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Protein Kinase C and Matrix Metalloproteinases Expression Using Phorbol Myristate Acetate in Degenerative Intervertebral Disc Cells

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Background: Degeneration of nucleus pulposus (NP) cells involves multiple factors. The relationship between the canonical Wnt/β-catenin signaling pathway and matrix metalloproteinases (MMPs) is important in cellular senescence. Protein kinase C (PKC), an intermediate of the non-canonical Wnt pathway stimulated by phorbol myristate acetate (PMA), possibly prevents NP cell senescence, although not yet demonstrated in human-based studies. This study aimed to investigate the effect of PMA stimulation on the non-canonical and canonical Wnt pathways and MMP expression in human NP cells to ascertain its inhibitory effects on the senescence of NP cells.

Methods: Human disc tissues of Pfirrmann grades 1 and 2 were collected from patients during spinal surgery and subsequently cultured. Protein and ribonucleic acid (RNA) were isolated from NP cells treated with PMA (400 nM) for 24 hours. Expression of MMP1, MMP13, tissue inhibitor of matrix metalloproteinase 1 (TIMP1), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), transient receptor potential vanilloid 4 (TRPV4), interleukin-6 (IL-6), and β-catenin were detected using western blot analysis. Messenger RNA (mRNA) expression of type II collagen and glycosaminoglycan (GAG) were analyzed using reverse transcription polymerase chain reaction. IL-6 and prostaglandin E2 (PGE_2) levels were measured using enzyme-linked immunosorbent assay.

Results: Expression of PKC-δ (intermediate of the non-canonical Wnt pathway) and β-catenin (intermediate of the canonical Wnt pathway) was increased by PMA treatment. The mRNA levels of type II collagen and GAG increased; however, their protein levels were not altered. PMA treatment increased the expression of MMP1, TIMP1, ADAMTS5, IL-6, PGE_2, and TRPV4; however, the expression of MMP13 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) was unaltered.

Conclusions: PMA activated PKC-δ, affecting the non-canonical Wnt pathway; however, its effect on β-catenin in the canonical Wnt pathway was limited. β-catenin activation through the TRPV4 channel led to increased expression of MMP1 and ADAMTS5 and that of IL-6 and PGE_2 owing to NF-κB expression. Consequently, the degeneration of NP cells was not prevented.

Keywords: *Protein kinase C-delta, Disc degeneration, Matrix metalloproteinase, Nucleus pulposus, Phorbol myristate acetate*

The intervertebral disc (IVD) comprises 3 main structural components, including the annulus fibrosus, nucleus pulposus (NP), and cartilaginous endplate, and each is vital in the disc function. These components and their main constituents, including proteoglycans and collagens, play a synergistic role in providing the spine with motion, protection against mechanical loading, and the ability to effectively absorb and distribute loads.[1] Changes in the composition of these glycoproteins lead to degenerative

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changes in the IVD, which is characterized by decreased oxygen pressure owing to the loss of ability to control osmotic pressure, fibrosis of type II collagen, loss of elasticity, and activation of proteolytic enzymes.[2] These effects lead to degenerative IVD disease, whose progression depends on the ability of IVD cells to produce proteins, such as type II collagen, which are necessary to maintain IVD homeostasis.[3]

The *Wnt* gene was discovered during the wing development of fruit flies and is reportedly involved in cell differentiation and tumor development; moreover, it affects the skeletal musculature system.[4] Among various Wnt signaling pathways, the canonical Wnt/β-catenin signaling pathway is a major regulator of maintaining bone and cartilage tissue homeostasis.[5-8] It is important in inducing osteoblast differentiation, proliferation, and survival. It is regulated by soluble antagonists, including dickkopf-1, secreted frizzled-related proteins, and sclerostin. Dysregulation of this pathway is implicated in various diseases, including cancer, fibrosis, and degenerative disorders.[9]

β-Catenin is involved in bone formation, promotes cellular senescence, and inhibits aggrecan synthesis in NP cells through the canonical Wnt/β-catenin pathway. Activation of Wnt/β-catenin signaling may lead to increased breakdown of the matrix, induce the expression of matrix metalloproteinases (MMPs), and thereby promote IVD degeneration.[10,11]

β-Catenin, which promotes NP cell aging in the Wnt/β-catenin pathway, is downregulated by protein kinase C (PKC) activated by phorbol myristate acetate (PMA) in the non-canonical pathway.[12] Therefore, senescence and degradation of NP cells might be prevented or slowed by activating PKC in the non-canonical pathway and reducing β-catenin. Nevertheless, studies on the interaction between PKC and β-catenin and the resulting changes in MMP levels are scanty. Additionally, the existing published studies have used rat NP cells, and similar studies using human NP cells are very few.[12]

In this study, we aimed to assess the effect of PMA on PKC activation in human NP cells, determine whether the role of PKC, an intermediate product of the non-canonical pathway in rat NP cells, is similar to that in human NP cells, and analyze MMP expression in human NP cells treated with PMA. The experiments aimed to investigate the interaction and role of PKC and Wnt/β-catenin signaling in NP cells and changes in signal transduction between the non-canonical Wnt and the canonical Wnt signaling pathways.

METHODS

The study was approved by the Institutional Review Board of Yonsei University College of Medicine in Seoul, Korea (IRB No. 3-2015-0029). Informed consent was obtained from each participant.

Patient Samples

NP samples were collected from 7 patients (3 men and 4 women; average age, 18.4 ± 3.2 years) suffering from disc disease including scoliosis. The extent of IVD degeneration was evaluated using the modified Pfirrmann grading system through magnetic resonance imaging. Samples from grade I (n = 2) and grade II (n = 5) were obtained.

Isolation and Expansion Of Human IVD Cells

The harvested IVD tissues were minced using a scalpel and digested in Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F12, Gibco-BRL, Thermo Fisher Scientific) containing collagenase type II (2.5 mg/mL, Sigma-Aldrich solutions) for 2 hours at 37 °C with gentle agitation. Cells were then filtered through a sterile nylon mesh filter (pore size, 100 μm; Falcon, Corning) and seeded in T-25 flasks (Nunc, Merck) at a density of approximately 1×10^5 cells/mL. Primary cultures were maintained for 2–3 weeks in DMEM/F12 supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Thermo Fisher Scientific), 1% v/v penicillin, streptomycin, and nystatin (Gibco-BRL, Thermo Fisher Scientific) in a humidified 37 °C incubator containing 5% CO_2.

Culturing of NP Cells

Only NP cells were used in this study. We collected NP cells only by excluding the annulus from the patient during surgery. We observed the typical shape of NP cells by immunostaining and assessed the expression of Paired box-1 (PAX1), Ovo Like Zinc Finger 2 (OVOL2), and Forkhead Box F1 (FOXF1) by quantitative polymerase chain reaction (Q-PCR). NP cells were cultured with 400 nM PMA in 60-mm culture dishes at a density of 5×10^4 cells at 37 °C within an incubator (Thermo Fisher Scientific) containing 5% CO_2 for 24 hours. Cells treated with bisindolylmaleimide I (BII; Sigma-Aldrich solutions, Merck) and saline were used as controls.

Cell Viability Test

The viability of NP cells was assessed after 48 hours using an EZ-Cytox Cell viability assay kit (Daeil Lab Service Co., Ltd.). The assays were performed in triplicates. Culture medium (200 μL) and assay reagent (30 μL) were added to

each well and incubated at 37 °C for 1 hour. The supernatant was transferred to a 96-well plate, and the absorbance was measured at 450 nM using an enzyme-linked immunosorbent assay (ELISA) reader (VersaMax, Molecular Devices).

RNA Extraction and Reverse Transcription-PCR

NP cells were cultured and treated with PMA for 24 hours, and total RNA was isolated using a RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Subsequently, complementary DNA (cDNA) was synthesized using a Maxime RT premix kit (Qiagen). The expression of type II collagen was evaluated in duplicates using 1 μg cDNA, power SYBR Green Master Mix (Applied Biosystems), and ribonuclease-free water in a total reaction volume of 20 μL using a real-time PCR machine (Applied Biosystems, Thermo Fisher Scientific). β-actin was used as a reference gene. Results are shown as relative values to control condition and β-actin. Primer details are presented in Supplementary Table 1.

Sulfated Glycosaminoglycan Assay

Sulfated glycosaminoglycan (GAG) was extracted from PMA-treated NP cells following lysis in a papain extraction buffer comprising 0.1 M sodium acetate, 0.01 M ethylenediaminetetraacetic acid (EDTA), and 5 mM cysteine-HCl (pH 6.4). Sulfated GAG samples were concentrated using a sulfated GAG assay protocol, and the absorbance at 650 nM was measured according to the manufacturer's instructions (Blyscan; Biocolor Ltd.). Sulfated GAG was quantified using a standard curve of bovine tracheal chondroitin 4-sulfate.

Protein Isolation and Western Blot Analysis

Cells were lysed in a buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5 mM EDTA (pH 7.4), 1 mM Tri-HCl (pH 7.4), and protease inhibitor cocktail (Complete Mini, Roche Diagnostics). After treatment of NP cells with PMA for 24 hours, nuclear and cytoplasmic proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce, Thermo Fisher Scientific). Proteins in cell lysates were separated using SDS-polyacrylamide gel electrophoresis and transferred onto 0.45-μm polyvinylidene difluoride membranes (Pierce, Thermo Fisher Scientific) using an electrophoretic transfer system (Mini Trans-Blot Cell and systems, Bio-Rad). Membranes were incubated with antibodies against β-catenin, PKC-δ, or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; Cell Signaling Technology). After incubation with the secondary antibodies, immunoreactive bands were visualized using a western blot detection system (EzWestLumi plus; ATTO Corp.). Blots were stripped to remove bound antibodies and probed using an antibody against actin (Abcam) to verify the amounts of loaded proteins.

Enzyme-Linked Immunosorbent Assay

NP cells were cultured with 400 nM PMA, and the medium was harvested. ELISA kits were used to quantify the levels of interleukin-6 (IL-6; Enzo, Enzo Life Sciences) and prostaglandin E2 (PGE_2; Enzo, Enzo Life Sciences) in the medium according to the manufacturer's instructions by measuring the absorbance of the samples at 450 nM (VERSA Max, Molecular Devices).

Immunofluorescence

NP cells were plated in a flat-bottom 14-mm microwell (3×10^4 cells/well) and incubated for 24 hours. After treatment, cells were fixed with 4% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 (v/v) in phosphate-buffered saline (PBS) for 10 minutes, blocked with PBS containing 10% FBS for 1 hours, and incubated overnight at 4 °C with antibodies against NF-κB, (Cell Signaling Technology), transient receptor potential vanilloid 4 (TRPV4; Abcam), and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5; Abcam). Cells were washed and incubated with anti-rabbit Alexa Fluor 488-conjugated secondary (green) antibodies (Invitrogen, Thermo Fisher Scientific) at a dilution of 1:200 and 10 μM 4',6-diamidino-2-phenylindole for 1 hour at room temperature (between 20 °C to 22 °C). Confocal microscopy (Zeiss LSM700, Carl Zeiss) was used to observe the samples.

Statistical Analyses

All experiments were performed in triplicates. Data are expressed as mean ± standard deviation. A paired *t*-test was performed to compare the results between the 2 groups, and the Mann-Whitney *U*-test was used for non-parametric data analysis using IBM SPSS Statistics for Windows version 21.0 (IBM Corp.). A p-value < 0.05 was considered statistically significant.

RESULTS

Cytotoxicity of PMA

NP cells (5.25×10^4 cell /well) were seeded in 24-well plates and treated with 50, 100, 200, 400, and 800 nM PMA. PMA did not affect cell viability as compared to the control (Supplementary Fig. 1). Since there was no differ-

ence in cytotoxicity according to PMA amount, 400 nM PMA was chosen for a stronger stimulus.

Effect of PMA on PKC-δ and β-Catenin Expression

Cells (2.5×10^5 cells/well) were incubated with PMA and BII for 24 hours. The expression of PKC-δ and β-catenin slightly increased by PMA treatment (Fig. 1). Additionally, the phosphorylation of β-catenin also increased by PMA treatment.

Effect of PMA on GAG Content

The GAG content was assayed to analyze whether PMA can dissolve the extracellular matrix of cells. Cells (2.5×10^5 cells/well) were seeded in the 60-mm culture dishes and treated with PMA for 3 days. Sulfated GAG and chondroitin-4 sulfate GAG were not affected by PMA (Fig. 2).

Effect of PMA on Collagen Type II Expression

We analyzed the expression of collagen type II at mRNA and protein levels. PMA enhanced the expression of its mRNA; however, its expression was not altered at the protein level (Fig. 3).

Effect of PMA on the Expression of MMPs and Tissue Inhibitor Metalloproteinases (TIMPs)

Cells (2.5×10^5) were incubated with PMA and BII for 24 hours. PMA treatment induced the expression of MMP1 and TIMP1 in cells. Moreover, the expression of ADAMTS5 increased following PMA treatment. However, MMP13 expression was not different between the control and PMA-treated cells (Fig. 4).

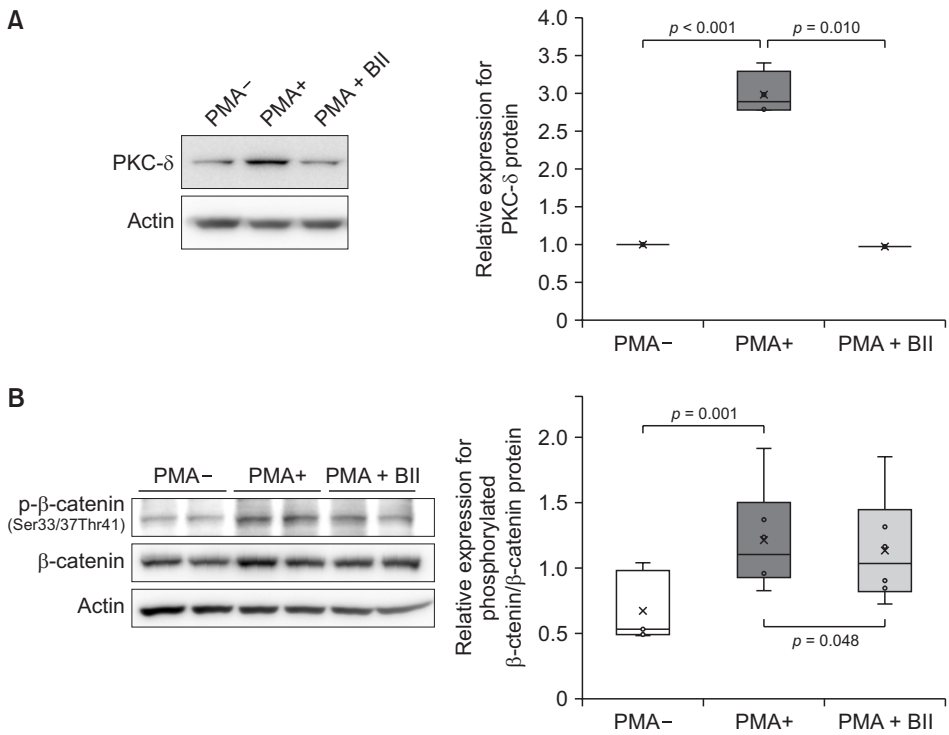

Fig. 1. Western blot analysis of the expression of protein kinase C (PKC)-δ (A) and β-catenin and its phosphorylated form (B). Values are presented as mean ± standard deviation. PMA: phorbol myristate acetate, BII: bisindolylmaleimide I. A *p*-value < 0.05 was considered significant.

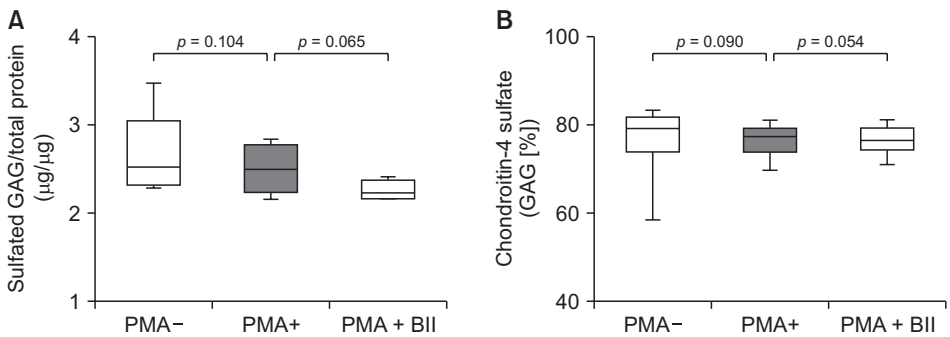

Fig. 2. Glycosaminoglycan (GAG) content in control and phorbol myristate acetate (PMA)-treated cells. (A) Total sulfated GAG. (B) Chondroitin-4 sulfate expression. Values are presented as mean ± standard deviation. BII: bisindolylmaleimide I. A *p*-value < 0.05 was considered significant.

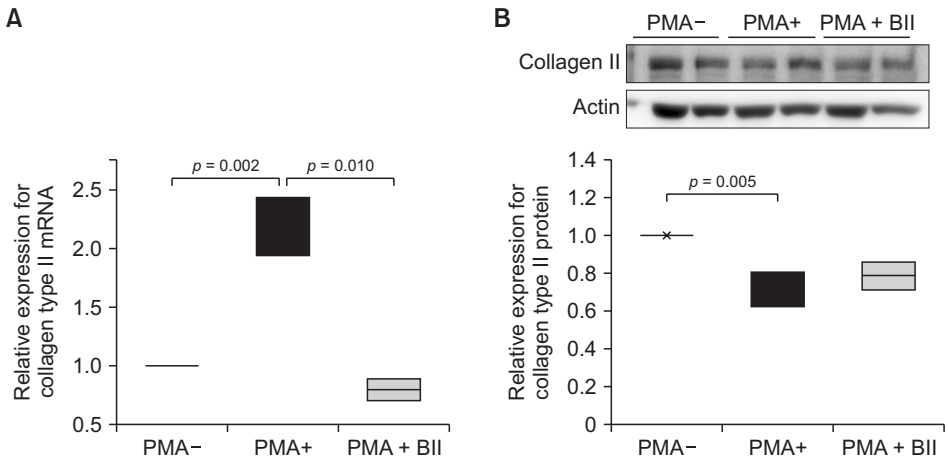

Fig. 3. Effect of phorbol myristate acetate (PMA) on the expression of collagen type II. (A) Expression of collagen type II messenger ribonucleic acid. (B) Expression of collagen type II protein. Values are presented as mean ± standard deviation. BII: bisindolylmaleimide I. A p-value < 0.05 was considered significant.

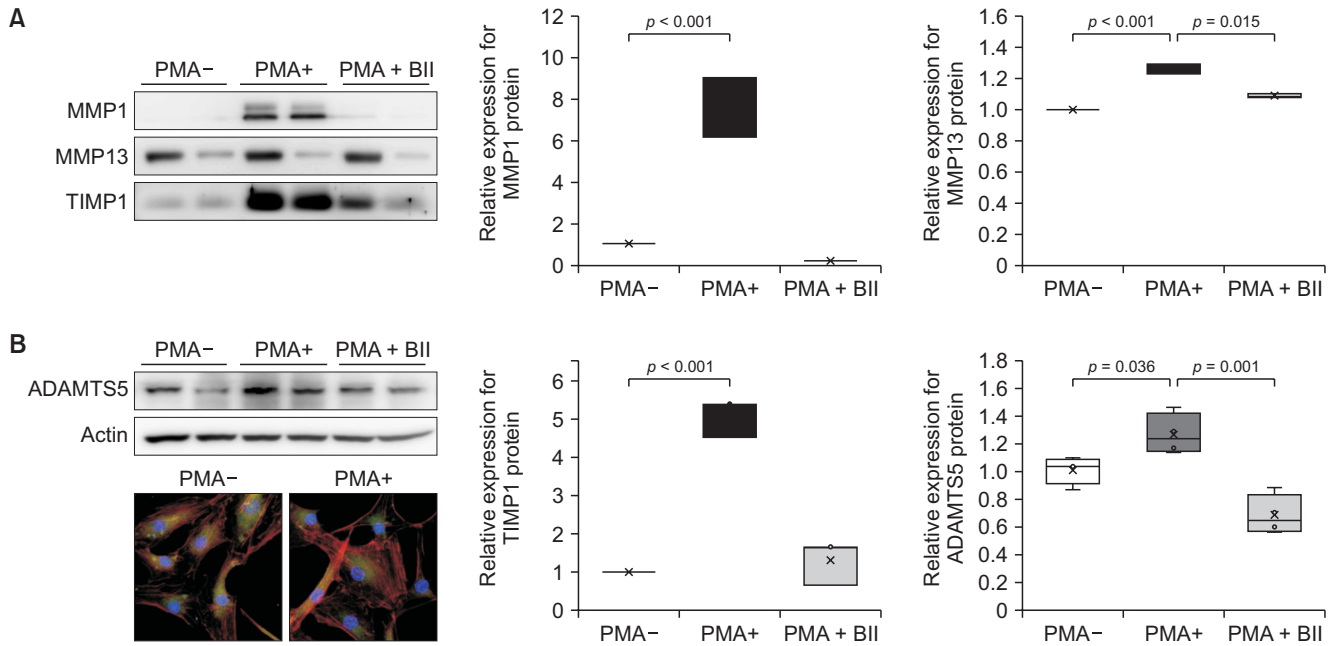

Fig. 4. Effect of phorbol myristate acetate (PMA) on the expression of matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs). (A) Western blot analysis of the expression of MMP1, MMP13, and TIMP1. (B) Western blot and immunofluorescent images showing the expression of a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) (the red, green, and blue colors indicate actin, ADAMTS5, and the nucleus, respectively). Values are presented as mean ± standard deviation. BII: bisindolylmaleimide I. A p-value < 0.05 was considered significant.

Effect of PMA on the Expression of the Proinflammatory Cytokines

IL-6 expression increased in NP cells treated with PMA, as was evident by western blot analysis. ELISA results further supported this result. Additionally, PGE_2 expression increased in NP cells upon PMA treatment (Fig. 5).

Effect of PMA on NF-κB Expression

NF-κB expression slightly increased in the cytosol upon PMA treatment (Fig. 6). Moreover, PMA treatment increased NF-κB phosphorylation in the nucleus.

Effect of PMA on TRPV4 Expression

TRPV4 expression increased in PMA-treated NP cells compared to that in control, as was evident by western blot analysis and immunofluorescence (Fig. 7).

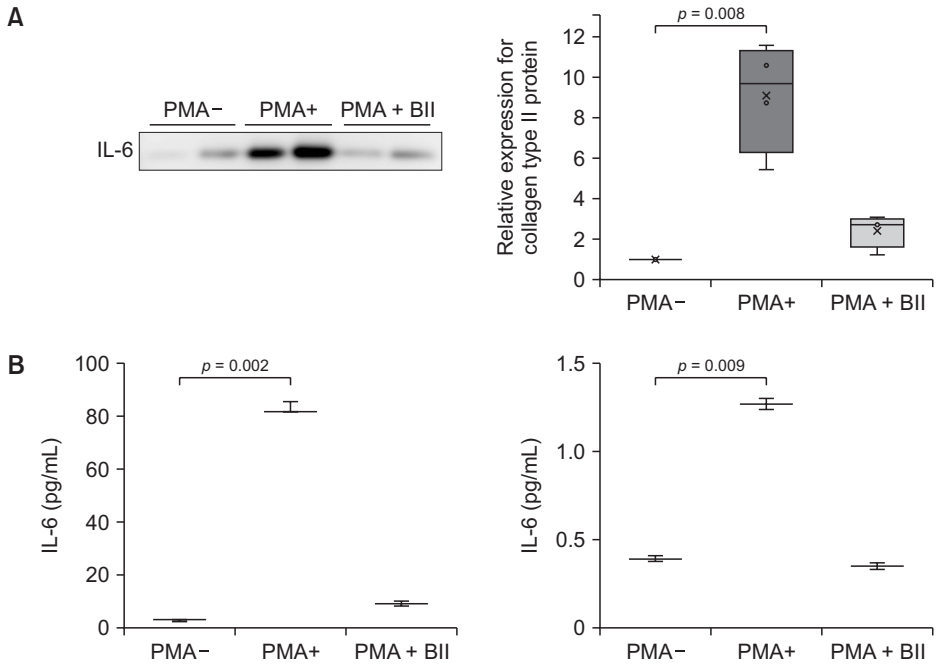

Fig. 5. Expression of interleukin-6 (IL-6) and prostaglandin E2 (PGE_2) in control and phorbol myristate acetate (PMA)-treated nucleus pulposus cells. (A) Analysis of IL-6 expression using western blotting. (B) Analysis of IL-6 and PGE_2 expression using enzyme-linked immunosorbent assay. Values are presented as mean ± standard deviation. BII: bisindolylmaleimide I. A *p*-value < 0.05 was considered significant.

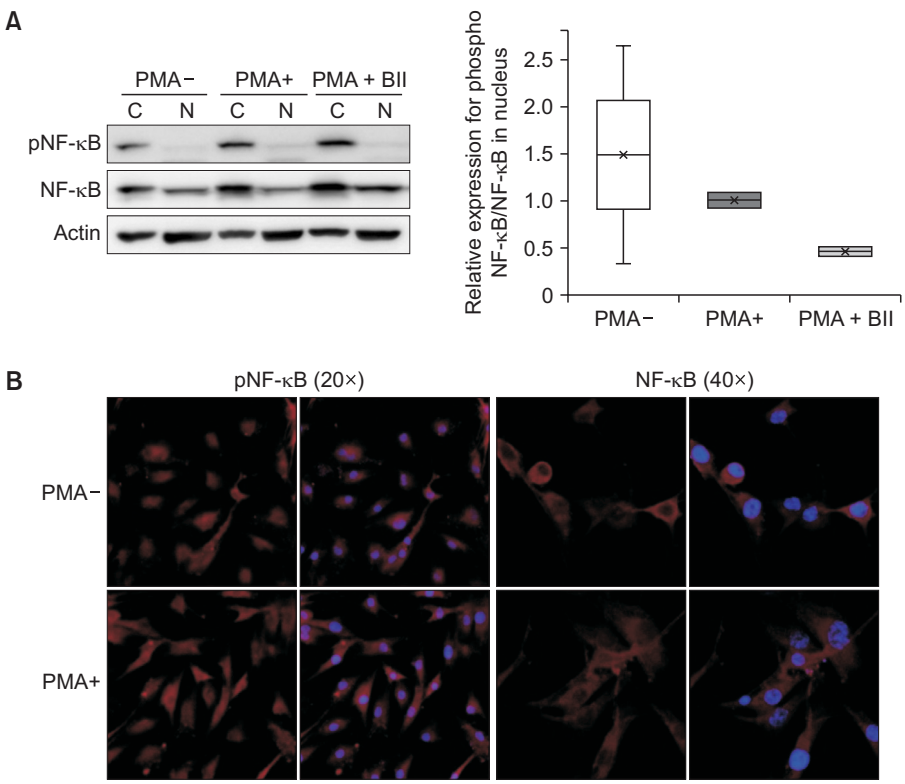

Fig. 6. Expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and phosphorylated NF-κB (pNF-κB) in the control and phorbol myristate acetate (PMA)-treated nucleus pulposus (NP) cells. (A) Western blot analysis of the expression of NF-κB and its phosphorylated form (pNF-κB). (B) Immunofluorescent images showing NF-κB expression in NP cells (magnification: 20× or 40×; the red and blue colors indicate NF-κB and the nucleus, respectively). Values are presented as mean ± standard deviation. BII: bisindolylmaleimide I, C: cytosolic, N: nuclear. A *p*-value < 0.05 was considered significant.

DISCUSSION

In this study, we assessed the effect of PMA on PKC activation in human NP cells and determined whether the role of PKC, an intermediate product of the non-canonical pathway observed in rat NP cells, is similar to that in human NP cells. We also analyzed MMP expression in human NP cells treated with PMA. Stimulation of human NP cells by PMA resulted in increased PKC-δ expression; however, the expression of other PKC isoforms did not increase. In addition, the phosphorylation of catenin in-

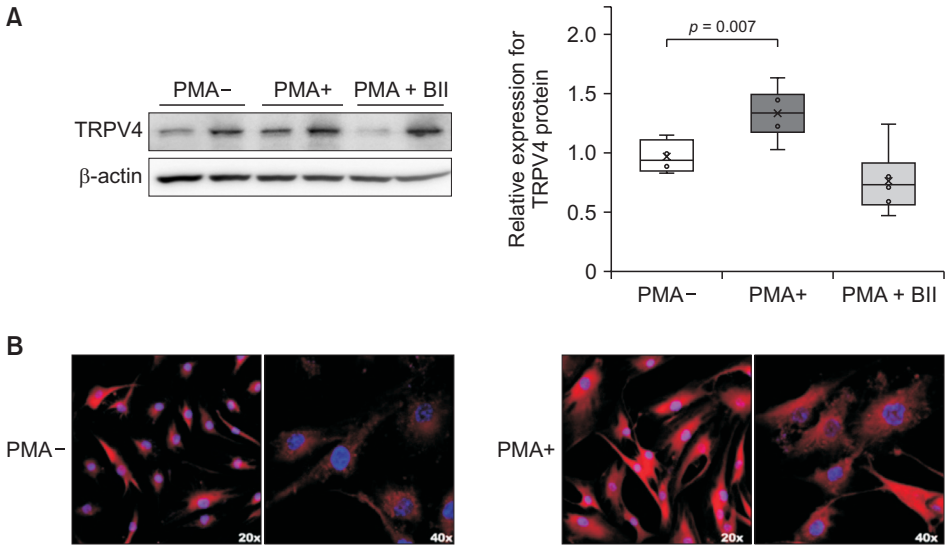

Fig. 7. Effect of phorbol myristate acetate (PMA) treatment on transient receptor potential vanilloid 4 (TRPV4) expression in nucleus pulposus (NP) cells. (A) Analysis of TRPV4 expression using Western blot. (B) Immunofluorescent images showing TRPV4 expression in NP cells (magnification: 20× or 40×; the red and blue colors indicate TRPV4 and the nucleus, respectively). Values are presented as mean ± standard deviation. BII: bisindolylmaleimide I. A p-value < 0.05 was considered significant.

creased, and an increase in intracellular β-catenin level was observed. Arai et al.[12] have reported different results regarding PKC expression in a similar experiment with rat NP cells; to confirm these differences, it will be necessary to identify substances that can selectively increase PKC-δ; however, they have observed similar trends of catenin expression and phosphorylation. These results indicate that although PKC signaling is slightly stimulated by PMA in human NP cells, the canonical Wnt pathway is not sufficiently downregulated compared to that in rat NP cells.[13,14] Probably, PMA upregulates other pathways, such as the TRPV1 channel,[15] to weaken the crosstalk between PMA and PKC.

PKC-δ expression was elevated in PMA-treated cells. PMA, a diester of phorbol, potent tumor promoter, and PKC agonist, activates PKC.[16] PMA-dependent activation of PKC signaling regulates the proliferation of NP cells, which may be strategized to treat degeneration.[13,17] PKC isoforms such as PKC-γ and PKC-δ may be involved in the activation of mitogen-activated protein kinases to promote the proliferation of rat NP cells and their matrix synthesis.[18] PKC-δ plays a pivotal pathophysiological role in NP cell homeostasis *in vitro, ex vivo, and in vivo*.[19]

In this study, human NP cells were treated with PMA to reduce the expression of β-catenin that is associated with disc degeneration. However, PMA treatment could not suppress the expression and phosphorylation of β-catenin; rather, β-catenin expression was slightly increased by PMA. β-catenin acts as a transcriptional coactivator after translocation into the nucleus[7,8,20,21] and is involved in cell proliferation, migration, and differentiation.[22] Increased activation of the canonical Wnt/β-catenin pathway is reportedly associated with catabolic effects in IVD tissue.[23] This could be owing to the increased expression of some MMPs that contribute to the degradation of extracellular matrix components within the disc.[24,25] In contrast, the Wnt/β-catenin pathway influences anabolic processes, such as the synthesis of extracellular matrix components like proteoglycans and collagens, for maintaining disc health.[26]

In the present study, the expression of ADAMTS5, MMP1, and TIMP1 significantly increased in PMA-treated human NP cells. MMPs play a key role in the decomposition of extracellular matrix, and specifically, ADAMTS5 degrades aggrecan.[27] The unaltered expression of total GAG, sulfate GAG, and collagen type II despite the expression of MMP1 and ADAMTS5 might be owing to high TIMP1 expression.

The TRPV4 expression confirmed the increased β-catenin expression and phosphorylation. TRPV4 expression increased by PMA treatment of human NP cells. TRPV4 is a calcium-permeable ion channel that plays multifaceted roles in NP cells, including mechanotransduction, osmoregulation, calcium signaling, inflammation, and pain signaling.[28] TRPV4 activation occurs by sustained physiological compression in NP cells with NF-κB signaling and IL-6 production.[15] Agonist-mediated TRPV4 activation alleviates disc degeneration and matrix disorganization.[29]

The levels of IL-6 and PGE_2 were analyzed to determine whether TRPV4 is involved in the inflammatory response in PMA-treated cells. Expression of IL-6 and PGE_2 increased by PMA treatment; however, IL-1β expression was not detected. In vertebral disc cells, PGE_2 and IL-6 are associated with proinflammatory and anti-inflammatory effects. These cytokines can promote inflammation by

increasing the production of proinflammatory cytokines, such as IL-1β and tumor necrosis factor-α, and by enhancing the expression of MMPs that contribute to tissue degradation in disc degeneration or herniation. In contrast, these cytokines can exert anti-inflammatory effects by inhibiting the production of proinflammatory cytokines in IVD cells.[30] Our finding indicates that IL-6 and PGE_2 may exert protective effects on IVD cells by increasing TIMP1 expression and not by reducing the expression of MMPs such as MMP1 or ADAMTS5. In the present study, IL-6 and PGE_2 exerted protective effects on IVD cells as we observed that the amount of total GAG and chondroitin-4 sulfate GAG were unaltered. Therefore, IL-6 and PGE_2 may affect the modulation of the extracellular matrix of IVD cells. IL-6 and PGE_2 may exert anti-inflammatory effects in human NP cells treated with PMA.

The expression and phosphorylation of NF-κB in the cytosol and nucleus were assessed to analyze its correlation with IL-6 and PGE_2 expression. However, the role of NF-κB could not be determined in this study, and further studies are needed to assess whether NF-κB canonically acts on the initiation of a regenerative response during tissue repair. Furthermore, in future studies, we will endeavor to further elucidate the relationship between PMA and NP cell senescence by confirming the expression of SA-β-gal staining and the expression of p53, p21, and p16. Future research could further investigate genes or proteins that, like the AQP1 gene, are involved in Wnt/β-catenin signaling and may contribute to the degradation of NP cells. In conclusion, the effects of PMA on human NP cells were not exactly similar to those on rat NP cells. Therefore, PMA cannot be utilized to prevent the degeneration of human NP cells.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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SUPPLEMENTARY MATERIAL

Supplementary material is available in the electronic version of this paper at the CiOS website, www.ecios.org.

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