Activin suppresses LPS-induced Toll-like receptor, cytokine and inducible nitric oxide synthase expression in normal human melanocytes by inhibiting NF-κB and MAPK pathway activation

YOUNG IL KIM[1], SEUNG-WON PARK[2], IN JUNG KANG[3], MIN KYUNG SHIN[3] and MU-HYOUNG LEE[3]

[1]Medical Science Research Institute, Kyung Hee University Medical Center and East-West Medical Research Institute, Kyung Hee University, Seoul 130-872; [2]Department of Biotechnology, Catholic University of Daegu, Daegu 712-702; [3]Department of Dermatology, College of Medicine, Kyung Hee University, Seoul 130-872, Republic of Korea

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Abstract. Activins are dimeric growth and differentiation factors that belong to the transforming growth factor (TGF)-β superfamily of structurally related signaling proteins. In the present study, we examined the mechanisms through which activin regulates the lipopolysaccharide (LPS)-induced transcription of Toll-like receptors (TLRs), cytokines and inducible nitric oxide synthase (iNOS) in human melanocytes, as well as the involvement of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling. Cell proliferation was analyzed by cell viability assay, mRNA expression was detected by RT-qPCR, and protein expression was measured by western blot analysis. LPS increased the mRNA expression of TLRs (TLR1-10) and cytokines [interleukin (IL)-1β, IL-6, IL-8 and TNF-α], as well as the mRNA and protein expression of iNOS. Activin decreased the LPS-induced TLR and cytokine mRNA expression, as well as the LPS-induced iNOS mRNA and protein expression. In addition, activin suppressed NF-κB p65 activation and blocked inhibitor of NF-κB (IκBα) degradation in LPS-stimulated melanocytes, and reduced LPS-induced p38 MAPK and MEK/ERK activation. On the whole, our results demonstrated that activin inhibited TLR and cytokine expression in LPS-activated normal human melanocytes and suppressed LPS-induced iNOS gene expression. Moreover, the anti-inflammatory effects of activin were shown to be mediated through the suppression of NF-κB and MAPK signaling, resulting in reduced TLR and iNOS expression, and in the inhibition of inflammatory cytokine expression.

Correspondence to: Dr Mu-Hyoung Lee, Department of Dermatology, College of Medicine, Kyung Hee University, 23 Kyungheedae-ro, Dongdaemun-gu, Seoul 130-872, Republic of Korea
E-mail: mhlee@khmc.or.kr

Abbreviations: IκB, inhibitors of NF-κB; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TLR, Toll-like receptor

Key words: normal human melanocytes, activin A, Toll-like receptor, cytokine, inducible nitric oxide synthase, nuclear factor-κB

Introduction

Inflammation is a multifaceted process involving changes at the cellular, tissue and systemic levels and it is coordinated through a complex network of cytokine pathways. Many of the key cytokines that are involved in inflammatory processes have been identified and their functions determined. Research is, however, continuing into the activation of the intracellular signaling pathways that are activated and the interactions between immunoregulatory factors and other cytokines and growth factors (1).

Pathogen recognition by innate immune cells is mediated by pattern recognition receptors that recognize conserved pathogen-associated molecular patterns. Humans have various pattern recognition receptors, which include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-1-like receptors (2,3). These receptors transduce signals leading to the activation of nuclear factor (NF)-κB, which subsequently drives the induction of several pro-inflammatory cytokines and chemokines (4-6). TLRs play a major role in microbial detection. The regulation of various genes, encoding inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, IL-12 and tumor necrosis factor (TNF)-α occurs through TLRs on macrophages and other immunocompetent cells (7). NF-κB is a key regulator of pro-inflammatory gene expression. In unstimulated cells, NF-κB is retained in the cytoplasm by binding to a family of inhibitory proteins, the inhibitors of NF-κB (IκB). Upon cell stimulation, the phosphorylation and degradation of IκBα leads to the translocation of free NF-κB to the nucleus (8,9). Nitric oxide (NO) is produced from L-arginine by the action of the enzyme nitric oxide synthase (NOS). Several isoforms of the enzyme exist, the most important of which are the constitutive form (cNOS), which is present in endothelial cells and neurons, and inducible NO synthase (iNOS), which is found in a variety of cells, including macrophages and neutrophils. The latter is not normally expressed, but it is induced by inflammatory cytokines and bacterial lipopolysaccharide (LPS) (10,11).

Activins are dimeric growth and differentiation factors that belong to the transforming growth factor (TGF)-β superfamily of structurally related signaling proteins. Activins are either heterodimers or homodimers of inhibin β subunits (βAβA, βBβB or βAβB). Biological signaling by activins is mediated by receptor complexes consisting of two different activin serine/threonine kinase receptors (ActRs): type I (ActR-I) and type II (ActR-II). Activin-responsive genes have been implicated in the control of homeostasis, development, proliferation, apoptosis, differentiation and inflammation in diverse cellular systems (12,13). Activin produced by microglia acts as an anti-inflammatory cytokine, presumably modulating inflammation in an autocrine manner (14). Activin A decreases the production of inflammatory factors and phagocytosis in activated macrophages by suppressing the maturation of LPS-stimulated macrophages or LPS-TLR4 signal transduction (15).

Human melanocytes are not merely pigment-producing cells; they also act as phagocytes that contribute to inflammatory responses (16), and secrete agents of a wide range of signaling molecules, including cytokines, pro-opiomelanocortin (POMC) peptides, catecholamines and NO in response to ultraviolet (UV) irradiation and other stimuli. Potential targets of these secretory products are keratinocytes, lymphocytes, fibroblasts, mast cells and endothelial cells, all of which express receptors for these signaling molecules (17). However, the regulatory effects of activin A on normal human melanocytes as anti-inflammatory factors remain unclear.

In this study, we examined the mechanisms through which activin regulates the LPS-induced transcription of TLRs, cytokines and NOS in normal human melanocytes, and its effects on NF-κB and mitogen-activated protein kinase (MAPK) signaling.

Materials and methods

Cell culture. Normal human melanocytes were purchased from Cascade Biologics (Gibco, Carlsbad, CA, USA) and cultured in Gibco Medium 254 containing human melanocyte growth supplement (Invitrogen, Grand Island, NY, USA). The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 in 95% air.

Cell viability assay. Cell proliferation was measured using CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA). The cells were seeded ($5x10^3$ cells/well) in 96-well plates and incubated with *Escherichia coli* LPS (*E. coli* 0111: B4; Sigma-Aldrich Co., St. Louis, MO, USA) and activin A (ProSpec (Protein-Specialists), East Brunswick, NJ, USA) for 24 h. Cell viability was determined by colorimetric assay using PMS/MTS solution. The absorbance was measured at 490 nm, with background subtraction at 650 nm.

Treatment with LPS and activin A. Normal human melanocytes were treated for 20 h with activin A (10 or 25 ng/ml) prior to exposure to LPS (1 μg/ml) for 4 h. At each time point, total RNA and protein were isolated from the cultured melanocytes.

RNA extraction and reverse transcription-quantitative-PCR (RT-qPCR). Total RNA was purified from the cultured cells using an RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). First-Strand cDNA synthesis was performed with 1 μg of total RNA, which was transcribed into cDNA using a reverse transcription system with random hexamers (Promega) according to the manufacturer's instructions. The primer sequences are listed in Table I. Quantitative PCR (qPCR) was performed on a StepOnePlus Real-Time PCR system with Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR was performed with

Table I. Primers used in RT-qPCR.

Gene	Primer sequences	Product length (bp)
TLR1	5'-GCCCAAGGAAAAGAGCAAAC-3' 5'-AAAGCAGCAATATCAACAGGAG-3'	135
TLR2	5'-TCTCCCATTTCCGTCTTTTT-3' 5'-GGTCTTGGTGTTCATTATCTTC-3'	125
TLR3	5'-TAAACTGAACCATGCACTCT-3' 5'-TATGACGAAAGGCACCTATC-3'	101
TLR4	5'-GAAGCTGGTGGCTGTGGA-3' 5'-TGATGTAGAACCCGCAAG-3'	213
TLR5	5'-TTGCTCAAACACCTGGACAC-3' 5'-CTGCTCACAAGACAAACGAT-3'	149
TLR6	5'-GTGCCATTACGAACTCTA-3' 5'-CTTGTTGGGAATGCTGTT-3'	109
TLR7	5'-CTGACCACTGTCCCTGAG-3' 5'-AACCCACCAGACAAACCA-3'	264
TLR8	5'-AACATCAGCAAGACCCAT-3' 5'-GACTCCTTCATTCTCCCT-3'	65
TLR9	5'-CGCCAACGCCCTCAAGACA-3' 5'-GGCGCTTACATCTAGTATTTGC-3'	79
TLR10	5'-CTCCCAACTTTGTCCAGAAT-3' 5'-TGGTGGGAATGCAATAGAAT-3'	132
IL-1β	5'-TGATGGCTTATTACAGTGGCAATG-3' 5'-GTAGTGGTGGTCGGAGATTCG-3'	140
IL-6	5'-GTGTTGCCTGCTGCCTTC-3' 5'-AGTGCCTCTTTGCTGCTTTC-3'	194
IL-8	5'-GACATACTCCAAACCTTTCCAC-3' 5'-CTTCTCCACAACCCTCTGC-3'	160
TNF-α	5'-ATCTTCTCGAACCCCGAGTG-3' 5'-GGGTTTGCTACAACATGGGC-3'	51
iNOS	5'-TGGATGCAACCCCATTGTC-3' 5'-CCCGCTGCCCCAGTTT-3'	59
β-actin	5'-GCGAGAAGATGACCCAGATC-3' 5'-GGATAGCACAGCCTGGATAG-3'	77

TLR, Toll-like receptor; IL, interleukin; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase.

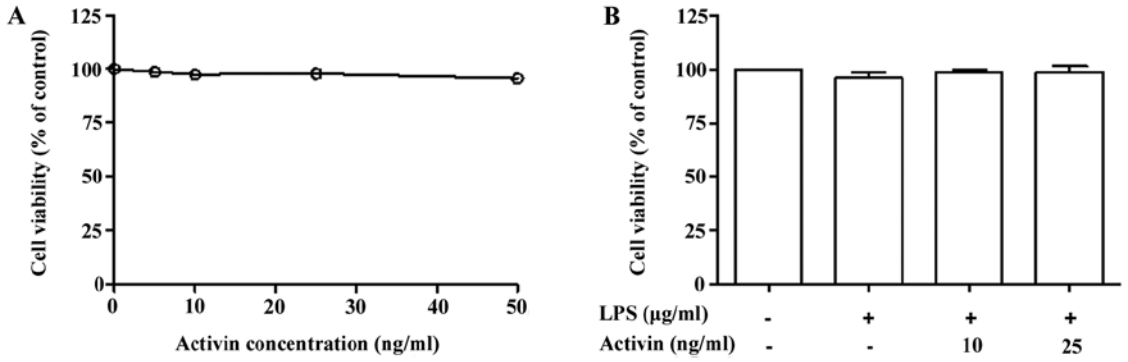

Figure 1. Effects of lipopolysaccharide (LPS) and activin on the proliferation of normal human melanocytes. Normal human melanocytes were treated with (A) various concentrations of activin A, and (B) a combination of LPS and activin A for 24 h. Cell viability was then determined by a PMS/MTS solution. The data represent the means ± SEM of 3 independent experiments repeated in triplicate.

1 μl of cDNA in a 20-μl reaction mixture containing 10 μl of Power SYBR-Green PCR Master Mix, 2 μl of primers, and 7 μl of PCR grade water. The reaction conditions were as follows: denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The crossing points of the target genes with β-actin were calculated using the formula $2^{-(\text{target gene - β-actin})}$ and the relative amounts were quantified.

Immunoblot analysis. The cells were collected and washed with cold PBS then lysed using lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin] containing 1 mM PMSF (Cell Signaling Technology, Inc., Boston, MA, USA). The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Protein (30 μg) were fractionated by 12% SDS-PAGE and transferred by electrophoresis onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then incubated overnight with antibodies against iNOS (AB5382), NF-κB p65 (#8242), phosphorylated (p-)NF-κB p65 (#3031), IκBα (#9242), p-IκBα (#9246), p38 MAPK (#9228), p-p38 MAPK (#9215), MEK (#4694), p-MEK (#9154), ERK (#4696) and p-ERK1/2 (#4376; Cell Signaling Technology) and β-actin (A5441; Sigma-Aldrich Co.), diluted 1:1,000 with Tris-buffered saline containing 0.05% Tween-20 (TBS-T). After washing with TBS-T for 1 h, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted 1:2,500 in TBS-T. The membranes were subsequently washed with TBS-T for 1 h, and proteins were detected using an Enhanced Chemiluminescence kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Protein expression was analyzed using a Davinch-Chemi™ Chemiluminescence Imaging system (Davinch-K Co., Ltd., Seoul, Korea).

Statistical analyses. The experiments were repeated 3 times independently. All values are expressed as the means ± SEM. Data were compared by a non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA). Values of $P<0.05$ and $P<0.01$ were considered to indicate statistically significant differences.

Results

Effects of activin on the proliferation of normal human melanocytes. Normal human melanocytes were treated with various concentrations of activin A (0-50 ng/ml) for 24 h and cell proliferation was examined by PMS/MTS solution. Activin did not influence the proliferation rate (cell viability) at concentrations of 10 and 25 ng/ml (Fig. 1A). Co-stimulation of the normal human melanocytes with LPS (1 μg/ml) and activin A (10 and 25 ng/ml) for 24 h did not produce any cytotoxic effects (Fig. 1B).

Activin inhibits the LPS-induced increase in the mRNA expression of TLRs in normal human melanocytes. To investigate the effects of activin on the expression of TLR1-10 in normal human melanocytes in the presence of LPS, the mRNA expression of TLR was measured by RT-qPCR. LPS increased the mRNA expression of TLR1-10 compared with the control. However, activin suppressed the LPS-induced increase in the mRNA expression of all TLRs except TLR3 (Fig. 2).

Activin inhibits the LPS-induced increase in the mRNA expression of cytokines in normal human melanocytes. We then determined whether activin A affects inflammatory cytokine mRNA transcription. The expression of cytokines was measured by RT-qPCR. LPS increased cytokine mRNA expression compared with the controls, while activin A suppressed the LPS-induced increase in the mRNA expression of IL-1β, IL-6, and IL-8. The decrease in the mRNA expression of TNF-α following the administration of activin A did not reach statistical significance (Fig. 3).

Activin inhibits the LPS-induced increase in the mRNA and protein expression of iNOS in normal human melanocytes. To examine the anti-inflammatory activity of activin, we examined its effects on the mRNA and protein expression of iNOS in the LPS-stimulated normal human melanocytes. The mRNA and protein expression of iNOS was measured by RT-qPCR and immunoblot analysis, respectively. LPS increased the mRNA and protein expression of iNOS compared with the controls. However, activin suppressed the LPS-induced increase in the mRNA and protein expression of iNOS (Fig. 4).

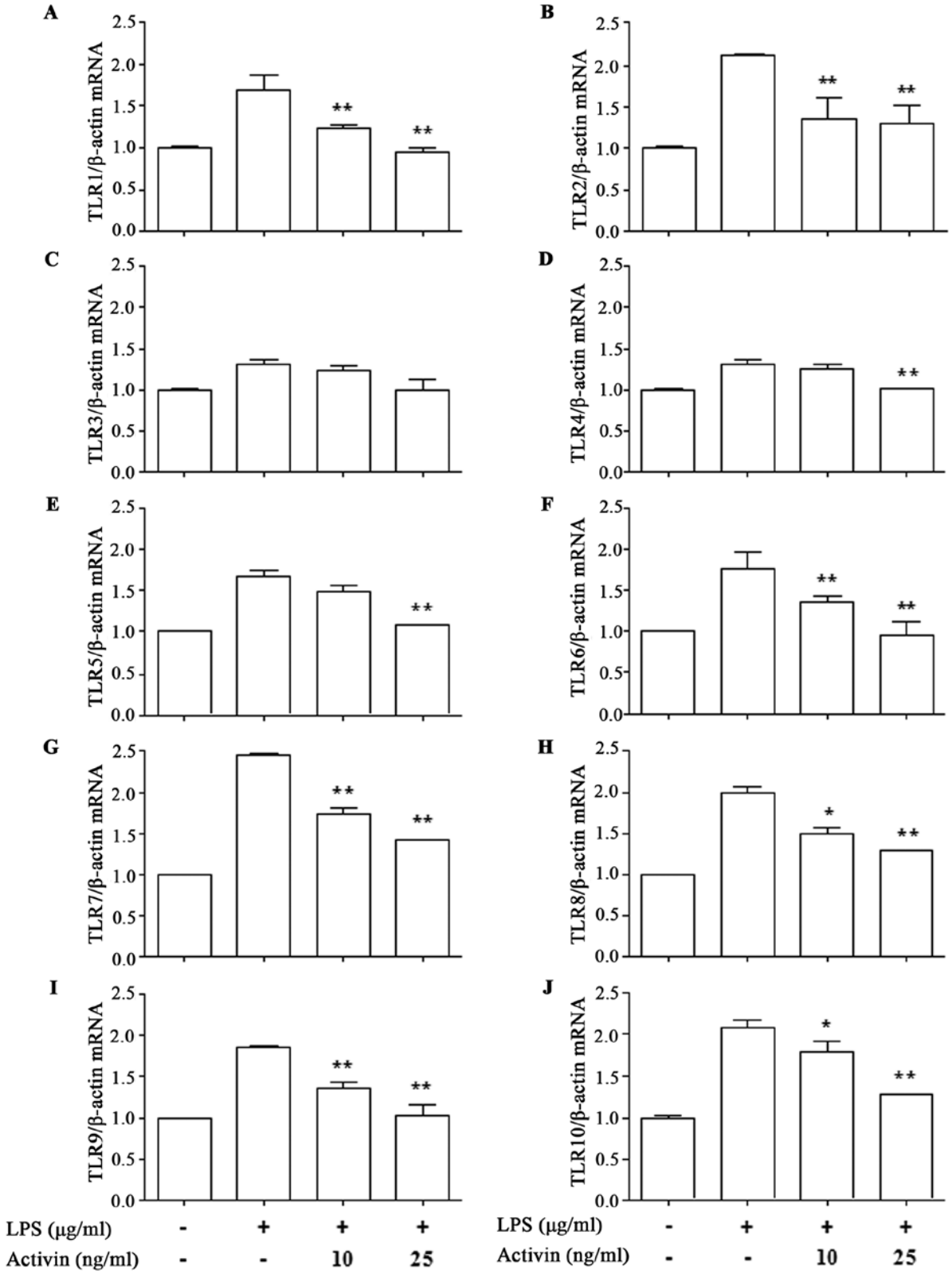

Figure 2. Effects of activin on the lipopolysaccharide (LPS)-induced increase in the mRNA expression of Toll-like receptors (TLRs) in normal human melanocytes. Normal human melanocytes were treated for 20 h with activin A (10 or 25 ng/ml) prior to exposure to LPS (1 μg/ml) for 4 h, and mRNA levels were measured by RT-qPCR (A-J). The crossing points of the TLRs with β-actin were entered into the formula $2^{-(\text{target gene - β-actin})}$, and relative amounts were quantified. The data represent the means ± SEM of 3 independent samples. *P<0.05 and **P<0.01 compared to stimulation with LPS alone.

Activin inhibits the LPS-induced activation of NF-κB signaling and IκBα degradation in normal human melanocytes. To investigate the phenomena involved in the inhibition of NF-κB activity, the effects of activin on IκBα degradation were examined. The NF-κB p65 and IκBα protein levels were measured by immunoblot analysis. LPS promoted the activa-

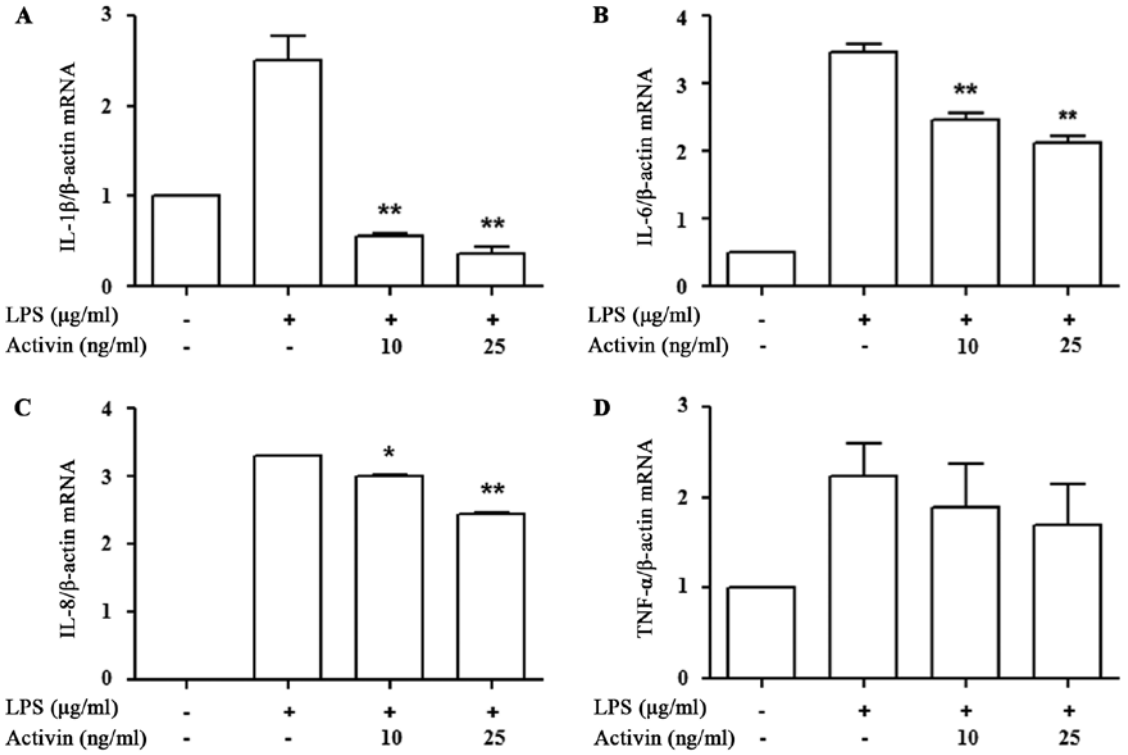

Figure 3. Effects of activin on the lipopolysaccharide (LPS)-induced increase in the mRNA expression of cytokines in normal human melanocytes. Normal human melanocytes were treated for 20 h with activin A (10 or 25 ng/ml) prior to exposure to LPS (1 μg/ml) for 4 h, and mRNA levels were measured by RT-qPCR. The crossing points of (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF-α with β-actin were entered into the formula $2^{-(\text{target gene - β-actin})}$, and relative amounts were quantified. The data represent the means ± SEM of 3 independent samples. *P<0.05 and **P<0.01 compared to stimulation with LPS alone.

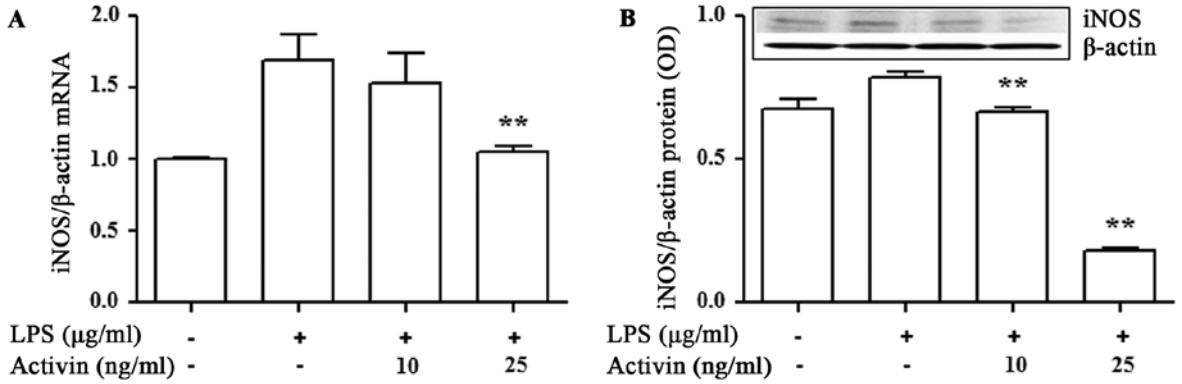

Figure 4. Effects of activin on the lipopolysaccharide (LPS)-induced increase in the mRNA and protein expression of inducible nitric oxide synthase (iNOS) in normal human melanocytes. Normal human melanocytes were treated for 20 h with activin A (10 or 25 ng/ml) prior to exposure to LPS (1 μg/ml) for 4 h. (A) mRNA levels of iNOS were measured by RT-qPCR. (B) The crossing points of iNOS with β-actin were entered into the formula $2^{-(\text{target gene - β-actin})}$, and relative amounts were quantified. iNOS protein expression was examined by immunoblot analysis. Densitometric analyses are presented as the relative ratios of iNOS and β-actin. The data represent the means ± SEM of 3 independent samples. **P<0.01 compared to stimulation with LPS alone.

tion of NF-κB p65 and IκBα compared with the controls. No phosphorylated IκBα was detected in the unstimulated normal human melanocytes. The LPS-induced phosphorylation of NF-κB and IκBα was inhibited by activin A (Fig. 5).

Activin inhibits the LPS-induced activation of p38 MAPK and MEK/ERK in normal human melanocytes. We then evaluated the activation of signaling molecules related to inflammation by activin. The expression levels of p38 MAPK, MEK and ERK were measured by immunoblot blot analysis. LPS promoted the activation of p38 MAPK, MEK and ERK compared with the controls, while activin inhibited the LPS-induced activation of p38 MAPK, MEK and ERK in normal human melanocytes (Fig. 6).

Discussion

Activin plays an important physiological role in cell differentiation and inflammation (18). However, its effects on normal human melanocytes remain unclear. In a previous study, LPS

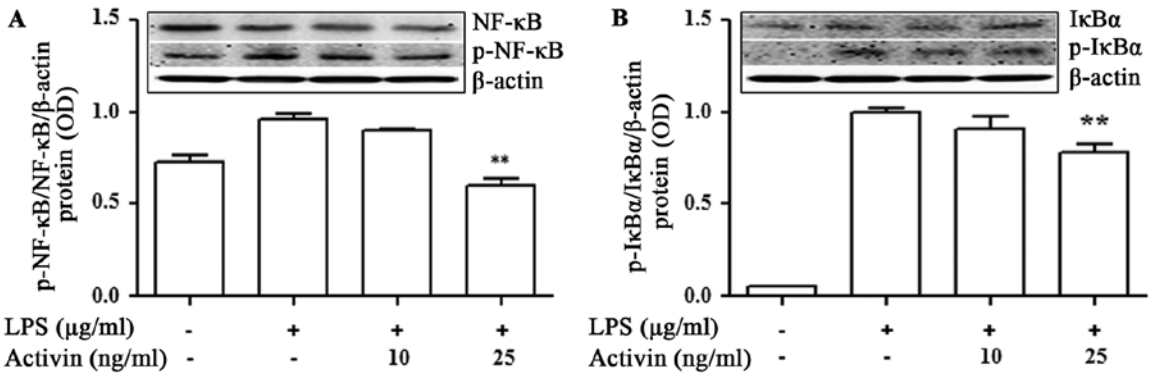

Figure 5. Effects of activin on the lipopolysaccharide (LPS)-induced phosphorylation of NF-κB p65 and IκBα degradation in normal human melanocytes. Normal human melanocytes were treated for 20 h with activin A (10 or 25 ng/ml) prior to exposure to LPS (1 μg/ml) for 4 h, and the protein expression of NF-κB p65 and IκBα was examined by immunoblot analysis. Densitometric analyses are presented as the relative ratios of (A) phosphorylated (p)-NF-κB p65/NF-κB p65 or (B) p-IκBα/IκBα and β-actin. The data represent the means ± SEM of 3 independent samples. **P<0.01 compared to stimulation with LPS alone.

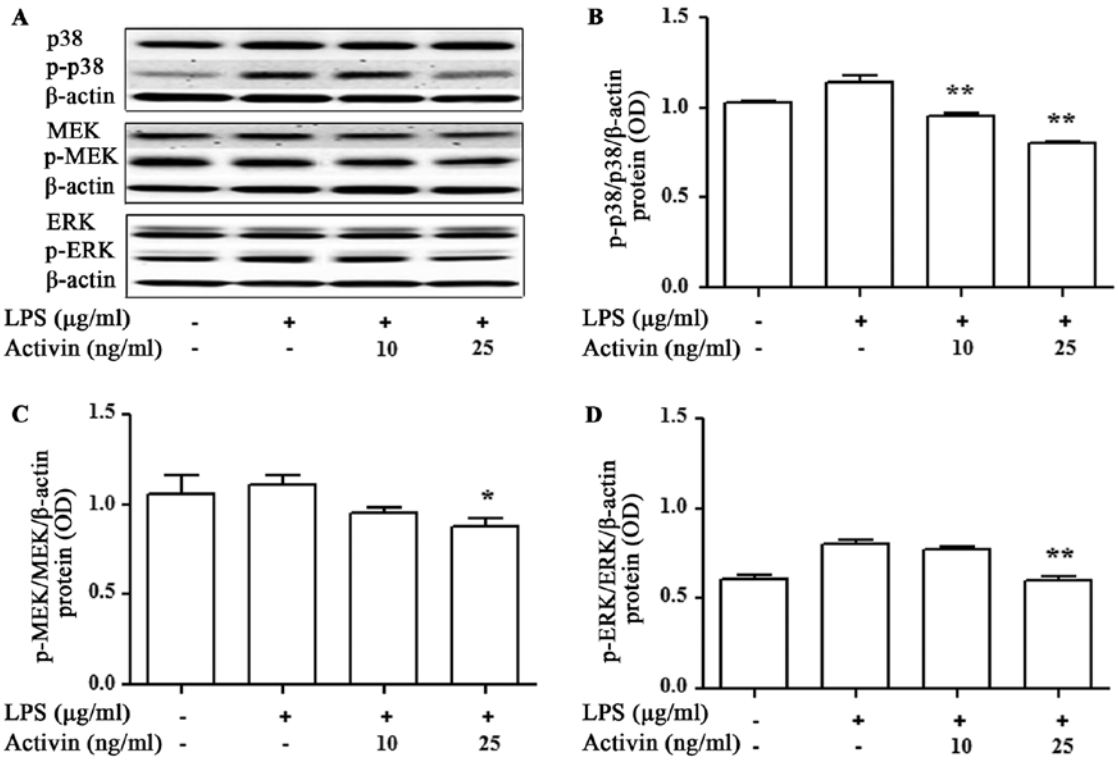

Figure 6. Effects of activin on the lipopolysaccharide (LPS)-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) and MEK/ERK in normal human melanocytes. (A) Normal human melanocytes were treated for 20 h with activin A (10 or 25 ng/ml) prior to exposure to LPS (1 μg/ml) for 4 h, and the protein expression of p38 MAPK, MEK and ERK was examined by immunoblot analysis. Densitometric analyses are presented as the relative ratios of (B) phosphorylated (p)-p38 MAPK/p38 MAPK, (C) p-MEK/ME, (D) p-ERK/ERK and β-actin. The data represent the means ± SEM of 3 independent samples. *P<0.05 and **P<0.01 compared to stimulation with LPS alone.

inhibited melanocyte proliferation in a dose-dependent manner when applied for 5 days (19). Activin (25 ng/ml) has been shown to suppress the growth of normal human melanocytes when applied for 4 days (20). In this study, we investigated the effects of LPS and activin A on the proliferation of normal human melanocytes. The cells stimulated with activin (10 and 25 ng/ml) for 24 h did not show any cytotoxicity. Therefore, these concentrations of activin were deemed to be appropriate for our experiments.

Humans have various pattern recognition receptors, including TLRs, NLRs and RIG-1-like receptors (21-23). Human melanocytes constitutively express mRNA and protein for TLRs 2-5, 7, 9 and 10 (19,24,25); however, the mRNA expression of NODs and RIG-1 in melanocytes is unclear. In the present study, we demonstrate that activin regulates TLR gene transcription in normal human melanocytes. Activin inhibited the LPS-induced mRNA expression of TLRs (except TLR3). These results suggest that activin plays a regulatory role in inflammation at the level of TLR transcription. However, to the best of our knowledge, the modulation of LPS-activated TLRs by activin has not been previously reported in normal human melanocytes.

Activin A acts as an anti-inflammatory cytokine produced by microglia and macrophages, and it is involved in regulation of the acute-phase response in inflammatory diseases, in an autocrine or paracrine manner (14,15). Activin was shown to significantly inhibit the LPS-induced production of IL-6, IL-18, iNOS and IL-1β-converting enzyme (ICE), *in vivo* and *in vitro* (14). In the present study, we found that activin suppressed the LPS-induced increase in the mRNA expression of IL-1β, IL-6 and IL-8, whereas the mRNA levels of TNF-α were not altered significantly. These results suggest that activin modulates the transcription of inflammatory cytokines in normal human melanocytes. In a previous study, no mRNA expression of IL-2, IL-3, IL-4, IL-9, IL-12p40, IFN-α, or IFN-γ was detected in human melanocytes (18).

Human melanocytes produce NO in response to UV radiation and bacterial LPS. NO is produced through the enzymatic action of NOS, of which both the constitutive (cNOS) and inducible (iNOS) isoforms exist. In the control unstimulated melanocytes, iNOS expression was detected, which suggests that, unlike in most cell types, low levels of iNOS are constitutively expressed in melanocytes (26). Normal dermal fibroblasts express both endothelial NOS (eNOS) and iNOS mRNA (27). Cultured normal human melanocytes have been shown to express iNOS when stimulated with LPS/cytokines (28). To determine whether activin exerts an inhibitory effect on iNOS production in melanocytes, we investigated its effects on iNOS mRNA expression in the presence of LPS. Our results revealed that, under these conditions, activin blocked the stimulatory effect of LPS on iNOS mRNA and protein expression. These findings suggest that activin inhibits iNOS at the transcriptional and translational levels in normal human melanocytes exposed to inflammatory stimuli, such as LPS.

NF-κB plays a crucial role in the expression of a number of the genes involved in immune and inflammatory responses (29). LPS has been shown to induce the nuclear translocation of NF-κB in human melanocytes (19). Activated NF-κB acts as a transcription factor that increases the expression of several inflammation-associated genes, including iNOS, COX-2, IL-1β, IL-6 and TNF-α (30). In this study, we observed that activin blocked the degradation of IκBα, suggesting that it attenuates the LPS-stimulated translocation of NF-κB in normal human melanocytes by blocking IκBα degradation. The phosphorylation and activation of 3 major MAPKs (p38 MAPK, JNK and ERK1/2) has been shown to initiate the expression inflammation-associated genes in LPS-stimulated macrophages. Activated p38 induces TNF-α and iNOS production by modulating NF-κB (31). It has been demonstrated that activin stimulates the production of IL-1β, IL-6 and TNF-α (32) by modulating IκB degradation, the nuclear translocation of NF-κB, and the phosphorylation of p38 MAPK and ERK1/2 (33,34). In this study, we found that activin inhibited the LPS-induced phosphorylation of p38 MAPK and MEK/ERK in normal human melanocytes. Taken together, our results demonstrated that in LPS-stimulated normal human melanocytes, activin exerted anti-inflammatory effects by modulating MAPK phosphorylation and inactivating NF-κB by blocking IκBα degradation.

In conclusion, the findings of the present study demonstrated that activin inhibited LPS-induced TLR, cytokine and iNOS expression in normal human melanocytes. Moreover, activin exerted anti-inflammatory effects on LPS-activated normal human melanocytes by modulating MAPK phosphorylation and NF-κB inactivation.

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