BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 22(4), 295-300 (2014)

Cytochalasin B Modulates Macrophage-Mediated Inflammatory Responses

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Abstract

The actin cytoskeleton plays an important role in macrophage-mediated inflammatory responses by modulating the activation of Src and subsequently inducing nuclear factor (NF)-κB translocation. In spite of its critical functions, few papers have examined how the actin cytoskeleton can be regulated by the activation of toll-like receptor (TLR). Therefore, in this study, we further characterized the biological value of the actin cytoskeleton in the functional activation of macrophages using an actin cytoskeleton disruptor, cytochalasin B (Cyto B), and explored the actin cytoskeleton's involvement in morphological changes, cellular attachment, and signaling events. Cyto B strongly suppressed the TLR4-mediated mRNA expression of inflammatory genes such as cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and inducible nitric oxide (iNOS), without altering cell viability. This compound also strongly suppressed the morphological changes induced by lipopolysaccharide (LPS), a TLR4 ligand. Cyto B also remarkably suppressed NO production under non-adherent conditions but not in an adherent environment. Cyto B did not block the co-localization between surface glycoprotein myeloid differentiation protein-2 (MD2), a LPS signaling glycoprotein, and the actin cytoskeleton under LPS conditions. Interestingly, Cyto B and PP2, a Src inhibitor, enhanced the phagocytic uptake of fluorescein isothiocyanate (FITC)-dextran. Finally, it was found that Cyto B blocked the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at 1 min and the phosphorylation of heat shock protein 27 (HSP27) at 5 min. Therefore, our data suggest that the actin cytoskeleton may be one of the key components involved in the control of TLR4-mediated inflammatory responses in macrophages.

Key Words: Actin cytoskeleton, Inflammation, Cytochalasin B, Macrophages, TLR4

INTRODUCTION

Inflammation is an important mechanism through which the host defense clears out infected materials. Macrophage cells are the primary regulators of this response (Seo *et al*., 2013). The macrophage-mediated reaction includes morphological changes, phagocytic uptake, and the secretion of inflammatory mediators [eg., nitric oxide (NO) and prostaglandin E_2 (PGE_2)] and pro-inflammatory cytokines [eg., tumor necrosis factor (TNF)-α] (Jeon *et al*., 2013). For these responses, macrophages require the activation of surface receptors [eg., pattern recognition receptor (PPR)], intracellular signaling cascades, and transcription factors [eg., nuclear factor (NF)-κB] to stimulate the expression of corresponding genes such as inducible nitric oxide synthase (iNOS), TNF-α, and cyclooxygenase (COX)-2 (Malyshev and Shnyra, 2003; Qureshi *et al*., 2005).

The importance of the actin cytoskeleton in macrophage-mediated inflammatory responses has been previously proposed (Kim *et al*., 2010; Diesel *et al*., 2013), in addition to other biological actions including modulating cell morphology (e.g., fillopodia), villi formation, cell division via contractile ring formation, cell migration, and intracellular signaling pathways (Disanza *et al*., 2005; Kustermans *et al*., 2008). Thus, fully

Open Access **http://dx.doi.org/10.4062/biomolther.2014.055**

Received May 12, 2014 **Revised** Jun 9, 2014 **Accepted** Jun 10, 2014
Published online Jul 31, 2014

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activated macrophages need flexible structural changes to engulf infected materials and to digest these in intracellular compartments. Indeed, the blockade of the actin cytoskeleton has been reported to suppress macrophage-mediated inflammatory responses (Kim *et al*., 2010). By exploring intracellular signaling events, it was found that Src activation is targeted by actin polymerization (Kim *et al*., 2010). Inhibition of Src in toll-like receptor 4 (TLR4) signaling events was linked to the suppression of NF-κB. In spite of these studies, it is not known which proteins act as linkers between actin polymerization and Src activation. Moreover, there is not yet sufficient evidence elucidating the involvement of the actin cytoskeleton in the inflammatory role of macrophages. Therefore, in this study, we further characterized the role of the actin cytoskeleton in modulating the inflammatory activation of macrophages by using cytochalasin B (Cyto B, Fig. 1), an actin polymerization inhibitor (Sutovsky *et al*., 1994).

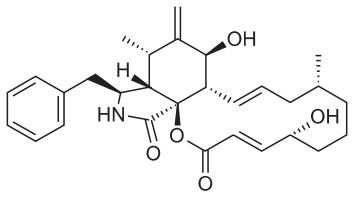

Fig. 1. The chemical structure of cytochalasin B (Cyto B).

Table 1. Primers used for quantitative real-time RT-PCR analysis

Gene		Primer sequences
TNF-α	F	5'-TGCCTATGTCTCAGCCTCTTC-3'
	R	5'-GAGGCCATTTGGGAACTTCT-3'
COX-2	F	5'-GGGAGTCTGGAACATTGTGAA-3'
	R	5'-GCACATTGTAAGTAGGTGGACTGT-3'
iNOS	F	5'-GGAGCCTTTAGACCTCAACAGA-3'
	R	5'-TGAACGAGGAGGGTGGTG-3'
GAPDH	F	5'-CAATGAATACGGCTACAGCAAC-3'
	R	5'- AGGGAGATGCTCAGTGTTGG-3'

F: forward, R: reverse.

MATERIALS AND METHODS

Materials

Cytochalasin B (from *Helminthosporium dematioideum*) and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PP2, a Src inhibitor, and piceatannol, a Syk inhibitor, were obtained from Calbiochem (La Jolla, CA, USA). Rhodamine phalloidin was purchased from Molecular Probes (Carlsbad, CA, USA). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells were purchased from the American Tissue Culture Center (Rockville, MD, USA). All other chemicals were of Sigma grade. Antibodies to phospho- or total proteins of β-actin (0.2 μg/blot, rabbit polyclonal, Cell Signaling, Beverly, MA, USA), myeloid differentiation protein 2 (MD2) (2 μg/blot, rabbit polyclonal, Abcam, Cambridge, MA, USA), vasodilator-stimulated phosphoprotein (VASP) (0.5 μg/blot, rabbit polyclonal, Cell Signaling, Beverly, MA, USA), and heat shock protein 27 (HSP27) (0.5 μg/blot, rabbit polyclonal, Cell Signaling, Beverly, MA, USA) were used. Alexa 488-conjugated secondary antibody was obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

RAW264.7 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C with 5% CO_2.

mRNA detection by quantitative real-time reverse transcription-PCR

Total RNA from LPS-treated-RAW264.7 cells (5×10^6 cells/ml) was prepared by adding TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol as reported previously (Sohn *et al*., 2013). The total RNA solution was stored at -70°C until used. For real-time PCR analysis, one microgram of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of cDNA obtained for each sample were submitted to a qPCR using the SYBR green Master Mix method (Applied Biosystems, Foster City, CA, USA) in the ABO sequence detection system. The results were normalized with the 18S transcript. The primers (Bioneer, Daejeon, Korea) used in this experiment are indicated in Table 1.

Cell viability test

After pre-incubation for 18 h, Cyto B (0-20 μM) was added to the RAW264.7 cells (1×10^6 cells/ml) and allowed to incubate for 24 h. The cytotoxic effect of Cyto B was evaluated by MTT assay, as reported previously (Gerlier and Thomasset, 1986). At 3 h before culture termination, 10 μl of MTT solution (10 mg/ml in phosphate buffered saline, pH 7.4) was added and cells were continuously cultured until 15% sodium dodecyl sulfate was added to each well, solubilizing the formazan (Kim and Cho, 2013a). The absorbance at 570 nm ($OD_{570-630}$) was measured using a Spectramax 250 microplate reader.

Morphological change test

LPS-treated RAW264.7 cells in the presence or absence of cyto B were incubated for the indicated times. Images of the cells in culture at each time point were obtained using an inverted phase contrast microscope attached to a video camera, and captured using NIH image software.

Determination of NO production

RAW 264.7 cells (1×10^6 cells/ml) under normal culture conditions and cells seeded on 0.5% agarose gel in culture plate were pre-incubated with Cyto B for 30 min and continuously activated with LPS (1 μg/ml) for 24 h (Kim *et al*., 2013b; Kim and Cho, 2013b). The nitrite in the culture supernatants was also measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl of sample medium for 10 min at room temperature. The OD at 570 nm (OD_{570}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve for NO was made with sodium nitrite. The detection limit of the assay was 0.5 μM.

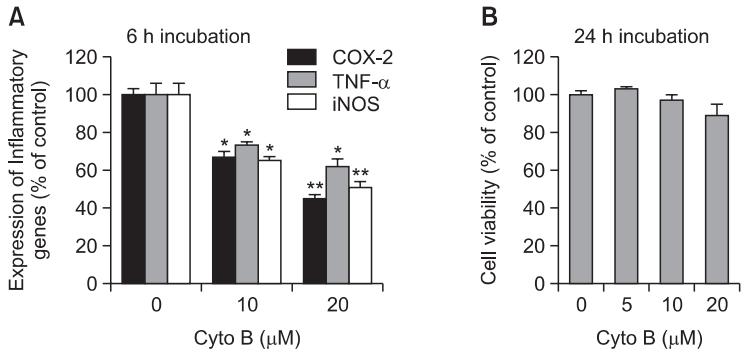

Fig. 2. The effect of cytochalasin B (Cyto B) on the mRNA expression of inflammatory genes in LPS-treated RAW264.7 cells and the viability of RAW264.7 cells. (A) RAW264.7 cells (5×10^6 cells/ml) were incubated with LPS (1 μg/ml) in the presence or absence of Cyto B for 6 h. The mRNA levels of iNOS, COX-2, and TNF-α were determined by quantitative real-time PCR. (B) Cell viability of RAW264.7 treated with Cyto B was determined by MTT assay. Data represent the mean ± SEM of three independent experiments performed in triplicate. **p*<0.05 and ***p*<0.01 compared to the control group.

Confocal microscopy

RAW264.7 cells were plated at a density of 2×10^5 cells/well in 12-well plates containing sterile cover slips and grown at 37°C for 12 h. Cells were treated with Cyto B for 30 min, followed by stimulation with LPS (1 μg/ml) for 2 min. After treatment, the cells were washed twice with PBS pre-warmed to 37°C and fixed to the cover slips by incubating in 3.7% formaldehyde for 10 min. Cells were then washed three times with PBS. The coverslips were blocked in 1% BSA for 1 h at room temperature with shaking. MD2 antibody was added in 1% BSA and incubated for 1 h with shaking at room temperature. For cytoskeleton staining, rhodamine phalloidin (Molecular Probes, 1:250) was added in 1% BSA and incubated for 1 h in the dark. Coverslips were then washed three times each with PBS. Alexa 488-conjugated secondary antibody (1:100) was then added in 1% BSA and incubated for 1 h with shaking at room temperature. Coverslips were washed three times with PBS and mounted onto slides using fluorescent mounting medium (DakoCytomation, Carpentaria, CA, USA). Intensity changes in MD2 and the cytoskeleton were imaged with an Olympus LX70 FV300 (Olympus, Tokyo, Japan).

Determination of phagocytotic uptake

To measure the phagocytic activity of RAW264.7 cells, we modified a method reported previously (Duperrier *et al*., 2000). RAW264.7 (5×10^4) cells treated with Cyto B, PP2, or piceatannol were re-suspended in 100 μl PBS containing 1% human AB serum and incubated with fluorescein isothiocynate (FITC)-dextran (1 mg/ml) at 37°C for 30 min. The doses of PP2 and piceatannol were previously determined by measuring inhibitory activities of both NO production and Src or Syk phosphorylation (Lee *et al*., 2009). The incubations were stopped by adding 2 ml ice-cold PBS containing 1% human serum and 0.02% sodium azide. The cells were then washed three times with cold PBS-azide and analyzed on a FACScan flow cytometer, as reported previously (Lee *et al*., 2008).

Preparation of cell lysates and immunoblotting analysis

RAW264.7 cells (5×10^6 cells/ml) were washed three times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM PMSF) for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 *g* for 10 min at 4°C and stored at -20°C until needed. Whole cell lysates were then analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to a polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membrane was incubated for 60 min with specific primary antibody at 4°C, washed three times with the same buffer, and incubated for an additional 60 min with HRP-conjugated secondary antibody. The total and phosphorylated levels of VASP, HSP27, and β-actin were visualized using the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) calculated from at least three independent experiments, each performed in triplicate, or are representative of three different experiments with similar results. For statistical comparisons, results were analyzed using the analysis of variance/Scheffe's post-hoc test and the Kruskal-Wallis/Mann-Whitney test. Values with $p<0.05$ were taken to indicate statistically significant differences. All statistical tests were carried out using the SPSS computer program (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Previously, we have reported that the actin cytoskeleton plays a critical role in macrophage-mediated inflammatory responses (Kim *et al*., 2010). Indeed, the actin cytoskeleton is linked to the activation of Src and the subsequent induction of NF-κB translocation (Kim *et al*., 2010). In this study, we further examined the biological value of the actin cytoskeleton in the functional activation of macrophages by exploring the subsequent morphological changes, cellular attachments, and relevant signaling events.

As reported previously (Kim *et al*., 2010), we could confirm

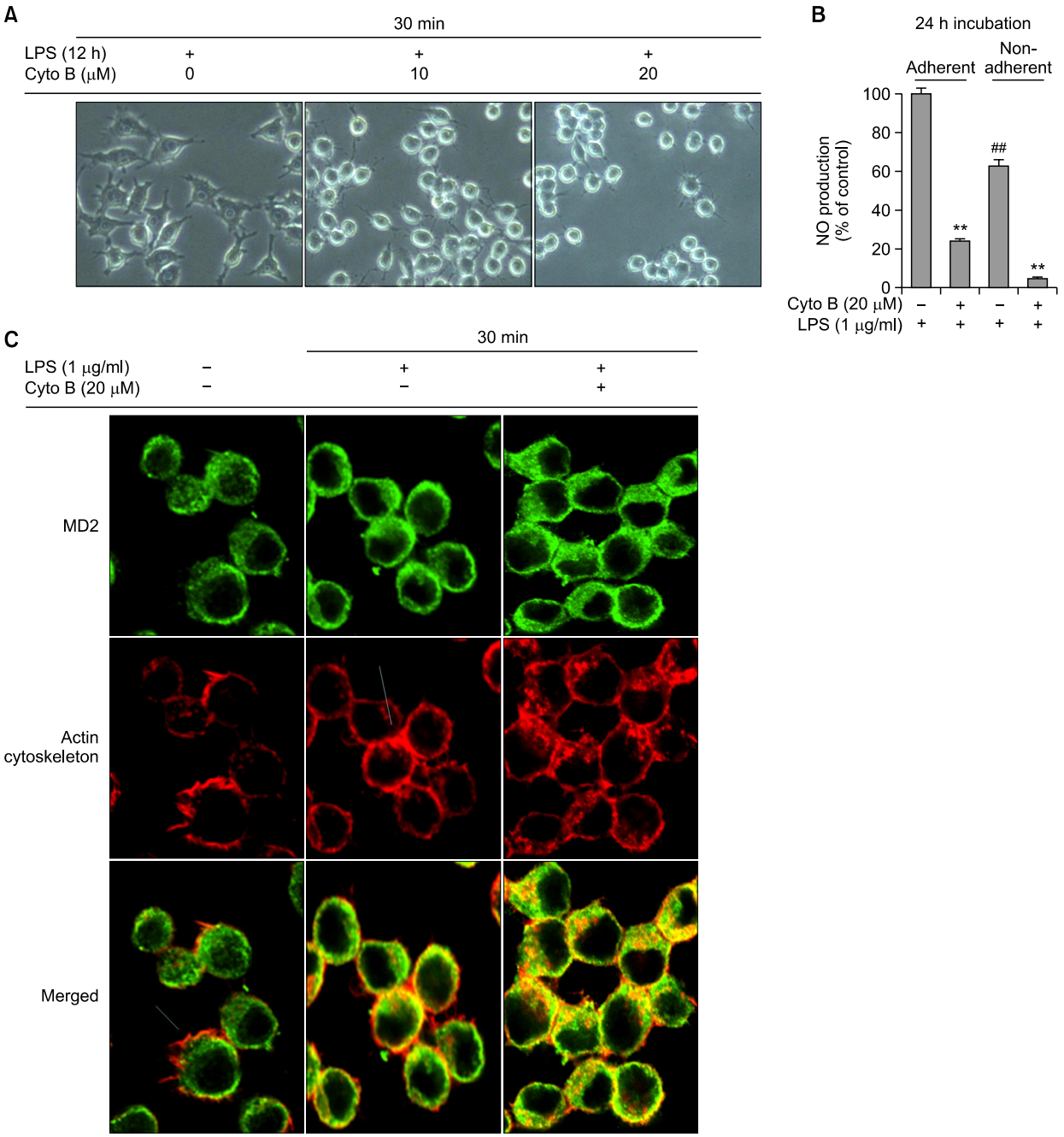

Fig. 3. The effect of cytochalasin B (Cyto B) on the morphological change and NO production in attached or unattached LPS-treated RAW264.7 cells. (A) Images of RAW264.7 treated with LPS (1 μg/ml) for 30 min in the presence or absence of Cyto B were obtained using an inverted phase contrast microscope attached to a video camera, and captured using NIH image software. (B) NO production was determined using Griess reagent from culture supernatants prepared by incubation of attached or unattached RAW264.7 cells (5×10^6 cells/ml) incubated with LPS (1 μg/ml) in the presence or absence of Cyto B for 24 h. (C) Molecular interaction between the actin cytoskeleton and MD2 in RAW264.7 cells during LPS exposure was determined by confocal microscopy. Data represent the mean ± SEM of three independent experiments performed in triplicate. $^{**}p<0.01$ compared to the control group, and $^{\#\#}p<0.01$ compared to LPS alone.

that Cyto B was able to strongly suppress the mRNA expression of inflammatory genes in LPS-treated RAW264.7 cells (Fig. 2A) without altering cell viability (Fig. 2B). Cyto B treatment also blocked LPS-induced morphological changes (Fig. 3A). In addition, 20 μM of Cyto B inhibited up to 80% of NO release in LPS-treated adherent RAW264.7 cells (Fig. 3B). Interestingly, unattached RAW264.7 cells cultured in a 0.5% agarose plate lost up to 40% of their NO-producing ability, while Cyto B more strongly suppressed up to 95% of the NO secretion (Fig. 3B). This result seems to suggest that the role of the actin cytoskeleton in inflammatory responses might be much larger under unattached conditions than during attached

states. Therefore, our data also indicate that this experimental model can be used for studying the functional role of the actin cytoskeleton in macrophage-mediated immune responses. By confocal microscopic analysis, it was found that MD2, a key surface glycoprotein for LPS signaling (Frleta *et al*., 2003), was not associated with the actin cytoskeleton under normal conditions, whereas LPS treatment induced strong interactions between the actin cytoskeleton and MD2 (Fig. 3C). Contrary to our expectations, Cyto B treatment did not strongly suppress the molecular association between MD2 and the actin cytoskeleton (Fig. 3C), implying that the inhibitory action of Cyto B in LPS-induced inflammatory responses might not be generated with the initial interaction of LPS to surface LPS binding proteins such as MD2, LBP, and CD14. Indeed, we found that the inhibitory target of Cyto B was Src via the suppression of actin polymerization (Kim *et al*., 2010), suggesting that the Src-mediated cellular signaling pathway could be linked to the actin cytoskeleton. More interestingly and unexpectedly, Src inhibition by both Cyto B and PP2, a Src inhibitor, up-regulated the phagocytic uptake of FITC-dextran (Fig. 4). In contrast, piceatannol, a Syk inhibitor, did not enhance but rather suppress dextran-induced phagocytosis (Fig. 4), indicating that PP2-mediated upregulation is not simply triggered by treatment with other protein tyrosine kinase (eg., Syk) inhibitor. This result could also imply that Src is an important regulator of phagocytic uptake in macrophages triggered by dextran. Therefore, we will further examine how actin polymerization and activated Src are able to cooperate to induce macrophage phagocytosis. Since TLR-dependent phagocytosis is modulated by the c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K), phospholipase C (PLC), and Janus kinase (JAK) (Lee *et al*., 2013; Nakanishi-Matsui *et al*., 2013), future experiments should explore the possibility that the non-TLR-mediated phagocytic ability of macrophages could be negatively controlled by Src activity.

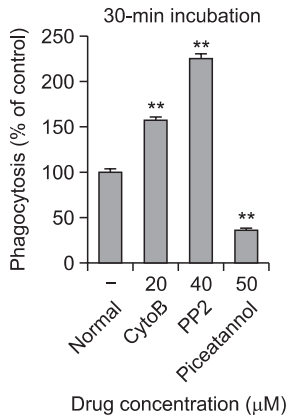

Fig. 4. Effect of cytochalasin B (Cyto B) on phagocytic uptake in RAW264.7 cells. (A) RAW264.7 cells pre-incubated with Cyto B were treated with FITC-dextran (1 mg/ml) for 2 h. The level of dextran uptake was determined by flow cytometric analysis. ***p*<0.01 compared with the normal group.

Finally, to examine the critical signaling events for the actin cytoskeleton in LPS-treated RAW264.7 cells, we further determined the phosphorylation patterns of actin cytoskeleton regulatory proteins. Since the upstream signaling events required for actin polymerization include HSP27 and VASP (Schneider *et al*., 1998; Huttelmaier *et al*., 1999), we tested the levels of phospho-HSP27 and phospho-VASP under LPS-treated conditions. As expected, LPS remarkably increased the phosphorylation of these proteins (Fig. 5A and 5B). However, interestingly, Cyto B strongly suppressed these phosphorylation events, indicating that actin cytoskeleton disruption itself or actin cytoskeleton disruption-linked molecular events may autologously participate in regulating upstream events for actin polymerization. Indeed, it has been reported that VASP and HSP27 are actin-binding proteins (Wang and Bitar, 1998; Gentry *et al*., 2012). Thus, the EVH2 domain is known as an important part of VASP for actin binding capacity (Gentry *et al*., 2012), indicating direct regulation between actin cytoskeleton and the activation of VASP and HSP27. Mean-

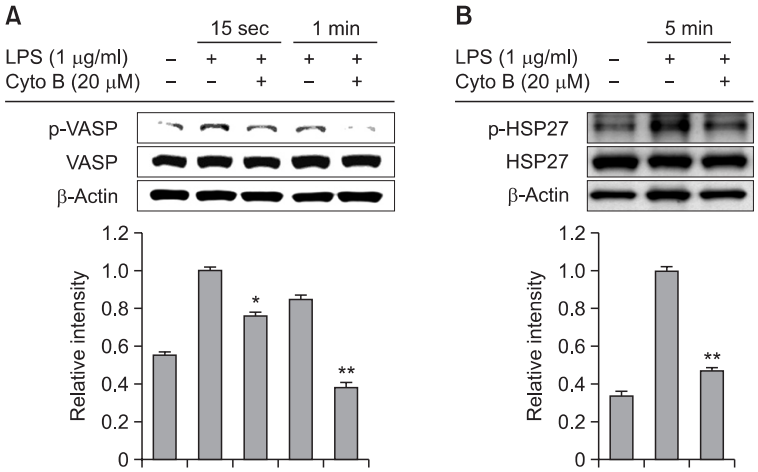

Fig. 5. The effect of cytochalasin B (Cyto B) on the phosphorylation of VASP and HSP27. (A and B) RAW264.7 cells (5×10^6 cells/ml) were stimulated with LPS (1 μg/ml) in the presence or absence of Cyto B. After immunoblotting, total or phospho-protein levels of VASP, HSP27, and β-actin were identified with their total protein- or phospho-specific antibodies. The results show one representative experiment of three. The relative intensity was calculated using total levels by the DNR Bio-Imaging system. **p*<0.05 and ***p*<0.01 compared with the control group.

while, considering that Src is a critical enzyme linked to actin cytoskeleton (Kim *et al*., 2010), a possibility that actin cytoskeleton-controlled Src is able to modulate the phosphorylation of VASP and HSP27 could be hypothesed. In fact, it has been proposed that the TRAIL-induced MEKK4/p38/HSP27/Akt survival network is modulated by Src (Kim *et al*., 2013a). Moreover, upon induction of phagocytosis, a large molecular complex was formed consisting in part of Ena/VASP proteins, the Fyn-binding/SLP-76-associated protein, Src-homology-2 (SH2)-domain-containing leukocyte protein of 76 kDa (SLP-76), Nck, and the Wiskott-Aldrich syndrome protein (WASP) (Coppolino *et al*., 2001). There is functional interaction between VASP and Src in IRSp53-mediated podosome formation (Oikawa *et al*., 2013). Therefore, our data and these reports imply that the Cyto B-induced inhibition of VASP and HSP27 phosphorylation might be managed by either direct actin disruption or its relevant inhibition of Src. Additional experiments to prove these possibilities will be further carried on.

In summary, we found that Cyto B exhibited inhibitory activity on the mRNA expression of iNOS, COX-2, and TNF-α in LPS-treated RAW264.7 cells. In addition, Cyto B blocked morphological changes and suppressed non-adherent cell-derived NO production under LPS stimulation conditions. Cyto B also reduced HSP27 and VASP phosphorylation, which is required for actin cytoskeleton rearrangement. Therefore, our data suggest that the actin cytoskeleton may play a central role in modulating the TLR4-mediated inflammatory responses of macrophages.

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea grant funded by the Korean government (MEST) (to J.Y.C., No. 2011-0016397).

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