japan) of Seoul center, Korea Basic Science Institute. The metal composition of PM was also analyzed by inductively coupled plasma mass spectrometer (ICP-MS, iCAP-RQ, Thermofisher Scientific, Bremen, Germany) of Seoul center, Korea Basic Science Institute. The PM extraction was always filtered with a 0.2 µm syringe filter before use and the particles were diluted with culture media into 75% of PM suspension (PM75%).

2.2. Cell culture

Human proximal renal tubular cells (HK-2) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco), 1% streptomycin (100 µg/mL) and penicillin (100 U/mL). The cells were maintained at 37 °C in a 5% CO₂ atmosphere and used for experiment in the second to fifth cell passages. When the cells were approximately 80% confluent, they were incubated with PM75% suspension for 2 days. To determine the effect of vitamin D against PM on the HK- cells, cells were stimulated with the PM75% suspension for 24 h (h) and then treated with 10 nM paricalcitol (19-Nor-1,25(OH)₂D₂, a selective VDR activator; Cayman Chemical, Ann Arbor, MI, USA) for another 24 h.

2.3. Groups and treatments

A day prior to treatment of PM extract, the HK-2 cells were incubated with serum-free medium overnight in order to synchronize cell growth. The cells were divided into the following four groups: normal culture media (NC) group, NC with vitamin D group, PM75% group, and PM75% with vitamin D group. The NC and PM75% groups were incubated with culture media or PM75% for 48 h, while the NC group treated with vitamin D and PM75% with vitamin D were initially exposed to culture media or PM75% for 24 h, followed by treatment with paricalcitol for 24 h. The morphology of live cells was observed using an inverted light microscope (ZEISS Axio Vert.A1; Carl Zeiss Microscopy GmbH, Jena, Germany) at x5 and x20 magnifications prior to cell lysis.

2.4. Cell counting kit-8 (CCK-8) assay for cell viability

Cell viability was determined using the CCK-8 cell assay kit (CCK-8; Dojindo, Kumamoto, Japan). HK-2 cells were seeded at a density of 1×10^4 cells/well in 96-well culture plates and treated with various concentrations of PM and incubated for 24 h. To determine the cytotoxic effect of PM samples in this study, we also tested the cytotoxicity of PBS. To establish the potential therapeutic effects of vitamin D, cells were incubated with different concentrations (10 nM and 100 nM) of paricalcitol for another 24h. After treatment with PM with or without paricalcitol, CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. Cell viability was assessed by measuring the optical density (OD) at 450 nm with a microplate reader (SpectraMax M2e; Bucher Biotec, Basel, Switzerland). The cell viability of the NC group was set to 100% and that of the treated group was expressed as a percentage relative to the NC group. Time-dependent cytotoxicity of PM with or without paricalcitol (10 nM) on HK-2 cells was determined at 6, 12, 24, and 48 h after exposure. All experiments were conducted in triplicate wells and repeated under each condition at least three times.

2.5. Western blot analysis

Cells were lysed with radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (all from ATTO, Tokyo, Japan). After collecting cell lysates, the total protein content was quantified using the BCA Protein Assay kit (Thermo Fisher Scientific). Proteins from each sample were separated via 4–20% polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (ATTO), and incubated overnight at 4 °C with primary antibodies against VDR (1:1000, ab109234; Abcam, Cambridge, MA, USA), CYP27B1 (1:2000, ab206655; Abcam), CYP24A1 (1:1000, PA5-9406; Thermo Fisher Scientific), renin (1:1000, sc-133145; Santa Cruz biotechnology, Santa Cruz, CA, USA), angiotensin-converting enzyme (ACE; 1:1000, PA5-78711; Thermo Fisher Scientific), angiotensin II type 1 receptor (AT1; 1:1000, PA5-20812; Thermo Fisher Scientific), nuclear factor erythroid 2-related factor 2 (Nrf2; 1:1000, ab89443; Abcam), nuclear factor-kappa B p65 (NF-κB p65; 1:1000, sc-8008; Santa Cruz), tumor necrosis factor (TNF)-α (1:1000, PA5-19810; Thermo Fisher Scientific), and interleukin (IL)-6 (1:1000, NB600-1131; Novus Biologicals, Centennial, CO, USA). After washing three times with tris-buffered saline containing 0.05% Tween-20 for 10 min each, membranes were incubated with the conjugated secondary antibody (anti-mouse IgG, HRP-linked Antibody or anti-rabbit IgG, HRP-linked Antibody 1:2000 dilution; Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 h. The protein bands were visualized using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by densitometry using Image Lab (Bio-Rad Laboratories). Anti-β-actin antibody (1:5000, sc-47778; Santa Cruz) was used to confirm equal protein loading.

2.6. Measurement of superoxide dismutase (SOD) and malondialdehyde (MDA)

To assess oxidative stress, the assays for SOD and MDA were carried out using Enzyme-Linked Immunosorbent Assay (ELISA) kits (DoGenBio, Seoul, South Korea) according to the manufacturer's instructions. The color changes were determined spectrophotometrically at a wavelength of 450 nm (SOD) and 540 nm (MDA), respectively. The activity levels of SOD and MDA in the samples were then calculated by comparing the sample ODs with each standard curve for the assays.

2.7. Statistical analysis

All of the experiments were conducted at least three times. The results were presented as means ± standard deviation. Statistical analysis was performed with GraphPad Prism v.7.0 (GraphPad Software, San Diego, CA, USA). All data were analyzed using one-way analysis of variance (ANOVA), two-way ANOVA, Turkey's multiple comparisons test or Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of PM

PM sample used in this study was collected on the day with high ambient concentrations of $PM_{2.5}$ (average $37 \mu\text{g}/\text{m}^3$). The composition of PM included WSOC, ions, and heavy metals. Nitrate (NO_3^-) were the most abundant ionic PM component, followed by WSOC. Sulfate (SO_4^{2-}), phosphate (PO_4^{3-}), and ammonium (NH_4^+) ions were also dominant in the PM suspension. Among the elemental components, the concentration of iron (Fe) was the highest. Increased levels of lead (Pb), copper (Cu), manganese (Mn), arsenic (As), chromium (Cr), nickel (Ni), and cadmium (Cd) in the PM extract were found in the order of quantity (Table 1).

3.2. Effects of PM on cell morphology

The effects of fine PM on the morphology of HK-2 cells were assessed using an inverted light microscope following incubation with PM75%

Table 1. The concentration of water soluble ions, WSOC and metal elements in PM samples ($\mu\text{g}/\text{L}$).

Ion	Concentration ($\mu\text{g}/\text{L}$)	Elements	Concentration ($\mu\text{g}/\text{L}$)
NO_3^-	114.1	Fe	2515
WSOC	90.1	Pb	1228
SO_4^{2-}	49.4	Cu	587
PO_4^{3-}	39.7	Mn	372
NH_4^+	23.6	As	183
F ⁻	2.28	Cr	162
Mg^{2+}	0.74	Ni	116
Ca^{2+}	0.46	Cd	39

The quantification of WSOC and heavy metal components was performed at the Seoul Center, Korea Basic Science Institute. WSOC, water-soluble organic carbon; PM, particulate matter.

with or without vitamin D for 48 h. Compared with the NC group, the PM group showed an apparent decrease in cell number, loss of physiological cell shape, and cell protrusions. Morphological changes and decreased cell number induced by PM exposure were attenuated by vitamin D treatment (Fig. 1a–h).

3.3. Cell viability

To establish the cytotoxic effect of PM sample in this study, the cell viability was determined in HK-2 cells after PBS or PM stimulation. PM exposure significantly decreased the viability of HK-2 cells in a dose- and time-dependent manner. As the concentration of PM increased, the cell viability significantly decreased. When exposed to the highest dose of PM (PM75%), the cell viability of HK-2 cells decreased to 54%. While viable cells were reduced with increasing concentrations of PBS, the cytotoxicity of PM50% and PM75% was greater than that of PBS50% and PBS75%, respectively (both $P < 0.05$) (Figure 2a). The HK-2 cell cytotoxicity also increased significantly with the increase in time of PM exposure. While the cell viability did not differ between 6 h and 12 h of exposure to PM75%, the cytotoxicity increased significantly at 24 h and 48 h after PM75% exposure (all $P < 0.05$) (Figure 2b). Vitamin D restored the cell viability in PM-treated HK-2 cells at different concentrations of fine particles and treatment time. The cell viability of PM50%, PM60% and PM75% was significantly improved after 24 h of treatment with 10 nM and 100 nM of vitamin D (all $P < 0.05$) (Figure 2c). The HK-2 cell cytotoxicity of PM75% was reduced significantly by treatment with 10 nM vitamin D for 6, 12, 24, and 48 h. The viable cells were mostly augmented after 24 h of treatment with vitamin D following 24 h of PM75% exposure (all $P < 0.05$) (Figure 2d).

3.4. Effects of PM on VDR, CYP27B1, and CYP24A1 in HK-2 cells

To investigate changes in vitamin D signaling during PM exposure, we measured the expression of VDR, CYP27B1, and CYP24A1 proteins in cultured human proximal tubular epithelial cells. Western blot showed that PM interfered with vitamin D signaling in HK-2 cells. Loss of VDR expression was clearly evident in HK-2 cells after PM exposure, while incubation of HK-2 cells with vitamin D, followed by PM stimulation, restored VDR expression dramatically (both $P < 0.05$) (Figure 3a, Fig. S1). In contrast, the expression of CYP27B1 was upregulated in proximal tubular epithelial cells after exposure to PM extract, compared with untreated control cells ($P < 0.05$). The increase in the expression of CYP27B1 gene induced by PM was reversed with subsequent vitamin D treatment ($P < 0.05$) (Fig. 3b, Fig. S2). The expression of CYP24A1 tended to increase with PM exposure, and to decrease with vitamin D treatment after PM stimulation; however, there were no statistically significant differences (Fig. 3c, Fig. S3).

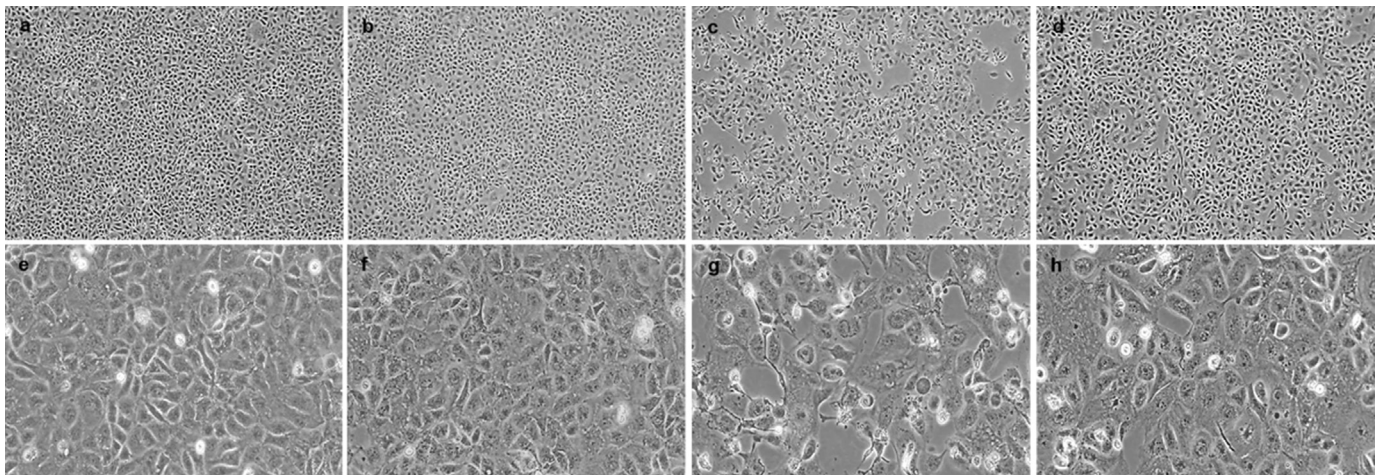


Figure 1. Effects of PM on the morphology of HK-2 cells. (a, e) NC group. (b, f) NC with vitamin D group. (c, g) PM group. (d, h) PM with vitamin D group. Representative pictures show the loss of physiological cell shape and decrease in cell number after PM exposure (c, g). Vitamin D treatment following PM stimulation restored the cellular morphology and reduced cell number (d, h) (a–d, x5 magnification; e–h, x20 magnification). PM, particulate matter; NC, normal culture media.

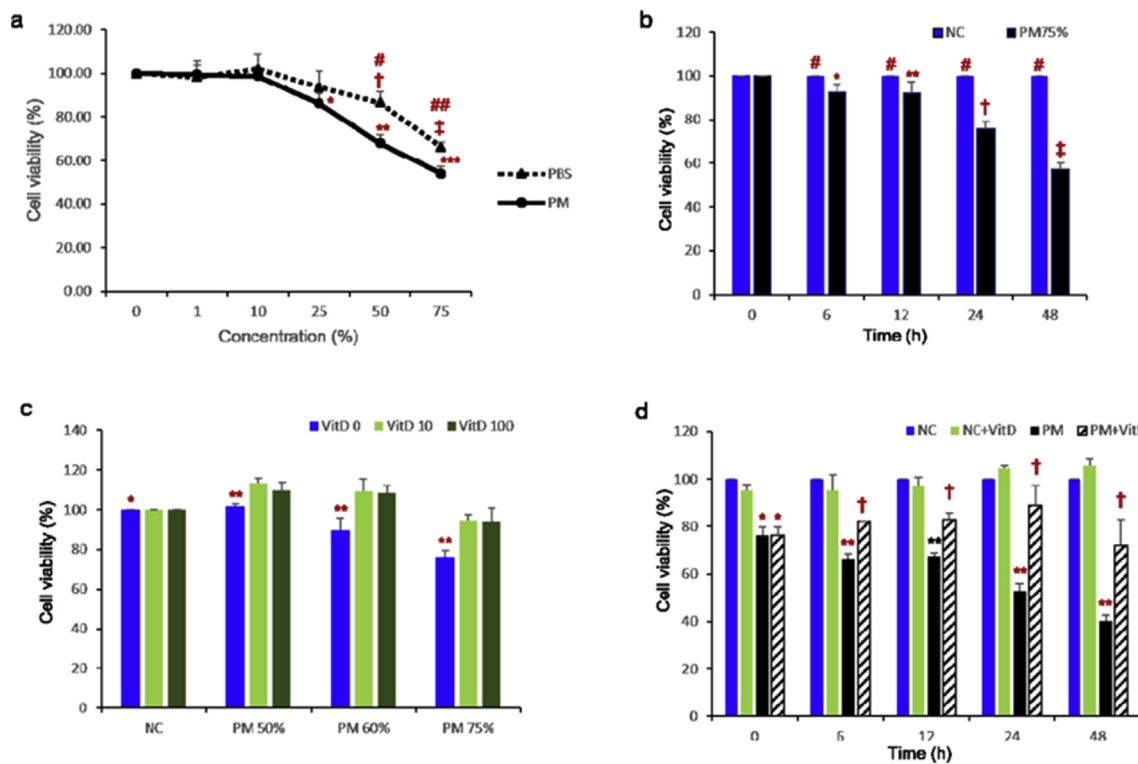


Figure 2. Effects of PM with or without vitamin D on the viability of HK-2 cells. (a) Dose-dependent cell viability after stimulation with different concentrations of PBS or PM (* $P < 0.05$, PM 25% vs. PM 0%, 1%, 10%, 50%, 75%; ** $P < 0.05$, PM 50% vs. PM 0%, 1%, 10%, 25%, 75%; *** $P < 0.05$, PM 75% vs. PM 0%, 1%, 10%, 25%, 50%; † $P < 0.05$, PBS 50% vs. PBS 0%, 10%, 75%; † $P < 0.05$, PBS 75% vs. PBS 0%, 1% 10%, 25%, 50%; # $P < 0.05$, PBS 50% vs. PM 50%; ## $P < 0.05$, PBS 75% vs. PM 75%). (b) Time-dependent cell viability after PM75% exposure (* $P < 0.05$, 6 h vs. 0, 24, 48 h; ** $P < 0.05$, 12 h vs. 0, 24, 48 h; † $P < 0.05$, 24 h vs. 0, 6, 12, 48 h; ‡ $P < 0.05$, 48 h vs. 0, 6, 12, 24 h; # $P < 0.05$, NC vs. PM75%). (c) Cell viability after treatment with different concentrations of vitamin D following PM stimulation (*with Vit D 0 nM, $P < 0.05$, NC vs. PM 60%, PM 75%; **with PM 50%, 60%, 75%, $P < 0.05$, Vit D, 0 nM vs. Vit D 10 nM, VitD 100 nM). (d) Cell viability after vitamin D (10 nM) treatment following PM75% exposure (* $P < 0.05$, PM, PM + Vit D vs. NC, NC + Vit D; ** $P < 0.05$, PM vs. NC, NC + Vit D, PM + Vit D; † $P < 0.05$, PM + Vit D vs. NC, NC + Vit D, PM). Values are expressed as the mean \pm standard deviation of three (or more) independent experiments. PM, particulate matter; PBS, phosphate-buffered saline; h, hour; NC, normal culture media; VitD, vitamin D.

3.5. Effects of PM on renin, ACE, and AT1 in HK-2 cells

Vitamin D is an important negative regulator of RAS (Chandel et al., 2017; Tiryaki et al., 2016). Therefore, we investigated whether PM stimulation increased the expression of RAS genes and determined if the addition of vitamin D following PM exposure reversed the activities of

the RAS components in proximal tubular epithelial cells. Compared with the control cells (NC group), PM exposure increased the expression of renin, ACE, and AT1 genes in HK-2 cells (all $P < 0.05$). Notably, the PM-mediated elevation of renin, ACE, and AT1 in proximal tubular epithelial cells was significantly attenuated by treatment with vitamin D (all $P < 0.05$). Paricalcitol treatment for 24 h after incubation with

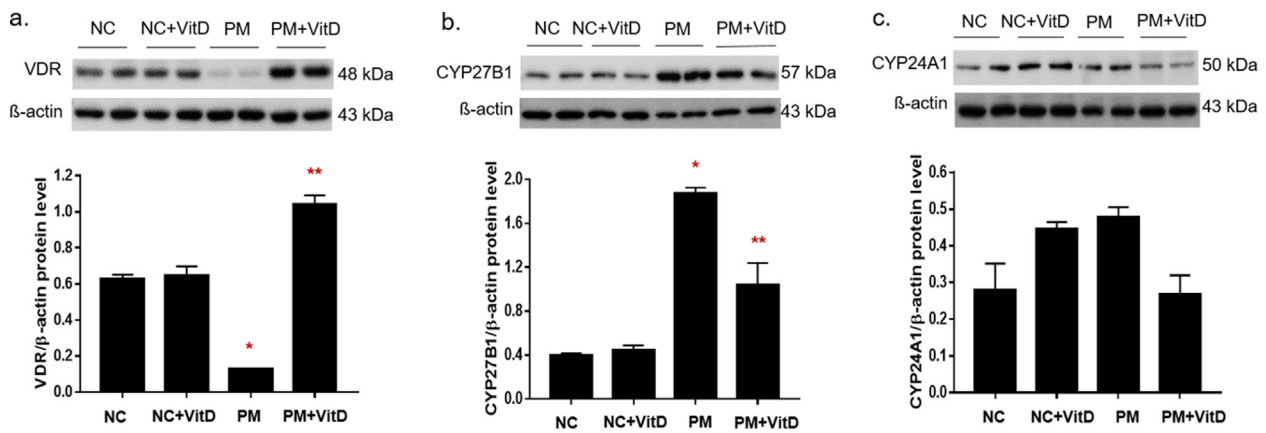


Figure 3. Effects of PM with or without vitamin D on the protein expression of VDR, CYP27B1, and CYP24A1 in HK-2 cells. (a) VDR (* $P < 0.05$, PM vs. NC, NC + Vit D, PM + Vit D; ** $P < 0.05$, PM + Vit D vs. NC, NC + Vit D, PM). (b) CYP27B1 (* $P < 0.05$, PM vs. NC, NC + Vit D, PM + Vit D; ** $P < 0.05$, PM + Vit D vs. NC, PM). (c) CYP24A1. Values are expressed as the mean \pm standard deviation of three (or more) independent experiments. PM, particulate matter; VDR, vitamin D receptor; CYP, cytochrome P450 mixed-function oxidase; NC, normal culture media; VitD, vitamin D.

culture media did not affect the expression of RAS proteins, compared with untreated control cells (NC group) (Fig. 4a–c, Figures S4, 5, and 6).

3.6. Effects of PM on oxidative stress and inflammatory response in HK-2 cells

We examined the PM-induced oxidative stress and inflammation on HK-2 cells by investigating protein levels, including Nrf2, NF- κ B p65, TNF- α , and IL-6. As shown in Figure 5, the protein expression of Nrf2 was reduced, while the activities of NF- κ B p65, TNF- α , and IL-6 were elevated after PM exposure in HK-2 cells (all $P < 0.05$). Vitamin D treatment after PM stimulation downregulated the level of TNF- α , compared with the PM exposure group ($P < 0.05$). However, the expression of Nrf2, NF- κ B p65, and IL-6 was not reversed by the addition of paricalcitol. The activity of Nrf2 was still lower in the PM treated with vitamin D and that of IL-6 was higher than in the NC group (Fig. 5a–d, Figures S7, 8, 9, and 10). Next, we evaluated the changes in oxidative stress markers along with the restoration of VDR expression. The levels of SOD and MDA in HK-2 cells after stimulation with fine particles were elevated, compared with untreated control cells (both $P < 0.05$). In contrast, vitamin D after PM stimulation did not reverse their activities. The concentration of SOD was persistently high in tubular cells treated with PM exposed to vitamin D, when compared with the NC group ($P < 0.05$). The activity of MDA

tended to decrease following vitamin D treatment after PM exposure; however, there was no statistically significant difference (Fig. 6a and b).

4. Discussion

This study investigated the effect of PM on renal proximal tubular cell injury and explored the potential underlying mechanisms *in vitro*. The main finding is that PM exposure significantly increased cytotoxicity, oxidative stress, and inflammatory response in tubular epithelial cells. Stimulation with PM also reduced the VDR expression, whereas it increased the activities of CYP27B1, renin, ACE, and AT1. Vitamin D analog reversed the RAS activation, while it restored VDR expression and reduced CYP27B1 activity. PM-induced cytotoxicity was attenuated by the addition of paricalcitol, a selective activator of VDR. Paricalcitol treatment following PM stimulation downregulated the expression of key inflammatory cytokine, TNF- α .

The chemical composition of PM varies geographically with time, and smaller particles have a more toxic effect on human health (Valavanidis et al., 2008). Organic particles and secondary inorganic aerosols of nitrate, sulfate, and ammonium are the major chemical components found in urban fine particles, and the acidic properties of secondary inorganic aerosol may contribute to oxidative stress and inflammation (Fang et al., 2017; Han et al., 2016). Fine PM also contains high levels of transition

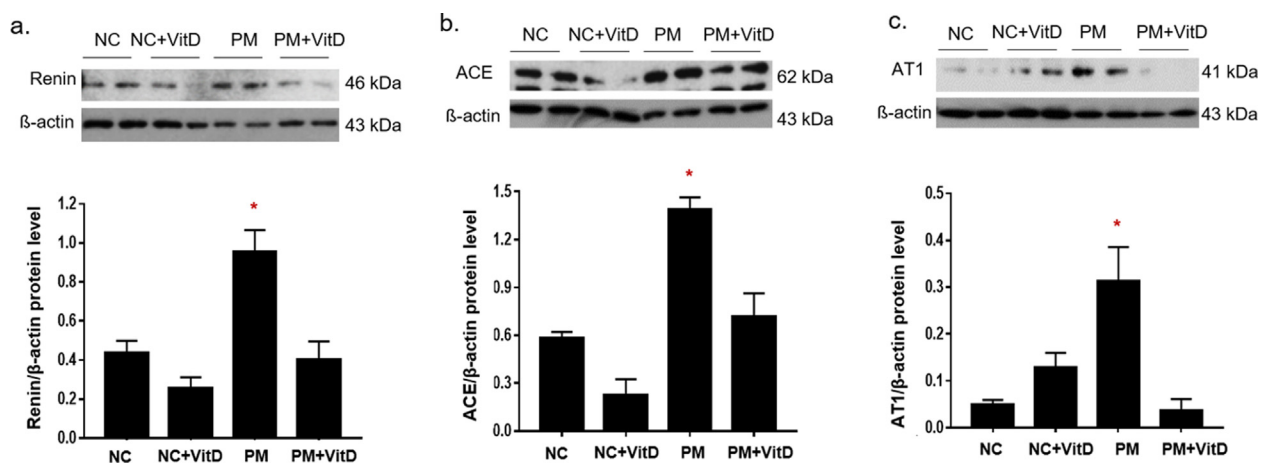


Figure 4. Effects of PM with or without vitamin D on the levels of renin, ACE, and AT1 in HK-2 cells. (a) renin (* $P < 0.05$, PM vs. NC, NC + Vit D, PM + Vit D). (b) ACE (* $P < 0.05$, PM vs. NC, NC + Vit D, PM + Vit D). (c) AT1 (* $P < 0.05$, PM vs. NC, PM + VitD). Values are expressed as the mean \pm standard deviation of three (or more) independent experiments. PM, particulate matter; ACE, angiotensin-converting enzyme; AT1, angiotensin II type 1 receptor; NC, normal culture media; VitD, vitamin D.

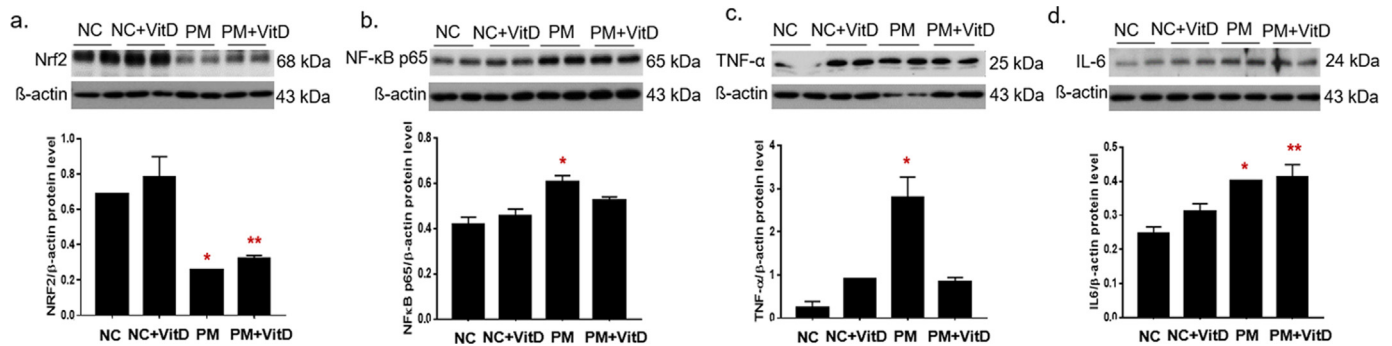


Figure 5. Effects of PM with or without vitamin D on the levels of Nrf2, NF-κB p65, TNF-α, and IL-6 in HK-2 cells. (a) Nrf2 (* $P < 0.05$, PM vs. NC, NC + Vit D; ** $P < 0.05$, PM + Vit D vs. NC, NC + Vit D). (b) NF-κB p65 (* $P < 0.05$, PM vs. NC). (c) TNF-α (* $P < 0.05$, PM vs. NC, NC + Vit D, PM + Vit D). (d) IL-6 (* $P < 0.05$, PM vs. NC; ** $P < 0.05$, PM + Vit D vs. NC). Values are expressed as the mean \pm standard deviation of three (or more) independent experiments. PM, particulate matter; Nrf2, nuclear factor erythroid 2-related factor 2; NF-κB p65, nuclear factor kappa B p65; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; NC, normal culture media; VitD, vitamin D.

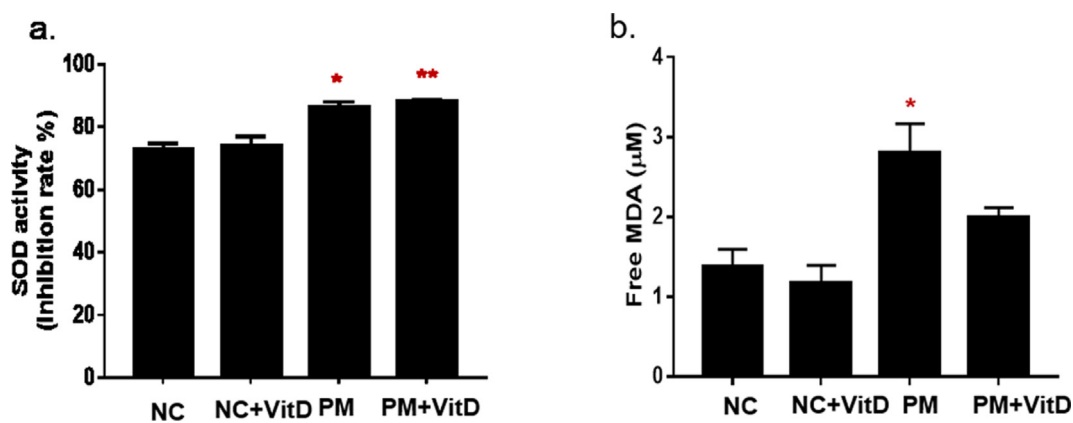


Figure 6. Effects of PM with or without vitamin D on the activities of SOD and MDA in HK-2 cells. (a) SOD (* $P < 0.05$, PM vs. NC, NC + Vit D; ** $P < 0.05$, PM + Vit D vs. NC, NC + Vit D). (b) MDA (* $P < 0.05$, PM vs. NC, NC + Vit D). Values are expressed as the mean \pm standard deviation of three (or more) independent experiments. PM, particulate matter; SOD, superoxide dismutase; MDA, malondialdehyde; NC, normal culture media; VitD, vitamin D.

metals with a great potential for oxidative stress in tissue and ultrafine PM can even cause direct damage to cellular macromolecules (Oberdörster et al., 2005). In the monocytic-macrophagic cell line, the elemental and organic carbons of fine and ultrafine particles were associated with the highest pro-inflammatory activity, and the transition metals of those extracts induced the largest decrease in cell viability (Steenhof et al., 2011). In human umbilical vein endothelial cells, urban fine PM enriched in Fe, Zn, and Pb contributed to an increase in reactive oxygen species (ROS) generation, disruption of mitochondrial transmembrane potential, NF-κB activation, and cell death (Han et al., 2011). Shafer et al. (2010) reported that transition metals, particularly iron, play a crucial role in ROS activity of the PM. Iron-rich nanoparticles in polluted atmosphere were also involved in myocardial mitochondrial dysfunction and cardiac oxidative stress (Maher et al., 2020). In the present study, we used fine particles smaller than 0.2 μm in diameter collected from an urban area of South Korea. The PM samples contained the highest Fe concentration, followed by Pb, Cu, Mn, and other elements. Organic carbon and inorganic ions such as NO³⁻, SO₄²⁻, PO₄³⁻, and NH₄⁺ were also abundant in our PM samples. We found that in HK-2 human renal proximal tubular cells, fine PM reduced the cell viability in a dose- and time-dependent manner. HK-2 cells stimulated with ambient PM showed damaged cell shape and size as well as reduced cell number. Exposure to fine PM resulted in Nrf2 depletion and activation of NF-κB p65, TNF-α, and IL-6. Significant increases in SOD and MDA were also observed in HK-2 cells after exposure to PM. The addition of VDR activator restored cell viability as well as changes in cell morphology. It reduced TNF-α expression in human renal proximal tubular cells.

Vitamin D exerts its anti-inflammatory and immunomodulatory effects by binding to VDR (Jang et al., 2014). The VDR in the proximal tubule cells of the kidney binds to filtered 1,25(OH)₂D₃ and acts as an inhibitor of CYP27B1 (Wang et al., 2015). Renal tubular cells express a high level of VDR under normal physiological conditions and inflammation per se hinders VDR expression (Yang et al., 2018). In cultured proximal tubular cells, proinflammatory TNF-α inhibited tubular VDR expression in a dose- and time-dependent manner (Xiong et al., 2012). VDR loss and TNF-α upregulation were early events preceding renal fibrogenesis in a mouse model of obstructive nephropathy (Xiong et al., 2012). In biopsy samples obtained from patients with different renal diseases, loss of VDR was only observed in the focal area characterized by significant inflammatory infiltration (Xiong et al., 2012). In the present study, PM exposure reduced the expression of VDR and increased the activity of the CYP27B1. Inflammatory cytokines were upregulated by PM stimulation. The addition of paricalcitol restored VDR activity and reduced the expression of CYP27B1 and TNF-α. The expression of catabolizing enzyme CYP24A1 tended to be higher in the PM group than in the control group and to be lower in the PM with vitamin D group than in the PM group. These findings indicate that fine PM exposure may be implicated in renal tubular injury partly via inhibition of VDR and imbalance in vitamin D metabolism by CYP27B1 and CYP24A1 in the proximal tubule cells. Similarly, rats chronically exposed to Pb showed substantial renal injuries mediated via downregulation of VDR and upregulation of CYP24A1 and vitamin D-binding protein (BaSalamah et al., 2018). The co-administration of vitamin D and Pb markedly mitigated renal injuries with restoration of the expression of vitamin D

related molecules and promotion of anti-inflammatory markers (BaSa-lamah et al., 2018).

In addition, we found that the expressions of renin, ACE, and AT1 increased under fine PM exposure, and these changes were all attenuated by paricalcitol treatment. These findings further demonstrate that vitamin D serves as a negative regulator of RAS. In rats subjected to 5/6 nephrectomy, paricalcitol reduces the renal expression of renin, angiotensinogen and AT1 and improves glomerular and tubulointerstitial damage (Freundlich et al., 2008). VDR activation directly inhibits renin gene transcription by interfering with the activity of cyclic AMP responsive element in the renin gene promoter (Yuan et al., 2007). Since RAS activation is an important determinant of renal disease progression, the fact that vitamin D suppresses the RAS has generated enormous excitement in the field (Yang et al., 2018). Our results indicate that exposure to PM affects endocrine responses via alteration of vitamin D metabolism and RAS elements. The addition of vitamin D alleviated PM-induced tubular cell injuries in parallel with the restoration of impaired vitamin D signaling and aberrant RAS activation. Consistent with our findings, Aztatzi-Aguilar et al. (2015, 2016) found that PM exposure induced expression of angiotensin/bradykinin endocrine system in the lungs, heart and kidneys. Zhang et al. (2018) also reported that the overexpression of ACE and AT1 after PM_{2.5} installation in mouse kidneys was accompanied by acute kidney damage. They speculated that the RAS activation might occur upstream of oxidative stress and inflammation induced by PM_{2.5} exposure. Given that RAS has multiple well-established pathophysiologic effects on the development and progression of various kidney diseases (Crowley and Rudemiller, 2017; Lee et al., 2021; Maneesai et al., 2017), it is plausible that RAS activation plays a crucial role in renal tubular cell injury after exposure to fine particles in the present study. Impaired vitamin D signaling may contribute to RAS activation, which mediates cellular toxicity by increasing oxidative damage and inflammatory response.

Emerging evidence revealed oxidative stress and inflammation as the potential mechanisms underlying the harmful effects of PM exposure. In mice, intratracheal instillation of PM_{2.5} suspension increased the levels of MDA and H₂O₂ as well as the mRNA expression of NF- κ B p65 and TNF- α in the kidney [6]. Radan et al. (2019) found that PM₁₀ exposure down-regulated the expression of Nrf2 and its upstream regulator genes in the lung, implying that Nrf2 plays a major role in PM₁₀-induced oxidative damage [39]. Notably, Nrf2 and NF- κ B are important transcription factors, which coordinate the cellular response to oxidative stress and inflammation (Wardyn et al., 2015). Disruption of Nrf2 leads to the activation of NF- κ B and its target genes, whereas NF- κ B also modulates Nrf2 activity (Ahmed et al., 2017; Lingappan, 2018). Various pro-inflammatory cytokines are overproduced following NF- κ B activation by oxidative stress (Lingappan, 2018). Several studies have reported that vitamin D regulates the activity of Nrf2 and NF- κ B (Chen et al., 2015; Nakai et al., 2014; Xu et al., 2015). Nakai et al. (2014) reported that the vitamin D analog attenuated oxidative stress together with the upregulation of Nrf2 and the inhibition of NF- κ B and NAD(P)H oxidase in diabetic rats with kidney damage. Vitamin D pretreatment reduced lipopolysaccharide-activated renal NF- κ B signaling as well as several inflammatory cytokines in mice with sepsis-induced acute kidney injury (Xu et al., 2015). In this study, PM exposure reduced the expression of Nrf2 and enhanced the expression of NF- κ B, TNF- α and IL-6. The addition of vitamin D following PM exposure downregulated the activity of TNF- α but did not reverse the activities of Nrf2, NF- κ B, and IL-6. These results indicate that fine PM exposure leads to oxidative stress and inflammation in human proximal renal tubular cells and vitamin D may attenuate PM-induced renal tubular cell inflammation by regulating TNF- α expression. The regulation of the activities of Nrf2, NF- κ B and IL-6 by vitamin D may be not obvious in this context. Likewise, PM stimulation also increased the levels of SOD and MDA, while vitamin D after PM exposure did not reduce their activities. SOD scavenges the highly toxic superoxide anion by converting it to hydrogen peroxide and SOD activity represents the severity of the stress (Patlolla et al., 2009). Since SOD acts against free radical-induced oxidative damage, its induction can

be considered as an antioxidant defense mechanism (Patlolla et al., 2009). MDA is the end-product of lipid peroxidation, which is one of the primary events in free radical-mediated cell and tissue injury; MDA level has long been used as a marker of oxidative stress and redox signaling (Morales and Munné-Bosch, 2019). Therefore, the upregulation of SOD and MDA levels by PM stimulation may not only reflect the intensity of oxidative damage but also the adaptive response against oxidative stress. The persistently high level of SOD in the presence of vitamin D following PM exposure suggests a protective role of SOD rather than an indicator of injury. Alternatively, the concentration of vitamin D in this study may be insufficient to restore the pro-oxidant/antioxidant balance efficiently in the absence of further studies investigating the anti-oxidant and anti-inflammatory effects of different concentrations of vitamin D. The detailed molecular mechanism of vitamin D in kidney protection following PM exposure has yet to be elucidated.

Studies suggest that PM has an impact on inflammation, oxidative stress, cell cycle regulation, endothelial dysfunction, and signaling pathways in different types of cells and tissues (Chin 2015; Valavanidis et al., 2008). In this study, we showed that exposure to airborne fine PM in an urban area of South Korea led to activation of cytotoxic responses in renal proximal tubular cells. Fine PM induced a decrease in cell viability together with an increase in oxidative stress and inflammatory response in HK-2 cells *in vitro*. These changes were closely associated with disrupted vitamin D signaling and excessive RAS activation. Particularly, VDR activation alleviated PM-induced tubular cell injury; paricalcitol inhibited the expression of renin, ACE, and AT1, reduced the levels of TNF- α , and improved the cytotoxicity of HK-2 cells. Paricalcitol may exert protective effects on PM-induced tubular cell injury, at least partially, by regulating the RAS elements and TNF- α activity. However, our findings should be interpreted with caution since the variation in chemical composition of PM contributes to the activation of different biological pathways and synergistic effects associated with specific renal outcomes. Since we treated high concentrations of fine particles in HK-2 cells directly *in vitro*, it is also difficult to determine whether our findings are representative of PM in human renal pathology. However, it is well known that acute and chronic exposure of heavy metals, major components of PM, leads to a variety of kidney injury including renal cell damage, tubular dysfunction, interstitial necrosis, and fibrosis of renal tissue (BaSalamah et al., 2018; Lentini et al., 2017). In addition, accumulating evidence suggests that kidney could be a toxicological target for exposure to PM (Bowe et al., 2017; Xu et al., 2022). More experimental and clinical studies on the possible impact of PM exposure on kidney disease are needed.

5. Conclusions

The present study investigated the cytotoxic response to airborne fine PM exposure in renal proximal tubular cells and the cytoprotective effects of vitamin D treatment during PM-induced renal cell injury *in vitro*. The increased levels of NF- κ B, TNF- α , IL-6, SOD, and MDA and the decrease in Nrf2 levels in PM-exposed renal cells suggest that fine particles are associated with oxidative stress and inflammation in renal tubular cells. Besides, VDR depletion and CYP27B1 elevation occurred concomitant with the activation of renin, ACE, and AT1. We found that treatment with active vitamin D analog ameliorated PM-induced tubular cell injury in addition to RAS blockade and TNF- α inhibition *in vitro*. Overall, this study may provide evidence that renal VDR is an important regulator of renal cytotoxic response in PM-induced renal tubular injury. Targeting aberrant RAS signaling and VDR loss in PM exposure is a potential therapeutic approach to combat PM-induced kidney injury.

Declarations

Author contribution statement

Eungu Kang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hyung Eun Yim: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sang Hoon Jeong: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ju Han Lee: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Kee Hwan Yoo: Conceived and designed the experiments; Wrote the paper.

Yoon Jeong Nam: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Min Hwa Son: Performed the experiments; Analyzed and interpreted the data.

Joo-Ae Kim: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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