Journal of Dental Sciences 21 (2026) 457–467

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Nuclear factor erythroid 2-related factor 2/heme oxygenase-1 activation by nattokinase reduces pro-inflammatory and matrix-degrading mediators in human gingival fibroblasts

Ya-Lun Li [a,b], Yang-Che Wu [c,d], Chien-Fu Tseng [e,f], Yung-Li Wang [c], Sin-Yu Li [c], Thi Thuy Tien Vo [g], Yuh-Lien Chen [h], Yin-Tzer Shih [a*], I-Ta Lee [c**]

[a] *Department of Applied Mathematics, National Chung Hsing University, Taichung, Taiwan*
[b] *Department of Chinese Medicine, Changhua Christian Hospital, Changhua, Taiwan*
[c] *School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan*
[d] *Department of Dentistry, Taipei Medical University-Shuang Ho Hospital, Ministry of Health and Welfare, New Taipei City, Taiwan*
[e] *Graduate Institute of Clinical Dentistry, School of Dentistry, College of Medicine, National Taiwan University, Taipei, Taiwan*
[f] *Department of Dentistry, Taoyunan General Hospital, Ministry of Health and Welfare, Taoyuan, Taiwan*
[g] *Faculty of Dentistry, Nguyen Tat Thanh University, Ho Chi Minh City, Viet Nam*
[h] *Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan*

Received 3 October 2025; Final revision received 16 October 2025
Available online 1 November 2025

KEYWORDS
Particulate matter;
Nattokinase;
Nrf2/HO-1;

Abstract *Background/purpose:* Particulate matter (PM) exposure is associated with inflammation and extracellular matrix degradation in periodontal tissues. Cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), and matrix metalloproteinase-1 (MMP-1) are key mediators in these processes. Nattokinase, a fibrinolytic enzyme derived from *Bacillus subtilis* fermentation, has recently gained attention for its potent anti-inflammatory and antioxidant effects.

* Corresponding author. Department of Applied Mathematics, National Chung Hsing University, No. 145, Xingda Road, Taichung, 402, Taiwan.
** Corresponding author. School of Dentistry, College of Oral Medicine, Taipei Medical University, No. 250, Wu-Xing Street, Taipei, 110, Taiwan.
E-mail addresses: yintzer_shih@nchu.edu.tw (Y.-T. Shih), itlee0128@tmu.edu.tw (I.-T. Lee).

https://doi.org/10.1016/j.jds.2025.10.022

COX-2;
Gingival fibroblasts

Materials and methods: Human gingival fibroblasts (HGF-1 cells) were exposed to PM, and the protective effects of nattokinase pretreatment were systematically evaluated. COX-2, PGE_2, and MMP-1 expression and release were analyzed using immunoblotting and enzyme-linked immunosorbent assay. The roles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived reactive oxygen species (ROS), phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), and mitogen-activated protein kinase (MAPK) pathways were examined using pharmacological inhibitors. The nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) axis was validated by inhibitors and antioxidant response element (ARE)-luciferase assays.

Results: PM stimulation induced COX-2 expression, PGE_2 release, and MMP-1 upregulation in HGF-1 cells through NADPH oxidase-mediated ROS generation, PI3K/Akt activation, and phosphorylation of p42/p44 MAPK and p38 MAPK. ROS and PI3K/Akt exhibited bidirectional regulation reinforcing COX-2 and MMP-1 induction. Nattokinase pretreatment markedly suppressed these pro-inflammatory and matrix-degrading responses. Mechanistically, nattokinase enhanced Nrf2 activation and HO-1 expression, thereby attenuating PM-induced signaling cascades and mediator release. Inhibition of Nrf2 or HO-1 abolished nattokinase's protective effects.

Conclusion: Nattokinase protects HGF-1 cells from PM-induced inflammation and matrix degradation by activating the Nrf2/HO-1 axis and suppressing NADPH oxidase-derived ROS, effectively interrupting the reciprocal regulation between ROS, PI3K/Akt, and MAPK pathways.

Introduction

Airborne particulate matter (PM) is a major environmental pollutant and has been strongly associated with cardiovascular and respiratory diseases through oxidative stress and inflammation.[1] Beyond systemic effects, epidemiological studies indicate that long-term exposure to fine PM increases the risk of periodontal disease.[2,3] This suggests that PM may act as an overlooked environmental risk factor for periodontitis, a chronic inflammatory condition characterized by gingival inflammation, connective tissue breakdown, and alveolar bone loss.

Gingival fibroblasts (GFs) are key stromal cells responsible for extracellular matrix synthesis and remodeling, and they act as sentinel cells that respond to environmental insults by producing cytokines and matrix metalloproteinases (MMPs).[4] Dysregulated activation of GFs amplifies inflammatory cascades, with mediators such as cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), and MMP-1 playing central roles in periodontal tissue destruction.[5] Oxidative stress has been recognized as a major mechanism of PM-induced cytotoxicity. Reactive oxygen species (ROS), generated mainly via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, activate phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) pathways and further drive pro-inflammatory and degradative responses.[6–8] While these signaling mechanisms have been well described in pulmonary and vascular systems, their contribution to gingival fibroblast-mediated periodontal pathology remains insufficiently studied.

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a central regulator of antioxidant defense, inducing cytoprotective genes such as heme oxygenase-1 (HO-1).[9,10] Recent studies show that activation of the Nrf2/HO-1 axis can alleviate oxidative stress and suppress inflammatory responses in periodontal disease models.[11,12] Natural compounds capable of modulating this pathway have attracted attention as adjunctive approaches in managing oral inflammation.[13,14] Nattokinase, a serine protease from *Bacillus subtilis* fermentation in "natto," is well known for its cardiovascular, antioxidative, and anti-inflammatory properties.[15,16] Experimental studies further demonstrated that nattokinase protects against oxidative and inflammatory damage by activating the Nrf2/HO-1 pathway in extraoral disease models.[17,18] However, whether nattokinase exerts similar protective effects in the oral cavity, particularly in PM-induced periodontal injury, remains unclear.

Therefore, this study investigated the mechanisms by which PM induces inflammatory and matrix-degrading responses in gingival fibroblasts, and whether nattokinase attenuates these effects via NADPH oxidase/ROS, PI3K/Akt, MAPK, and Nrf2/HO-1 signaling.

Materials and methods

Urban particulate matter water-soluble extracts

Fifty milligrams of urban PM (NIST 1648a, Sigma, St. Louis, MO, USA) was suspended in 5 ml of phosphate-buffered saline (PBS, pH 7.4) and sonicated for 20 min in an ice bath to ensure complete dispersion. The suspension was then centrifuged at 20,000 rpm for 10 min at 4 °C, and the supernatant was filtered through a 0.22-μm syringe filter to

obtain the water-soluble fraction. PBS was used instead of culture medium to prevent interference from serum components and to ensure reproducibility when preparing the aqueous extract of this certified reference material (NIST SRM 1648a).

Reagents

Diphenyleneiodonium chloride (DPI, Cat. #D2926), N-acetyl-L-cysteine (NAC, Cat. #A9165), apocynin (APO, Cat. #178385), and ML385 (Cat. #SML1833) were purchased from Sigma—Aldrich (St. Louis, MO, USA). Heme Oxygenase-1-IN-1 hydrochloride (Cat. #HY-111798A), nattokinase (Cat. #HY-P2373), PD98059 (Cat. #HY-12028), NS-398 (Cat. #HY-13913), LY294002 (Cat. #HY-10108), SH-5 (Cat. #HY-14766), and SB-242235 (Cat. #HY-16518) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Primary antibodies against HO-1 (Cat. #ab13243), GAPDH (Cat. #ab9485), and COX-2 (Cat. #ab15191) were purchased from Abcam (Cambridge, UK). Phospho-p38 MAPK (Thr180/Tyr182; Cat. #9211), phospho-Akt (Ser473; Cat. #9271), and phospho-p42/p44 MAPK (Thr202/Tyr204; Cat. #9101) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Human gingival fibroblasts (HGF-1, ATCC CRL-2014; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30—2002) supplemented with 10 % fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin, at 37 °C in a humidified 5 % CO_2 atmosphere. Cells at passages 3—7 were used for experiments.

Cell viability

HGF-1 cells were treated with PM or nattokinase at various concentrations for different durations to assess dose-dependent cytotoxicity. Cell viability was determined using the PrestoBlue™ Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA; Cat. #A13262) according to the manufacturer's instructions. Results were expressed as the percentage of viable cells relative to untreated controls.

Real-time PCR

Total RNA was extracted under each experimental condition using TRIzol reagent and reverse-transcribed into cDNA. Real-time PCR was carried out using SYBR™ Green Universal Master Mix (Applied Biosystems, Branchburg, NJ, USA; Cat. #4309155) with specific primers for human GAPDH, COX-2, MMP-1, and HO-1. Relative mRNA levels of COX-2, MMP-1, and HO-1 were normalized to GAPDH expression.

Luciferase reporter gene activity

The transcriptional activity of Nrf2 was assessed using the antioxidant response element (ARE) Reporter Kit for Nrf2 (BPS Bioscience, Cat. #60514). Luciferase activity was measured according to the manufacturer's protocol.

Western blot

HGF-1 cells were treated according to the experimental design and processed as previously described.[19] Following incubation, cells were rapidly washed, harvested, and denatured by heating at 95 °C for 15 min, then centrifuged at 45,000×*g* at 4 °C to obtain whole-cell extracts. Proteins were separated on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4 °C with primary antibodies (1:1000), followed by HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:2000) for 1 h at room temperature. After extensive washing with Tris-buffered saline containing 0.05 % Tween 20 (TTBS), immunoreactive bands were visualized using enhanced chemiluminescence reagents. Images were captured with an Azure Biosystems 260 imager (Dublin, CA, USA), and band intensities were quantified using UN-SCAN-IT gel software (Orem, UT, USA).

Determination of nicotinamide adenine dinucleotide phosphate oxidase activity

HGF-1 cells were scraped, centrifuged (400×*g*, 10 min, 4 °C), and resuspended in ice-cold RPMI-1640 medium. A 5 μl aliquot of the suspension (0.2×10^5 cells) was added to 200 μl of pre-warmed medium containing 1 μM NADPH or 20 μM lucigenin. Chemiluminescence was measured using an Appliskan luminometer (Thermo Fisher Scientific, Waltham, MA, USA) for 12 min, and activity was expressed as counts per 10^6 cells.

Measurement of intracellular reactive oxygen species generation

Intracellular ROS levels were measured using the CellROX™ Green Reagent (Molecular Probes, Eugene, OR, USA; Cat. #C10444). HGF-1 cells were washed with warm Hank's Balanced Salt Solution (HBSS) and incubated at 37 °C for 30 min in HBSS containing 5 μM CellROX Green. After incubation, the reagent was removed and replaced with fresh medium, followed by treatment with PM under different experimental conditions. Cells were then washed twice with PBS, detached with trypsin/EDTA, and analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA) with excitation/emission wavelengths of 485/520 nm.

Measurement of prostaglandin E_2 and matrix metalloproteinase-1 release

After incubation of HGF-1 cells with PM for the indicated times at 37 °C, culture media were collected. PGE_2 levels were quantified using a PGE_2 ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA; Cat. #ADI-900-001), and MMP-1 levels were measured using a human MMP-1 ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA; Cat. #EHMMP1), following the manufacturers' instructions.

Statistical analysis

All data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Quantitative results are expressed as mean ± S.D., with individual data points shown as dot plots to illustrate biological variability. Statistical significance was determined by one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons. A P-value <0.05 was considered statistically significant.

Results

Particulate matter triggers cyclooxygenase-2-driven inflammatory and matrix-degrading responses

Environmental PM has been shown to trigger oxidative stress and inflammation,[20,21] but its role in periodontal tissue remodeling remains incompletely understood. To establish the working concentration, HGF-1 cells were treated with various concentrations of PM for 24 h. Cell viability assays showed that only the highest concentration (100 μg/cm^2) caused a significant reduction in viability, whereas lower concentrations, including 50 μg/cm^2, had no effect (Fig. 1A). Time-course experiments further confirmed that treatment with 50 μg/cm^2 PM for up to 48 h did not affect cell viability (Fig. 1B). Thus, 50 μg/cm^2 was used for subsequent experiments. Under this condition, PM exposure markedly induced COX-2 protein expression (Fig. 1C) and significantly increased the release of PGE_2 (Fig. 1D). Both MMP-1 mRNA expression and protein secretion were also elevated in a time-dependent manner (Fig. 1E and F). Importantly, pretreatment with the selective COX-2 inhibitor NS-398 for 1 h suppressed PM-induced MMP-1 expression and release (Fig. 1G), confirming that MMP-1 induction in HGF-1 cells occurs via a COX-2-dependent mechanism.

Nattokinase attenuates particulate matter-induced pro-inflammatory and degradative responses

Nattokinase has been reported to exert anti-inflammatory and antioxidative activities,[22,23] but its effects on periodontal fibroblasts under environmental stress remain poorly defined. To examine this, HGF-1 cells were treated with nattokinase at concentrations ranging from 0 to 100 μM for 12—48 h. Cell viability assays revealed no cytotoxicity at any concentration or time point, confirming its cellular safety (Fig. 2A). Pretreatment with nattokinase for 1 h markedly suppressed PM-induced COX-2 protein expression (Fig. 2B) and mRNA levels (Fig. 2C), accompanied by a significant reduction in PGE_2 release (Fig. 2D). Furthermore, nattokinase treatment attenuated MMP-1 mRNA expression and protein secretion (Fig. 2E). These findings indicate that nattokinase effectively counteracts PM-induced pro-inflammatory signaling and matrix degradation in HGF-1 cells.

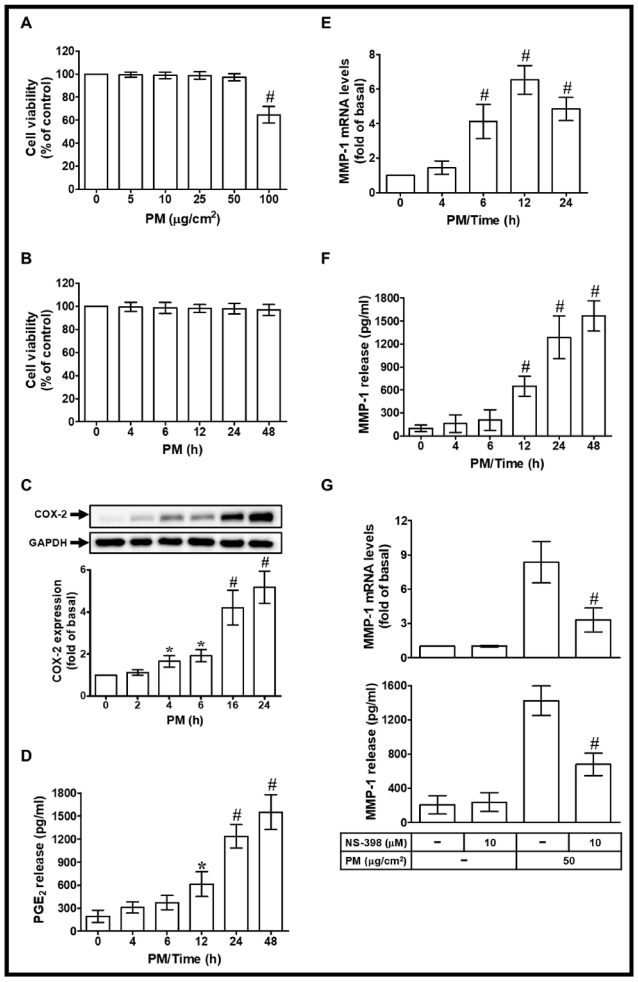

Figure 1 PM induces COX-2-dependent PGE_2 and MMP-1 production. (A and B) HGF-1 cells were treated with different concentrations of PM for 24 h, or with 50 μg/cm^2 of PM for various time points, and cell viability was then measured. HGF-1 cells were treated with 50 μg/cm^2 of PM for different time points, and (C) COX-2 protein levels, (D) PGE_2 release, (E) MMP-1 mRNA expression, and (F) MMP-1 release were measured. (G) HGF-1 cells were pretreated with NS-398 and then exposed to PM, after which MMP-1 mRNA expression and release were examined. Data are presented as mean ± SD from at least three independent experiments. $^*P < 0.05$, $^{\#}P < 0.01$ compared with the control group (A, C—F). $^{\#}P < 0.01$ compared with cells exposed to PM alone. PM: particulate matter; COX-2: cyclooxygenase-2; PGE_2: prostaglandin E_2; MMP-1: matrix metalloproteinase-1; HGF-1: human gingival fibroblast.

Nattokinase inhibits nicotinamide adenine dinucleotide phosphate oxidase/reactive oxygen species-mediated cyclooxygenase-2 expression

ROS generated via NADPH oxidase are important mediators of PM-induced inflammation.[24,25] To evaluate their involvement, HGF-1 cells were pretreated with ROS modulators prior to PM exposure. Pretreatment with NAC (a general ROS scavenger), APO (an NADPH oxidase inhibitor),

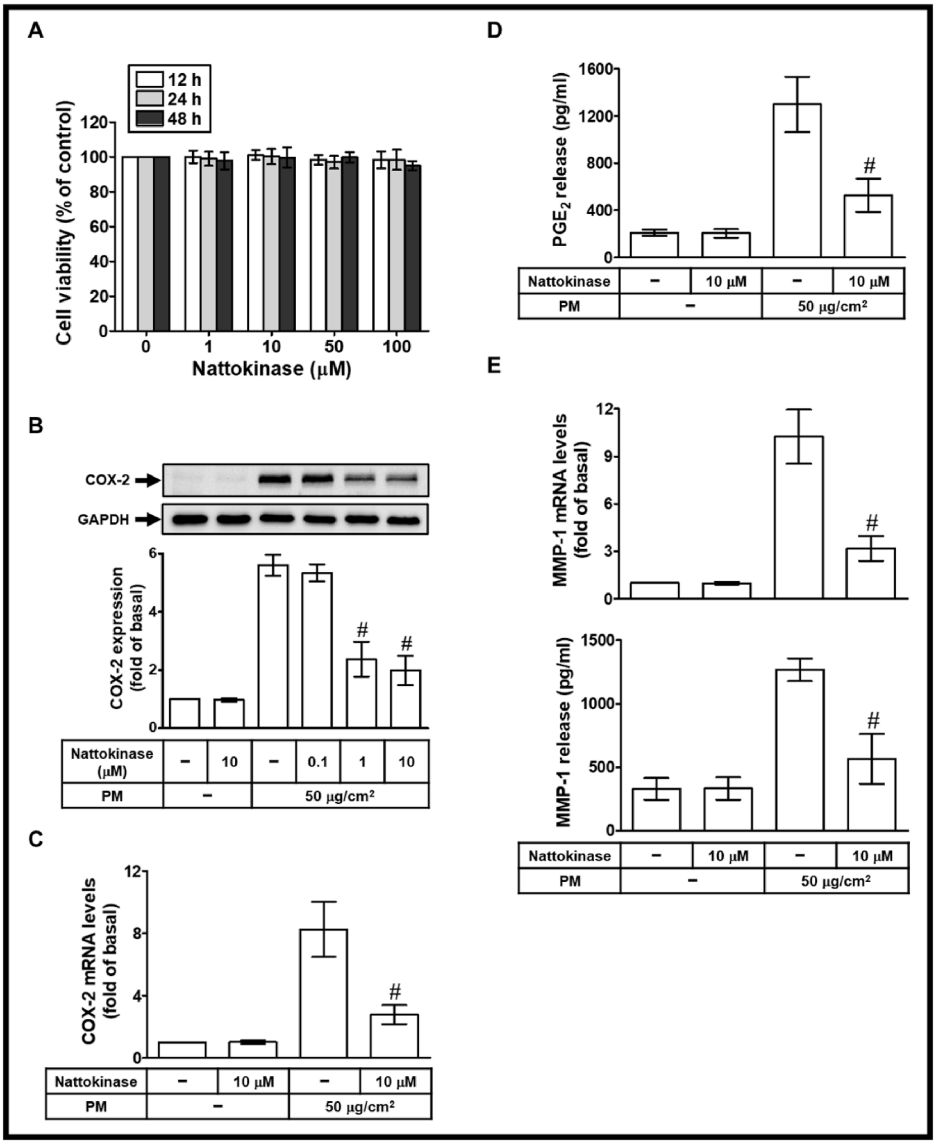

Figure 2 Nattokinase inhibits PM-induced COX-2 and MMP-1 expression. (A) Cells were exposed to different concentrations of nattokinase for various durations, after which cell viability was measured. Cells were pretreated with nattokinase for 1 h, followed by PM exposure, and then (B) COX-2 protein expression, (C) COX-2 mRNA levels, (D) PGE_2 release, and (E) MMP-1 mRNA expression and MMP-1 release were measured. Data are presented as mean ± SD from at least three independent experiments. $^{\#}P < 0.01$ compared with cells exposed to PM alone. PM: particulate matter; COX-2: cyclooxygenase-2; PGE_2: prostaglandin E_2; MMP-1: matrix metalloproteinase-1.

or DPI (a flavoprotein inhibitor that blocks NADPH oxidase activity) for 1 h significantly reduced COX-2 protein and mRNA expression, attenuated PGE_2 release, and decreased MMP-1 expression (Fig. 3A—E). Time-course analysis further showed that PM stimulation induced a rapid and sustained increase in intracellular ROS levels and NADPH oxidase activity (Fig. 3F and G). Notably, pretreatment with nattokinase markedly suppressed both ROS generation and NADPH oxidase activity (Fig. 3H). These results demonstrate that PM-induced COX-2 and MMP-1 expression is closely linked to NADPH oxidase-derived ROS, and that nattokinase attenuates this response by inhibiting ROS production.

Particulate matter activates protein kinase B phosphorylation through reactive oxygen species and establishes reciprocal regulation

The PI3K/Akt pathway is closely associated with redox signaling in inflammatory responses.[26,27] In HGF-1 cells, inhibition of PI3K/Akt with LY294002 or SH-5 (PI3K/Akt inhibitors) significantly reduced PM-induced COX-2 expression, PGE_2 release, and MMP-1 production (Fig. 4A—E). Time-course analysis showed that PM induced rapid Akt phosphorylation, which peaked at 10 min and remained elevated for at least 60 min (Fig. 4F). Pretreatment with

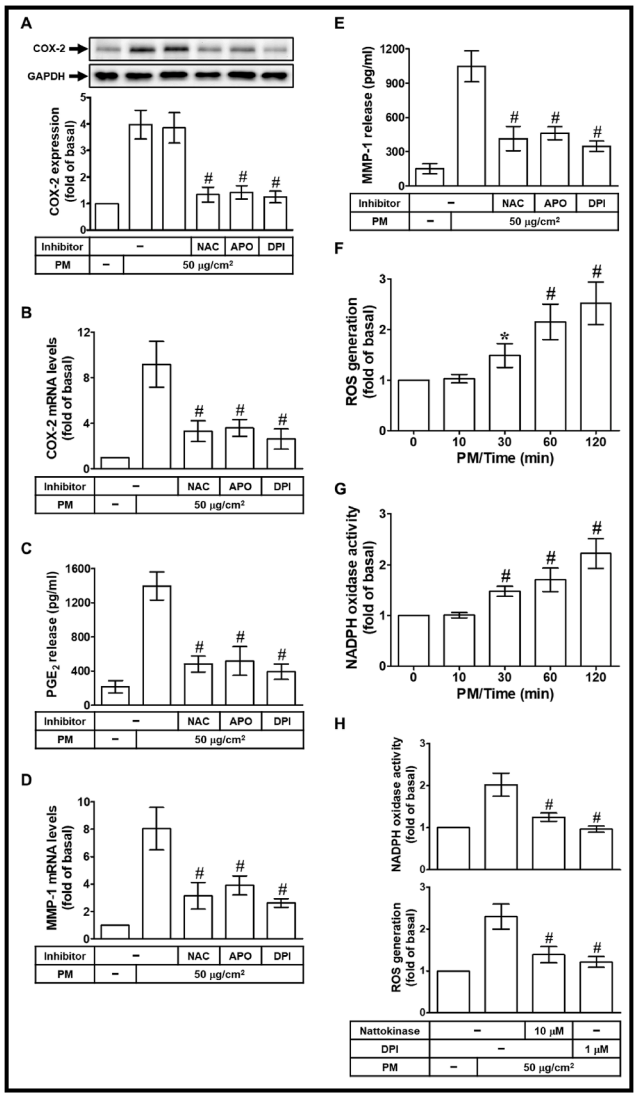

Figure 3 Nattokinase inhibits NADPH oxidase/ROS-mediated COX-2 expression. Cells were pretreated with NAC, APO, or DPI for 1 h, followed by PM exposure, and then (A) COX-2 protein expression, (B) COX-2 mRNA levels, (C) PGE_2 release, (D) MMP-1 mRNA expression, and (E) MMP-1 release were examined. After cells were treated with PM for different time points, (F) intracellular ROS generation and (G) NADPH oxidase activity were examined. (H) Cells were pretreated with nattokinase or DPI for 1 h, followed by PM exposure, and intracellular ROS generation and NADPH oxidase activity were examined. Data are presented as mean ± SD from at least three independent experiments. $^{\#}P < 0.01$ compared with cells exposed to PM alone (A-E and H). $^{*}P < 0.05$, $^{\#}P < 0.01$ compared with the control group (F and G). NADPH: nicotinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; COX-2: cyclooxygenase-2; NAC: N-acetyl-L-cysteine; DPI: diphenyleneiodonium chloride; APO: apocynin; PM: particulate matter; PGE_2: prostaglandin E_2; MMP-1: matrix metalloproteinase-1.

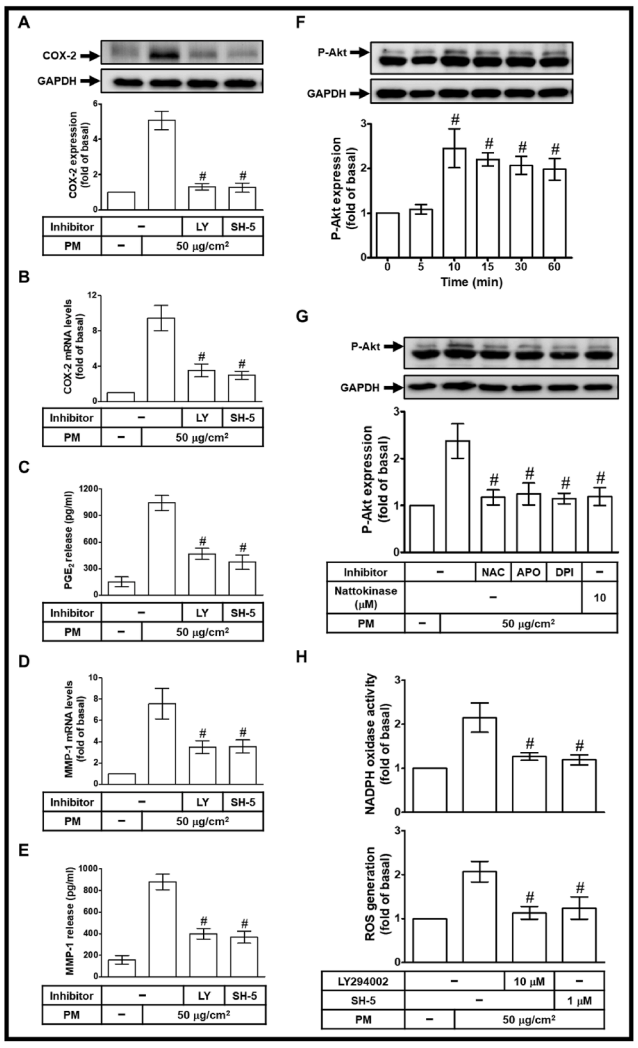

Figure 4 Bidirectional regulation of PI3K/Akt and ROS in PM-induced COX-2 and MMP-1 expression. Cells were pretreated with LY294002 (LY) or SH-5 for 1 h, followed by PM exposure, and (A) COX-2 protein expression, (B) COX-2 mRNA levels, (C) PGE_2 release, (D) MMP-1 mRNA expression, and (E) MMP-1 release were examined. (F) Cells were treated with PM for different time points, or (G) pretreated with NAC, APO, DPI, or nattokinase for 1 h followed by PM exposure, and Akt phosphorylation was examined. (H) Cells were pretreated with LY294002 or SH-5 for 1 h, followed by PM exposure, and intracellular ROS generation and NADPH oxidase activity were examined. Data are presented as mean ± SD from at least three independent experiments. $^{\#}P < 0.01$ compared with cells exposed to PM alone (A-E, G, and H). $^{\#}P < 0.01$ compared with the control group (F). PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; ROS: reactive oxygen species; PM: particulate matter; COX-2: cyclooxygenase-2; MMP-1: matrix metalloproteinase-1; PGE_2: prostaglandin E_2; NAC: N-acetyl-L-cysteine; APO: apocynin; DPI: diphenyleneiodonium chloride; NADPH: nicotinamide adenine dinucleotide phosphate.

ROS inhibitors or nattokinase for 1 h markedly suppressed PM-induced Akt phosphorylation (Fig. 4G), indicating that ROS contribute to Akt activation. Conversely, inhibition of PI3K/Akt reduced PM-induced ROS generation and NADPH oxidase activity (Fig. 4H), suggesting a reciprocal regulatory relationship between ROS and PI3K/Akt. These results indicate that PM-induced COX-2 and MMP-1 expression requires both ROS and PI3K/Akt signaling, and that nattokinase effectively interrupts this amplification loop.

Nattokinase inhibits p42/p44 mitogen-activated protein kinase-mediated cyclooxygenase-2 expression

MAPK cascades, particularly p42/p44 MAPK, are important mediators of inflammatory signaling.[28,29] To assess their involvement, HGF-1 cells were pretreated with the MEK inhibitor PD98059 before PM exposure. Inhibition of p42/p44 MAPK significantly suppressed PM-induced COX-2 protein and mRNA expression, reduced PGE_2 release, and decreased MMP-1 expression (Fig. 5A–E). Time-course analysis showed that PM rapidly induced p42/p44 MAPK phosphorylation, with a clear increase detected at 5, 10, and 15 min, and the highest response observed at 5 min (Fig. 5F). This phosphorylation was markedly inhibited by ROS and PI3K/Akt inhibitors, as well as by nattokinase pretreatment (Fig. 5G). These findings show that p42/p44 MAPK is activated downstream of ROS and PI3K/Akt, and that nattokinase effectively suppresses this pathway.

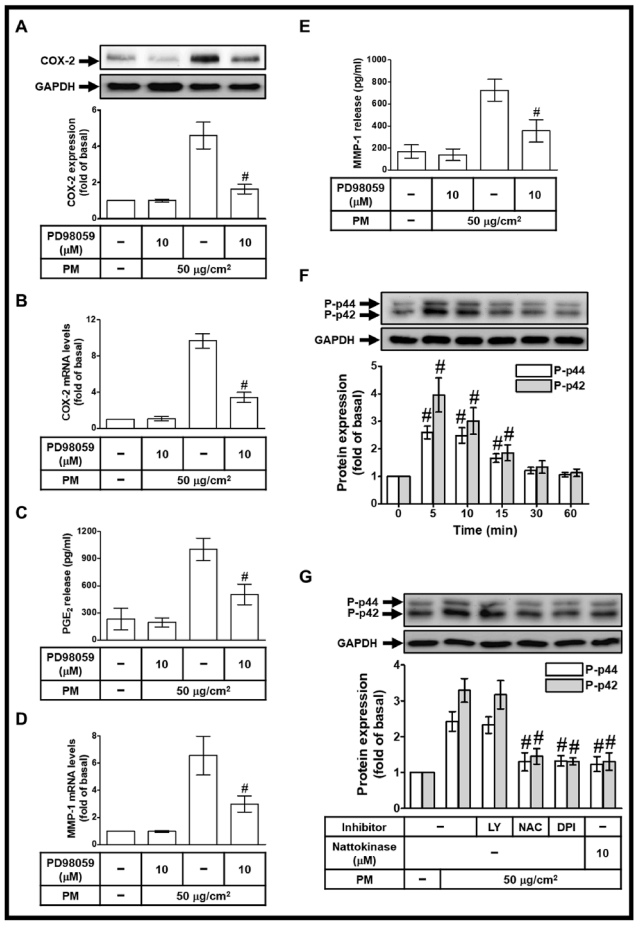

Figure 5 Nattokinase inhibits p42/p44 MAPK-mediated COX-2 expression. Cells were pretreated with PD98059 for 1 h, followed by PM exposure, and (A) COX-2 protein expression, (B) COX-2 mRNA levels, (C) PGE_2 release, (D) MMP-1 mRNA expression, and (E) MMP-1 release were examined. (F) Cells were treated with PM for different time points, or (G) pretreated with LY294002, NAC, DPI, or nattokinase for 1 h followed by PM exposure, and p42/p44 MAPK phosphorylation was examined. Data are presented as mean ± SD from at least three independent experiments. $^{\#}P < 0.01$ compared with cells exposed to PM alone (A-E and G). $^{\#}P < 0.01$ compared with the control group (F). MAPK: mitogen-activated protein kinase; COX-2: cyclooxygenase-2; PM: particulate matter; PGE_2: prostaglandin E_2; MMP-1: matrix metalloproteinase-1; NAC: N-acetyl-L-cysteine; DPI: diphenyleneiodonium chloride.

Nattokinase inhibits p38 mitogen-activated protein kinase-mediated cyclooxygenase-2 expression

The p38 MAPK pathway is another important regulator of inflammatory gene expression.[30,31] To evaluate its role in PM-induced responses, HGF-1 cells were pretreated with the p38

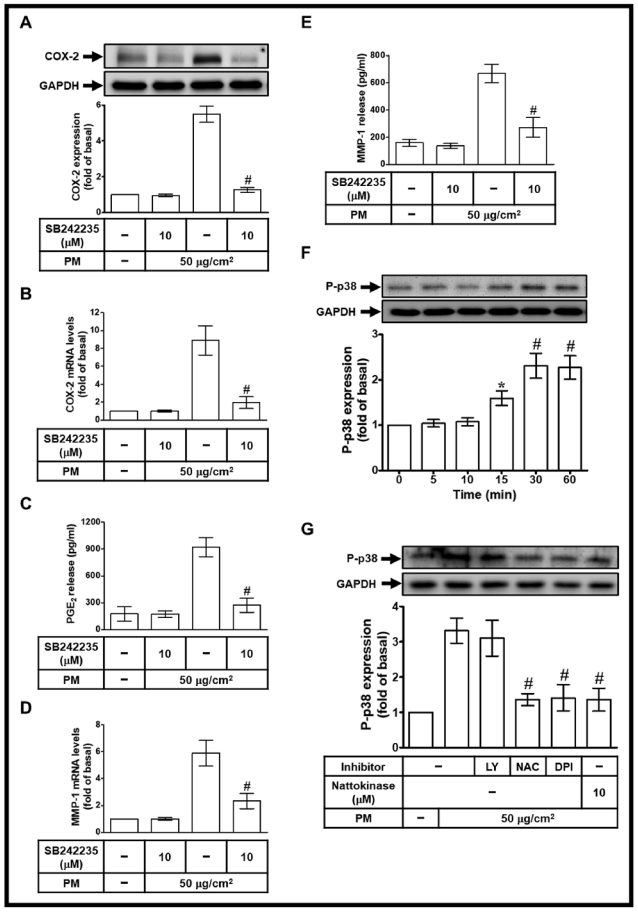

Figure 6 Nattokinase inhibits p38 MAPK-mediated COX-2 expression. Cells were pretreated with SB242235 for 1 h, followed by PM exposure, and (A) COX-2 protein expression, (B) COX-2 mRNA levels, (C) PGE_2 release, (D) MMP-1 mRNA expression, and (E) MMP-1 release were examined. (F) Cells were treated with PM for different time points, or (G) pretreated with LY294002, NAC, DPI, or nattokinase for 1 h followed by PM exposure, and p38 MAPK phosphorylation was examined. Data are presented as mean ± SD from at least three independent experiments. $^{\#}P < 0.01$ compared with cells exposed to PM alone (A-E and G). $^{*}P < 0.05$, $^{\#}P < 0.01$ compared with the control group (F). MAPK: mitogen-activated protein kinase; COX-2: cyclooxygenase-2; PM: particulate matter; PGE_2: prostaglandin E_2; MMP-1: matrix metalloproteinase-1; NAC: N-acetyl-L-cysteine; DPI: diphenyleneiodonium chloride.

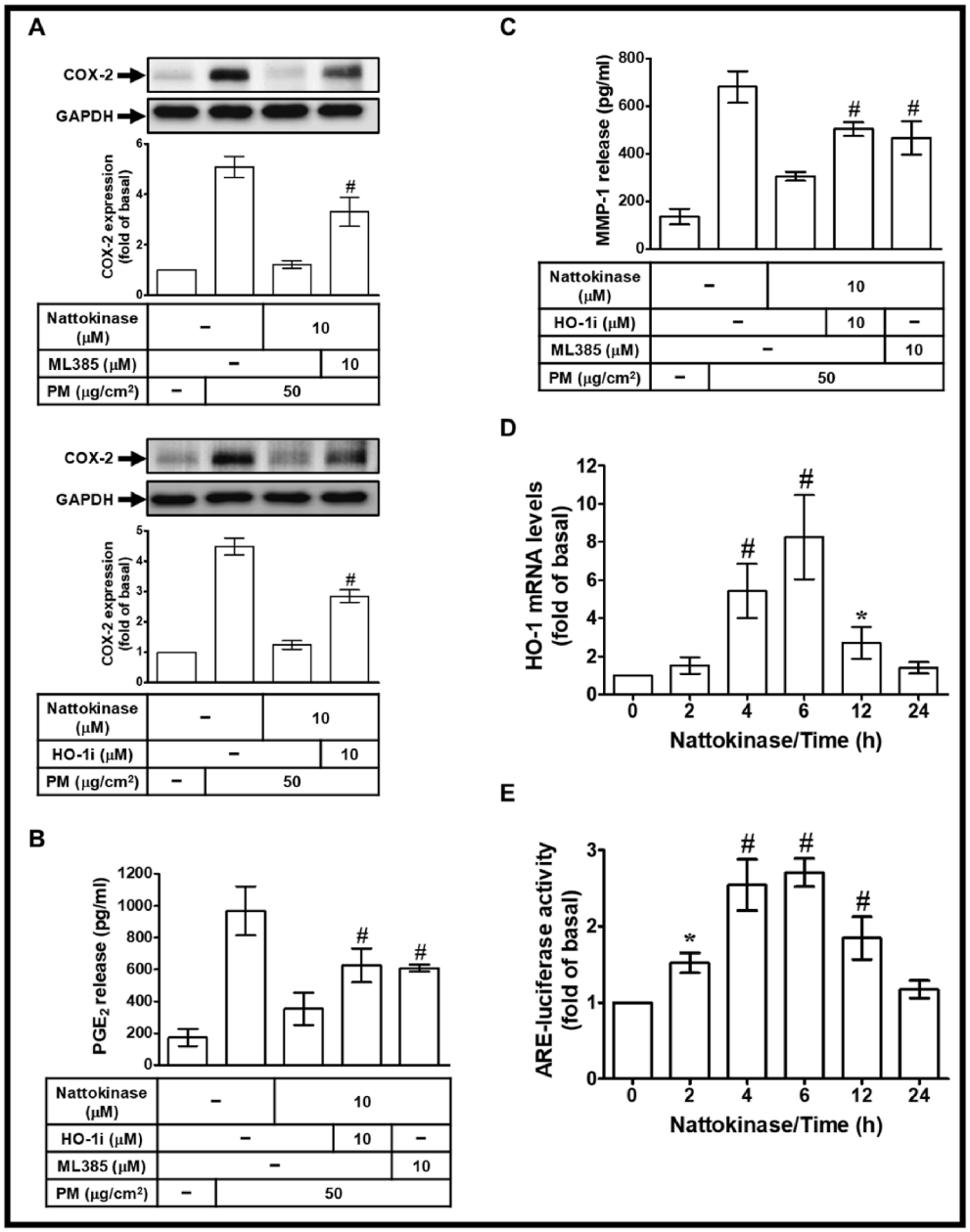

Figure 7 Nattokinase suppresses COX-2 and MMP-1 via Nrf2/HO-1 activation. Cells were pretreated with ML385 or Heme Oxygenase-1-IN-1 hydrochloride (HO-1i) for 1 h, with or without nattokinase, followed by PM exposure. (A) COX-2 protein expression, (B) PGE_2 release, and (C) MMP-1 release were examined. Cells were treated for different time points, and (D) HO-1 mRNA levels and (E) ARE-luciferase activity were examined. Data are presented as mean ± SD from at least three independent experiments. $^{\#}P < 0.01$ compared with cells treated with PM plus nattokinase (A—C). $^{*}P < 0.05$, $^{\#}P < 0.01$ compared with the control group (D and E). COX-2: cyclooxygenase-2; MMP-1: matrix metalloproteinase-1; Nrf2: nuclear factor erythroid 2-related factor 2; HO-1: heme oxygenase-1; PM: particulate matter; PGE_2: prostaglandin E_2; HO-1i: heme oxygenase-1 inhibitor; ARE: antioxidant response element.

MAPK inhibitor SB242235 before PM exposure. Inhibition of p38 MAPK significantly reduced PM-induced COX-2 protein and mRNA expression, attenuated PGE_2 release, and suppressed MMP-1 expression (Fig. 6A—E). Time-course experiments showed that PM induced phosphorylation of p38 MAPK, with clear increases observed at 15, 30, and 60 min (Fig. 6F). This phosphorylation was markedly reduced by ROS inhibitors, PI3K/Akt blockade, and nattokinase pretreatment (Fig. 6G). These results indicate that p38 MAPK activation contributes to PM-induced COX-2 and MMP-1 expression, and that nattokinase suppresses this process through inhibition of upstream ROS and PI3K/Akt signaling.

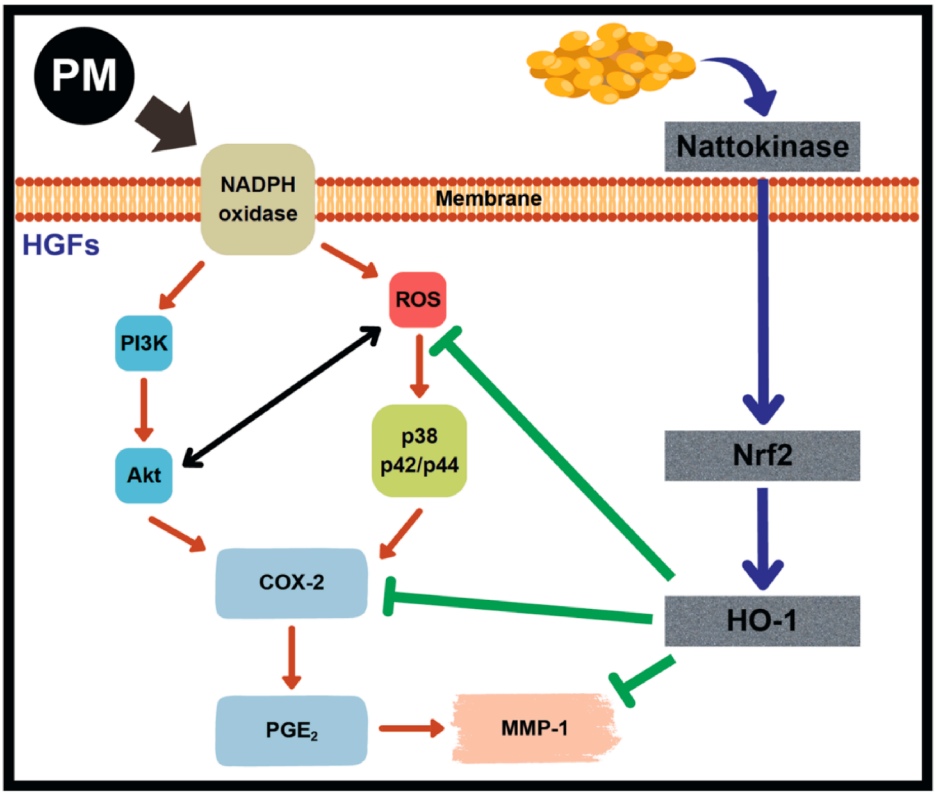

Figure 8 Schematic illustration of the proposed mechanism. PM stimulation activates multiple signaling pathways, including ROS generation, NADPH oxidase activation, and PI3K/Akt as well as MAPK cascades, leading to the upregulation of COX-2 expression and PGE_2 release, which in turn promote MMP-1 mRNA expression and protein release. Pretreatment with nattokinase attenuates these PM-induced responses through the induction of the Nrf2/HO-1 signaling pathway, thereby suppressing downstream inflammatory and matrix-degrading processes.

Nattokinase suppresses cyclooxygenase-2 and matrix metalloproteinase-1 via nuclear factor erythroid 2-related factor 2/heme oxygenase-1 activation

The Nrf2/HO-1 pathway is a major antioxidant defense mechanism that regulates cellular adaptation to oxidative stress.[32,33] Since nattokinase is known to exert antioxidative effects, we investigated whether its protective action involves Nrf2/HO-1 activation. Nattokinase pretreatment markedly suppressed PM-induced COX-2 expression, PGE_2 release, and MMP-1 production. However, these inhibitory effects were reversed when cells were cotreated with the Nrf2 inhibitor ML385 or the HO-1 inhibitor Heme Oxygenase-1-IN-1 (Fig. 7A–C), demonstrating that Nrf2/HO-1 activity is indispensable for nattokinase's protective role. Time-course analysis further demonstrated that nattokinase significantly increased HO-1 mRNA expression at 4, 6, and 12 h, while ARE-luciferase activity was enhanced as early as 2 h and remained elevated at 4, 6, and 12 h (Fig. 7D and E). Collectively, these findings demonstrate that nattokinase counteracts PM-induced inflammatory signaling through activation of the Nrf2/HO-1 pathway.

Discussion

This study demonstrates that PM induces inflammatory and degradative responses in HGF-1 cells through NADPH oxidase-derived ROS, PI3K/Akt, and MAPK signaling, and that nattokinase effectively counteracts these effects by activating the Nrf2/HO-1 pathway. We found that ROS and PI3K/Akt formed a reciprocal regulatory loop, amplifying downstream activation of p42/p44 and p38 MAPKs and leading to COX-2 expression, PGE_2 release, and MMP-1 upregulation. The data further demonstrate that nattokinase exerts a potent inhibitory effect on PM-induced oxidative and inflammatory cascades. Pretreatment with nattokinase markedly reduced ROS generation and NADPH oxidase activity, attenuated COX-2 expression, PGE_2 release, and MMP-1 induction. Mechanistically, nattokinase enhanced Nrf2 activation and HO-1 expression, and blocking either Nrf2 or HO-1 reversed its protective effects, confirming that the Nrf2/HO-1 pathway is indispensable. The early induction of ARE-luciferase activity, followed by sustained increases in HO-1 mRNA, suggests that nattokinase primes an antioxidant defense program that effectively neutralizes PM-induced stress.

The integrated mechanism is summarized in Fig. 8, which illustrates how PM activates NADPH oxidase-derived ROS, leading to PI3K/Akt and MAPK phosphorylation, resulting in COX-2 and MMP-1 induction and subsequent matrix degradation. Nattokinase counters this process by enhancing Nrf2/HO-1 activity, thereby suppressing ROS generation, disrupting the reciprocal loop between ROS and PI3K/Akt, and attenuating downstream MAPK activation and mediator release. This schematic underscores the central role of Nrf2/HO-1 activation in conferring protection against pollutant-induced injury.

These findings carry important implications for periodontal health. Periodontitis is a multifactorial disease, and while microbial dysbiosis remains central, environmental exposures such as PM represent overlooked risk factors that may exacerbate tissue destruction. Given that nattokinase is safe, orally bioavailable, and already utilized in cardiovascular health, its potential application as a preventive or adjunctive therapeutic agent in oral diseases deserves attention. Functional foods, supplements, or formulations containing nattokinase may provide additional benefits in populations exposed to high levels of PM.

Nonetheless, limitations of this study should be noted. The work was performed *in vitro* using immortalized HGF-1 cells, which cannot fully replicate the complexity of periodontal tissues *in vivo*. The composition of urban PM is heterogeneous, and real-world exposures may involve additional organic or microbial components that were not captured in this model. Furthermore, the pharmacokinetics of nattokinase in gingival tissues remain unclear. Future investigations using *in vivo* models of PM-induced periodontal damage are required to confirm these protective effects, and translational studies should evaluate whether nattokinase can synergize with conventional periodontal therapies to improve treatment outcomes.

In conclusion, our results reveal that PM promotes COX-2 expression, PGE_2 release, and MMP-1 induction in HGF-1 cells through NADPH oxidase/ROS, PI3K/Akt, and MAPK signaling, while nattokinase protects against these responses by activating the Nrf2/HO-1 pathway. See Fig. 8 for the proposed mechanism, which highlights how nattokinase interrupts oxidative and inflammatory cascades, thereby protecting periodontal tissues from pollutant-induced injury. These findings support the potential use of nattokinase as a therapeutic strategy for managing PM-related periodontal tissue damage.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This study was supported by the National Science and Technology Council of Taiwan (Grant No. 114-2313-B-038-002-MY3).

References

1. Krittanawong C, Qadeer YK, Hayes RB, et al. PM2.5 and cardiovascular diseases: State-of-the-Art review. *Int J Cardiol Cardiovasc Risk Prev* 2023;19:200217.
2. Li W, Li N, Liang Z, et al. Long-term exposure to ambient fine particulate matter and periodontitis: an observational study using nationally representative survey data. *J Clin Periodontol* 2024;51:596—609.
3. Marruganti C, Shin HS, Sim SJ, Grandini S, Laforí A, Romandini M. Air pollution as a risk indicator for periodontitis. *Biomedicines* 2023;11:443.
4. Wielento A, Lagosz-Cwik KB, Potempa J, Grabiec AM. The role of gingival fibroblasts in the pathogenesis of periodontitis. *J Dent Res* 2023;102:489—96.
5. Sakaki H, Matsumiya T, Kusumi A, et al. Interleukin-1β induces matrix metalloproteinase-1 expression in cultured human gingival fibroblasts: role of cyclooxygenase-2 and prostaglandin E_2. *Oral Dis* 2004;10:87—93.
6. Lee IT, Yang CM. Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced airway and pulmonary diseases. *Biochem Pharmacol* 2012;84:581—90.
7. Lee IT, Yang CM. Inflammatory signalings involved in airway and pulmonary diseases. *Mediat Inflamm* 2013;2013:791231.
8. Lim EY, Kim GD. Particulate matter-induced emerging health effects associated with oxidative stress and inflammation. *Antioxidants* 2024;13:1256.
9. Saha S, Buttari B, Panieri E, Profumo E, Saso L. An overview of Nrf2 signaling pathway and its role in inflammation. *Molecules* 2020;25:5474.
10. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cell Mol Life Sci* 2016;73:3221—47.
11. Lv W, Hu S, Yang F, et al. Heme oxygenase-1: potential therapeutic targets for periodontitis. *PeerJ* 2024;12:e18237.
12. Saha S, Sachivkina N, Lenchenko E, Pilshchikova O, Muraev A. Activation of the Nrf2 signaling pathway as a therapeutic strategy against periodontal disease: a narrative review. *Dent J* 2025;13:314.
13. Arabpour M, Zareanshahraki M, Albadr RJ, et al. The role of Nrf2 in the regulation of periodontitis, peri-implantitis, dentin infection, and apical periodontitis. *Biol Proced Online* 2025;27:23.
14. Yin Y, Ma Z, Shi P. Nootkatone mitigates periodontal inflammation and reduces alveolar bone loss via Nrf2/HO-1 and NF-κB pathways in a rat model of periodontitis. *Folia Histochem Cytobiol* 2024;62:145—53.
15. Granito M, Alvarenga L, Ribeiro M, et al. Nattokinase as an adjuvant therapeutic strategy for non-communicable diseases: a review of fibrinolytic, antithrombotic, anti-inflammatory, and antioxidant effects. *Expert Rev Cardiovasc Ther* 2024;22:565—74.
16. Yuan L, Liangqi C, Xiyu T, Jinyao L. Biotechnology, bioengineering and applications of *Bacillus* nattokinase. *Biomolecules* 2022;12:980.
17. Huang Z, Ng TK, Chen W, et al. Nattokinase attenuates retinal neovascularization via modulation of Nrf2/HO-1 and glial activation. *Investig Ophthalmol Vis Sci* 2021;62:25.
18. Elbakry MMM, Mansour SZ, Helal H, Ahmed ESA. Nattokinase attenuates bisphenol A- or γ-irradiation-mediated hepatic and neural toxicity by activation of Nrf2 and suppression of inflammatory mediators in rats. *Environ Sci Pollut Res Int* 2022;29:75086—100.

19. Cheng CY, Chen YH, Vo TTT, et al. CORM-2 prevents human gingival fibroblasts from lipoteichoic acid-induced VCAM-1 and ICAM-1 expression by inhibiting TLR2/MyD88/TRAF6/PI3K/Akt/ROS/NF-κB signaling pathway. *Biochem Pharmacol* 2022;201:115099.
20. Li N, Xia T, Nel AE. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic Biol Med* 2008;44:1689—99.
21. Zhai X, Wang J, Sun J, Xin L. PM2.5 induces inflammatory responses via oxidative stress-mediated mitophagy in human bronchial epithelial cells. *Toxicol Res* 2022;11:195—205.
22. Li X, Long J, Gao Q, et al. Nattokinase supplementation and cardiovascular risk factors: a systematic review and meta-analysis of randomized controlled trials. *Rev Cardiovasc Med* 2023;24:234.
23. Wu H, Wang Y, Zhang Y, et al. Breaking the vicious loop between inflammation, oxidative stress and coagulation: a novel anti-thrombus insight of nattokinase by inhibiting LPS-induced inflammation and oxidative stress. *Redox Biol* 2020;32:101500.
24. Kampfrath T, Maiseyeu A, Ying Z, et al. Chronic fine particulate matter exposure induces systemic vascular dysfunction via NADPH oxidase and TLR4 pathways. *Circ Res* 2011;108:716—26.
25. Kang KA, Piao MJ, Fernando PDSM, et al. Particulate matter stimulates the NADPH oxidase system via AhR-mediated epigenetic modifications. *Environ Pollut* 2024;347:123675.
26. Koundouros N, Poulogiannis G. Phosphoinositide 3-kinase/Akt signaling and redox metabolism in cancer. *Front Oncol* 2018;8:160.
27. He X, Li Y, Deng B, et al. The PI3K/Akt signalling pathway in inflammation, cell death and glial scar formation after traumatic spinal cord injury: mechanisms and therapeutic opportunities. *Cell Prolif* 2022;55:e13275.
28. Arthur JSC, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol* 2013;13:679—92.
29. Matthews JS, O'Neill LA. Distinct roles for p42/p44 and p38 mitogen-activated protein kinases in the induction of IL-2 by IL-1. *Cytokine* 1999;11:643—55.
30. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 2005;15:11—8.
31. Branger J, van den Blink B, Weijer S, et al. Anti-inflammatory effects of a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *J Immunol* 2002;168:4070—7.
32. Tarek H, Cho SS, Hossain MS, Yoo JC. Attenuation of oxidative damage via upregulating Nrf2/HO-1 signaling pathway by protease SH21 with exerting anti-inflammatory and anticancer properties *in vitro*. *Cells* 2023;12:2190.
33. He M, Pan H, Chang RCC, So KF, Brecha NC, Pu M. Activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects of *Lycium barbarum* polysaccharides in the rodent retina after ischemia-reperfusion-induced damage. *PLoS One* 2014;9:e84800.