

## Activin suppresses the expression of inflammatory genes and signaling proteins in human leukemia monocytic THP-1 cells

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### ABSTRACT

Activin regulates inflammation, cell proliferation, immune response, wound repair, and endocrine function. In this study, we investigated the effect of activin on inflammatory genes in THP-1 cells and the involvement of NF- $\kappa$ B, AKT, and mitogen-activated protein kinase (MAPK) signaling. Cell viability was determined using a colorimetric assay with the MTS/PES solution. The mRNA levels were analyzed using reverse transcription-quantitative polymerase chain reaction. The expression of NF- $\kappa$ B, AKT, and MAPK signaling proteins was measured using immunoblot analysis. Activin A did not affect THP-1 cell viability at concentrations below 50 ng/ml. Activin decreased the mRNA expression of cytokines (interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), toll-like receptor 4 (TLR4), and matrix metallo-proteinases (MMP)-9 proteins but did not affect IL-8 expression. Activin increased the expression of TLR2 and MMP-2. In addition, activin inhibited the phosphorylation of NF- $\kappa$ B p65, AKT, and MAPK (c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK) signaling proteins. Our results suggest that activin may be involved in anti-inflammation by inhibiting inflammatory gene expression and regulating NF- $\kappa$ B and AKT/MAPK signaling.

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### Introduction

Activin and inhibin are closely related protein complexes that have different biological effects. Inhibin suppresses follicle-stimulating hormone (FSH) synthesis and secretion. Activin promotes FSH biosynthesis and secretion regulating the menstrual cycle (1). Activin is a dimer that consists of two inhibin  $\beta$  subunits. Four  $\beta$ -subunits ( $\beta$ A,  $\beta$ B,  $\beta$ C, and  $\beta$ E) have been identified in mammals;  $\beta$ A and  $\beta$ B are primarily expressed in all tissues (2, 3). Activin receptors are transmembrane serine/threonine kinases that include type II ligand-coupled and type I signal transduction receptors. Activin phosphorylates these receptors by first binding to type II and subsequently to type I (4). Type I receptors then phosphorylate Smad2 or Smad3 or both. Phosphorylated R-Smad forms a complex with Smad4 and is translocated to the nucleus (5).

Activin belongs to the transforming growth factor- $\beta$  (TGF- $\beta$ ) family and regulates several functions, including cell proliferation, differentiation, apoptosis, metabolism, homeostasis, immune response, wound repair, and endocrine functions (6). The mRNA expression of cytokines, toll-like receptor (TLR), and nitric oxide is stimulated by lipopolysaccharide (LPS) and suppressed by activin. Blocking the action of activin using follistatin has shown significant therapeutic potential in reducing the severity of inflammatory diseases (7). Activin and its receptors are co-expressed in human adrenocortical cells (8). Additionally, activin functions as an anti-inflammatory cytokine

by regulating the production of mature IL-1 $\beta$  and IL-1 receptors at inflammation sites. Furthermore, activin inhibits the release of pro-inflammatory cytokines, suggesting that it plays an important role in the inflammatory response (9). Studies on the effects of activin on the expression of inflammatory genes in human monocytic leukemia THP-1 cells are unclear.

We aimed to investigate the effect of activin on inflammatory genes in THP-1 cells under basal conditions without inducing inflammation and with the involvement of NF- $\kappa$ B, AKT, and mitogen-activated protein kinase (MAPK) signaling.

### Materials and Methods

#### Cell culture

Human monocytic leukemia THP-1 cells were supplied by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI 1640 medium (Corning; Mediatech, Inc. Manassas, VA, USA) containing 10% fetal bovine serum and antibiotic-antimycotic solution (Gibco; Life Technologies Corp., Grand Island, NY, USA). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> in 95% air. Cells were treated with 100 nM phorbol myristate acetate (PMA, Sigma-Aldrich Co., St Louis, MO, USA) for 24 h to induce their differentiation into macrophages. Subsequently, the non-adherent cells were removed using aspiration. Adherent macrophages were washed three times with RPMI 1640 medium. Sub-

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**Table 1.** Primers used for real-time PCR amplification.

Genes	Primer sequences	Product size (bp)	Annealing Temperature (°C)
IL-1 $\beta$	5'-TGATGGCTTATTACAGTGGCAATG-3' 5'-GTAGTGGTGGTCGGAGATTTCG-3'	140	60
IL-6	5'-GTGTTGCCTGCTGCCTTC-3' 5'-AGTGCCTCTTTGCTGCTTTC-3'	194	60
IL-8	5'-GACATACTCCAAACCTTTCCAC-3' 5'-CTTCTCCACAACCCTCTGC-3'	160	60
TNF- $\alpha$	5'-GAACCAAGACCCAGACATC-3' 5'-CATTCTTCACCTGCTCCAC-3'	137	60
TLR2	5'-TCTCCCATTTCCGTCTTTTT-3' 5'-GGTCTTGGTGTTCATTATCTTC-3'	125	60
TLR4	5'-GAAGCTGGTGGCTGTGGA-3' 5'-TGATGTAGAACCCGCAAG-3'	213	60
NOD1	5'-TGATGCTGTTTCTGCCTCTC-3' 5'-AATTTGACCCCTGCGTCTAG-3'	374	56
NOD2	5'-AGCCATTGTCAGGAGGCTC-3' 5'-CGTCTCTGCTCCATCATAGG-3'	319	56
$\beta$ -actin	5'-GCGAGAAGATGACCCAGATC-3' 5'-GGATAGCACAGCCTGGATAG-3'	77	60

PCR, polymerase chain reaction; IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor; NOD, nucleotide-binding oligomerization domain.

sequently, the washed macrophages were incubated in a cell culture medium at 37 °C.

#### Activin A treatment

THP-1 cells were incubated in a serum-free medium with activin A (0, 2, 5, or 10 ng/ml; R&D Systems, Minneapolis, MN, USA) for 24 h. At each time point, total RNA and protein were isolated from cultured THP-1 cells.

#### Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 Aqueous One Solution (Promega Corporation, Madison, WI, USA). Cells were seeded at a density of  $2 \times 10^4$  cells/well in 96-well plates and incubated with activin A for 24 h. Cell viability was determined using the minoxidil topical solution (MTS) colorimetric assay. The absorbance was measured at 492 nm using background subtraction at 650 nm.

#### RNA isolation and RT-qPCR

Total RNA was purified from cultured cells using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) following the manufacturer's protocol. cDNA was transcribed from 1  $\mu$ g of total RNA using a RevertAid First Strand cDNA synthesis kit with Oligo (dT)<sub>18</sub> (Thermo Fisher Scientific Inc., Vilnius, Lithuania), according to the manufacturer's protocol. The primer sequences used are listed in Table 1. Quantitative polymerase chain reaction (qPCR) was performed using a StepOnePlus real-time PCR system with Power SYBRGreen PCR Master Mix (Applied Biosystems, Foster, CA, USA). The PCR mixture consisted of 1  $\mu$ l cDNA in 20  $\mu$ l reaction mixture, 10  $\mu$ l Power SYBR Green PCR Master Mix, 2  $\mu$ l primers, and 7  $\mu$ l PCR-grade water. The reactions were performed with a denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The crossing point of target genes with  $\beta$ -actin was calculated using the formula  $2^{-(\text{target}^{\text{gene}} - \beta\text{-actin})}$ . The relative amounts were quantified.

#### Immunoblot analysis

Cells were washed with cold phosphate-buffered saline (PBS) and lysed using a buffer (Cell Signaling Technology, Inc.) containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The protein concentration was determined using the bicinchoninic acid protein assay. Proteins (10  $\mu$ g) were separated using 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and subsequently incubated overnight at 4 °C with antibodies against matrix metalloproteinases (MMP)-2, MMP-9, NF- $\kappa$ B, AKT, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, and  $\beta$ -actin (Cell Signaling Technology, Inc.) at a 1:1000 dilution. After washing with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 1 h, the membranes were incubated for 1 h at room temperature with anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T (1:2,500). The membranes were subsequently washed with TBS-T for 1 h; proteins were detected using Amersham ECL prime western blotting detection reagent (GE Healthcare Life Sciences, UK). Protein levels were analyzed using Amersham Imager 600 (GE Healthcare Bio-Sciences, UK). Protein band densities were measured using the ImageJ software (version 1.44; National Institutes of Health, Bethesda, MA, USA).

#### Statistical analyses

Data are expressed as the mean  $\pm$  standard error. Data were compared using a one-way analysis of variance and Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). \* $p < 0.05$  and \*\* $p < 0.01$  indicated statistical significance.

#### Results

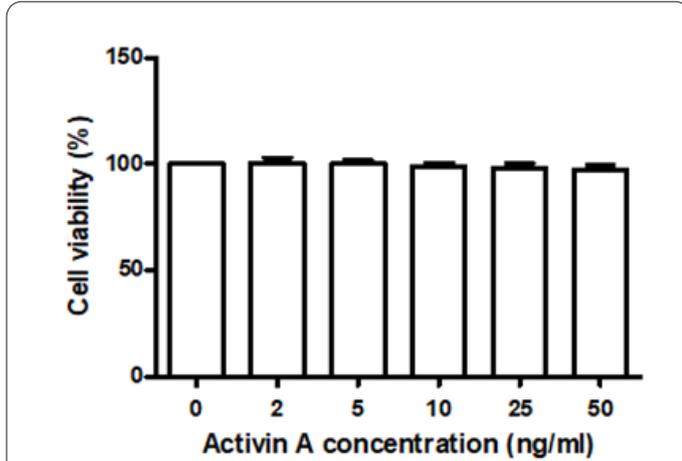
##### Effect of activin A on cell proliferation

THP-1 cells were treated with various concentrations

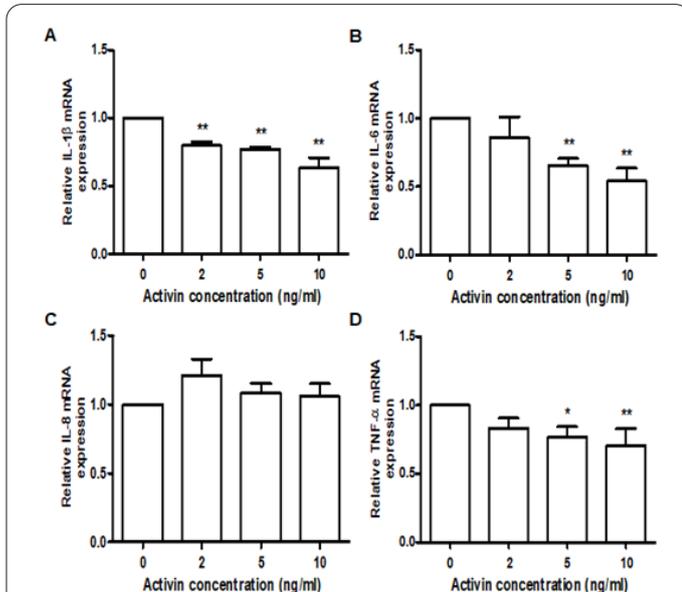
of activin A (0–50 ng/ml) for 24 h. Cell viability was measured using an MTS colorimetric assay. Activin A did not affect cell viability at concentrations below 50 ng/ml (Figure 1).

### Effect of activin A on cytokine mRNA expression

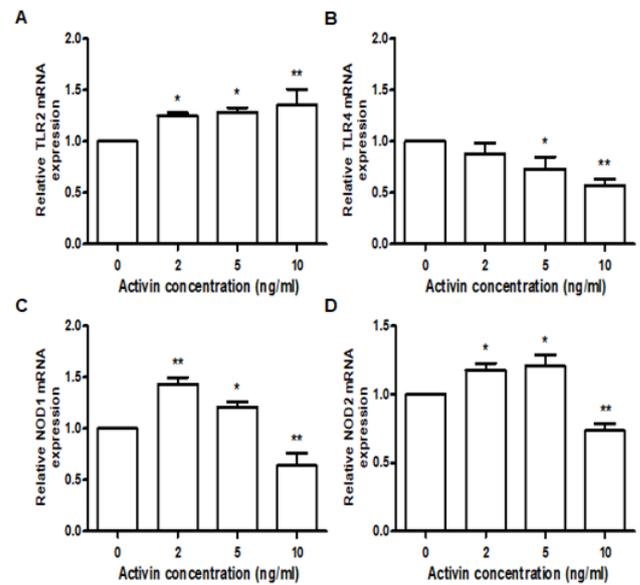
We evaluated whether activin affects the mRNA transcription of inflammatory cytokines. THP-1 cells were treated with various concentrations (0, 2, 5, and 10 ng/ml) of activin A for 24 h. The mRNA levels of cytokines (IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ) were determined using qPCR. Activin A significantly decreased the mRNA expression of cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) compared with that in the control. IL-8 mRNA expression increased at low activin A doses but decreased at high doses (Figures 2A–D).



**Figure 1.** Effect of activin on THP-1 cell growth. THP-1 cells were treated with various concentrations of activin A (0–50 ng/ml) for 24 h. Cell viability was determined using the MTS/PES solution. The data are shown as mean  $\pm$  SD of the triplicate samples.



**Figure 2.** Effect of activin on cytokine mRNA expression in THP-1 cells. Cells were treated with various concentrations of activin A (0, 2, 5, and 10 ng/ml) for 24 h. mRNA levels were measured using quantitative polymerase chain reaction (qPCR). The crossing point of cytokines interleukin (IL)-1 $\beta$  (A), IL-6 (B), IL-8 (C), and tumor necrosis factor (TNF)- $\alpha$  (D) with  $\beta$ -actin was entered into the formula:  $2^{-(\text{target gene} - \beta \text{ actin})}$ ; the relative amounts were quantified. The data represent the mean  $\pm$  SD of three independent samples. \* $p < 0.05$  and \*\* $p < 0.01$ , compared with that of the control.



**Figure 3.** Effect of activin on the expression of toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs) mRNA in THP-1 cells. Cells were treated with various concentrations of activin A (0, 2, 5, and 10 ng/ml) for 24 h. mRNA levels were measured using qPCR. The crossing point of TLR2 (A), TLR4 (B), NOD1 (C), and NOD2 (D) with  $\beta$ -actin was entered into the formula:  $2^{-(\text{target gene} - \beta \text{ actin})}$ ; the relative amounts were quantified. The data represent the mean  $\pm$  SD of three independent samples. \* $p < 0.05$  and \*\* $p < 0.01$ , compared with that of the control.

### Effects of activin A on TLR and nucleotide-binding oligomerization domain (NOD) mRNA expression

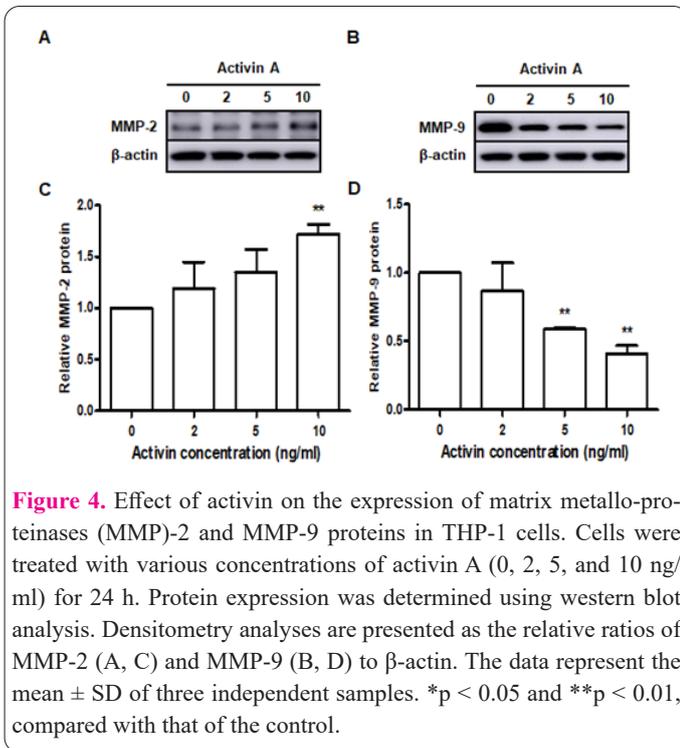
We evaluated whether activin affected TLR and NOD mRNA transcription using qPCR. THP-1 cells were treated with activin as described in the Methods section. Activin A reduced TLR4, NOD1, and NOD2 mRNA expression compared with that in the control and activated TLR2 mRNA expression. (Figures 3A–D).

### Effect of activin A on MMP-2 and MMP-9 protein expression

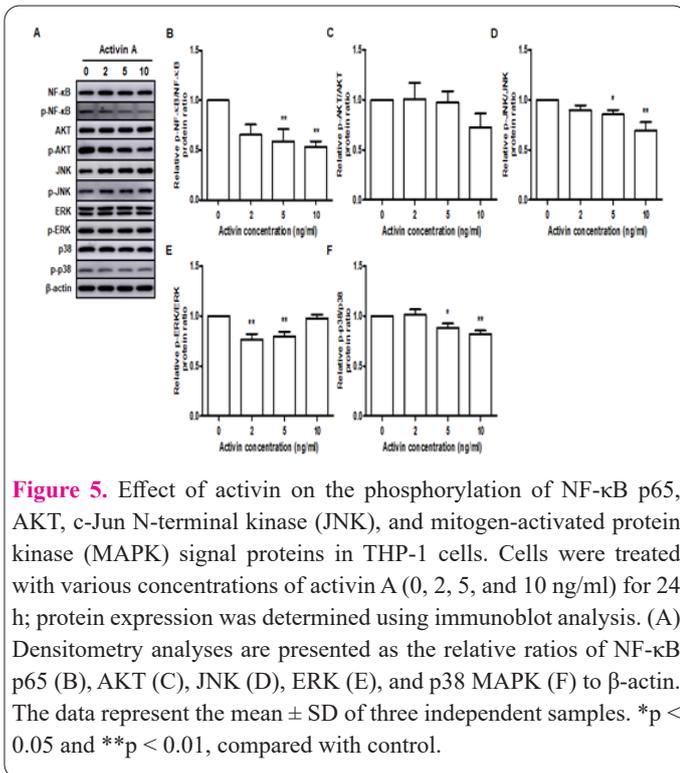
We examined the effects of activin A on MMP-2 and MMP-9 protein expression in THP-1 cells. Cells were treated with activin as described in the Methods section; protein expression was measured using immunoblot analysis. Activin A suppressed the expression of MMP-9 compared with that in control cells (Figures 4A and C). In contrast, activin A increased MMP-2 protein expression (Figures 4B and D).

### Effect of activin A on NF- $\kappa$ B, AKT, and MAPK signal phosphorylation

We evaluated the phosphorylation of signal transduction molecules by activin in relation to inflammation. THP-1 cells were treated with activin, as described in the Methods section. The protein expression levels of NF- $\kappa$ B, AKT, and MAPK (JNK, ERK, and p38) were analyzed using immunoblotting. Activin A suppressed the phosphorylation of NF- $\kappa$ B, AKT, c-JNK, and p38 MAPK signaling proteins compared with the control. ERK levels decreased at low activin doses but returned to control levels at higher doses (Figures 5A–F).



**Figure 4.** Effect of activin on the expression of matrix metallo-proteinases (MMP)-2 and MMP-9 proteins in THP-1 cells. Cells were treated with various concentrations of activin A (0, 2, 5, and 10 ng/ml) for 24 h. Protein expression was determined using western blot analysis. Densitometry analyses are presented as the relative ratios of MMP-2 (A, C) and MMP-9 (B, D) to  $\beta$ -actin. The data represent the mean  $\pm$  SD of three independent samples. \* $p < 0.05$  and \*\* $p < 0.01$ , compared with that of the control.



**Figure 5.** Effect of activin on the phosphorylation of NF- $\kappa$ B p65, AKT, c-Jun N-terminal kinase (JNK), and mitogen-activated protein kinase (MAPK) signal proteins in THP-1 cells. Cells were treated with various concentrations of activin A (0, 2, 5, and 10 ng/ml) for 24 h; protein expression was determined using immunoblot analysis. (A) Densitometry analyses are presented as the relative ratios of NF- $\kappa$ B p65 (B), AKT (C), JNK (D), ERK (E), and p38 MAPK (F) to  $\beta$ -actin. The data represent the mean  $\pm$  SD of three independent samples. \* $p < 0.05$  and \*\* $p < 0.01$ , compared with control.

## Discussion

Activin regulates several aspects of the inflammatory response, including the release of pro-inflammatory cytokines, nitric oxide production, and immune cell activity (10). However, the effect of activin on THP-1 cells remains unclear. We examined the effect of activin on inflammatory gene expression in THP-1 cells and THP-1 cell proliferation without inducing an inflammatory response. Cytotoxicity was not observed at 10 ng/ml when THP-1 cells were treated with activin at several concentrations for 24 h. Therefore, activin concentration was determined to be appropriate for our experiments.

In this study, we found that activin suppressed the mRNA expression of cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ )

but did not affect IL-8 expression. These results indicate that activin regulated the transcription of inflammatory cytokines and acted as an anti-inflammatory agent in THP-1 cells. Activin A induced the expression of tissue fibrosis-related factor  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and TGF- $\beta$ 1 release in L929 fibroblasts but did not affect pro-inflammatory IL-6 production (11). Phytohemagglutinin and LPS-induced inflammatory responses in neonatal peripheral blood lymphocytes significantly increased IL-1 $\beta$ , IL-6, CXCR8, and IL-10 production compared with that of unstimulated cells. Activin is an immunomodulatory agent that controls inflammatory responses in neonates with sepsis (12). Activin A has profound inhibitory effects on T- and B-cell functions and IL-1- and IL-6-mediated inflammatory responses (13).

TLRs are proteins that play important roles in innate immunity. They recognize pathogen-associated molecular patterns derived from microorganisms. They activated signal transduction pathways through adapter proteins, including TLRs, RIG-I-like receptors, and nucleotide-binding and oligomerization domain-like receptors (14, 15). In this study, we demonstrated that activin was regulated by TLR transcription in THP-1 cells. Activin inhibited the mRNA expression of TLR4 and enhanced the mRNA levels of TLR2. These results suggest that activin downregulated inflammation at the transcriptional level in TLR4 cells. Activin suppressed the expression of TLR4 in LPS-stimulated mouse peritoneal macrophages in vivo and in vitro, whereas no change was observed in the expression of TLR2 (16).

MMP expression is transcriptionally regulated by cell growth factors, hormones, cytokines, and cell transformation (17). MMP act on inflammation to regulate inflammatory cytokine and chemokine activity and production (18). Our results suggest that activin upregulates MMP-2 and downregulates MMP-9 in THP-1 cells. Stimulation of MMP-2 activity by activin A in TCam-2 cells suggests that activin affects TGCT by regulating the tumor niche (19). Activin stimulates MMP-2 expression in macrophages, with or without LPS. Conversely, MMP-9 production was affected (20). Activin enhances MMP-2 mRNA expression in L929 cells but does not affect MMP-9 mRNA expression (21).

The NF- $\kappa$ B pathway is activated by extracellular signaling factors and induces the expression of various pro-inflammatory genes, such as cytokines (22). MAPKs play an important regulatory role in the production of pro-inflammatory cytokines and are composed of the ERK, JNK, and p38 MAPK subfamilies, which are involved in gene regulation by transducing extracellular signals into the nucleus (23). We found that activin inhibited the phosphorylation of the NF- $\kappa$ B, AKT, JNK, and p38 MAPK signaling pathways in THP-1 cells. In contrast, ERK phosphorylation increased at low activin doses but returned to normal levels at high doses. Activin inhibits LPS-induced activation of NF- $\kappa$ B, MEK, ERK, and p38 MAPK phosphorylation in normal melanocytes (24). When treatment with activin, phosphorylation of MEK, ERK, and PI3K/AKT signaling pathways was not induced in simian virus 40 large T antigen-immortalized human granulosa cells (25). Our results suggest that activin may regulate the phosphorylation of NF- $\kappa$ B, AKT, JNK, and MAPK without inducing inflammation. The overall results of our study revealed that activin regulates anti-inflammatory effects via the

modulation of MAPK phosphorylation and inactivation of NF- $\kappa$ B in THP-1 cells.

In conclusion, our results demonstrated that activin inhibits the expression of inflammatory genes, including cytokines, in THP-1 cells. Thus, activin regulates inflammatory response genes via inhibiting NF- $\kappa$ B and AKT/JNK/MAPK signaling.

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### Interest conflict

The authors declare no competing interests.

### Author's contribution

Hyung Joo Ahn contributed to the conception of the study. Sang Oh Yun and Young Il Kim performed the experiment and manuscript preparation and contributed to data analysis. All authors have read and approved this version of the article.

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