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Role of actin cytoskeleton in LPS-induced NF-κB activation and nitric oxide production in murine macrophages

Sandeepa M. Eswarappa, Vidhi Pareek, Dipshikha Chakravortty

Centre for Infectious Disease Research and Biosafety Laboratories, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, Karnataka, India

Lipopolysaccharide (LPS) is a major cell wall component of Gram-negative bacteria and is known to cause actin cytoskeleton reorganization in a variety of cells including macrophages. Actin cytoskeleton dynamics influence many cell signaling pathways including the NF-κB pathway. LPS is also known to induce the expression of many pro-inflammatory genes via the NF-κB pathway. Here, we have investigated the role of actin cytoskeleton in LPS-induced NF-κB activation and signaling leading to the expression of iNOS and nitric oxide production. Using murine macrophages, we show that disruption of actin cytoskeleton by either cytochalasin D (CytD) or latrunculin B (LanB) does not affect LPS-induced NF-κB activation and the expression of iNOS, a NF-κB target gene. However, disruption of actin cytoskeleton caused significant reduction in LPS-induced nitric oxide production indicating a role of actin cytoskeleton in the post-translational regulation of iNOS.

Keywords: Actin, cytochalasin D, iNOS, latrunculin B, lipopolysaccharide, NF-κB

Introduction

Lipopolysaccharide (LPS) is a major cell wall component of Gram-negative bacteria that induces activation of macrophages. Activated macrophages produce several inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-12 (IL-12) and nitric oxide (NO). All these inflammatory mediators, together, when in excess, lead to a serious systemic disorder known as septic shock which has a high mortality rate.[1] Thus, it is important to understand LPS-induced signaling events in macrophages.

LPS signaling is mediated via the NF-κB pathway.[2] The NF-κB family of transcription factors regulates the expression of many genes involved in immune and inflammatory responses of mammalian cells.[2,3] Improper functioning of this family of transcription factors has been implicated in many human diseases ranging from cancer to degenerative diseases like arthritis.[4–6] Normally, NF-κB is kept inactive in the cytoplasm by proteins belonging to IκB family. Pathogen-associated molecular patterns like LPS or pro-inflammatory cytokines cause proteosomal degradation of IκB-α and subsequent mobilization of NF-κB into the nucleus and induction of many genes that encode inflammatory mediators (*e.g.* iNOS and TNF-α).

LPS is also known to cause re-organization of the actin cytoskeleton in macrophages, neutrophils, endothelial cells, mesangial cells, ureteral epithelial cells and fibroblasts.[7–12] In murine peritoneal macrophages,

Abbreviations: CytD, cytochalasin D; LanB, latrunculin B; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NO, nitric oxide; p-p38, phospho-p38

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Correspondence to: Dipshikha Chakravortty, Centre for Infectious Disease Research and Biosafety Laboratories, Department of Microbiology and Cell Biology, Indian Institute of Science, C.V. Raman Avenue, Bangalore 560012, Karnataka, India
Tel: +91-80-22932842/22932986; Fax: +91-80-23602697; E mail: dipa@mcbl.iisc.ernet.in

LPS causes actin polymerization within 30 min.[8] Actin cytoskeleton is involved in many mechanical functions of eukaryotic cells such as motility, phagocytosis, and muscular movement. In addition to these, the dynamics of actin cytoskeleton influence many intracellular signaling events.[13–17] However, the role of actin cytoskeleton in LPS signaling has not been studied so far. Recently, the effect of actin dynamics on the NF-κB signaling pathway has been studied in human myelomonocytic cells and epithelial cells.[18–20] These studies report that disruption of actin cytoskeleton activates the NF-κB pathway.

NO is one of the many inflammatory mediators produced by macrophages upon activation by LPS and it acts as a vasodilator, a neurotransmitter and a pro-inflammatory molecule. NO production occurs via constitutively expressed NO synthases (eNOS and nNOS) and an inducible isoform of NO synthase (iNOS). Production of NO via iNOS is regulated in a complex manner by various stimuli.[21,22] Inhibition of iNOS-mediated NO production will be beneficial for the treatment of some inflammatory diseases.[23] Production of NO upon LPS induction is dependent on the expression of iNOS, whose expression is, in turn, mediated by a series of signaling events like activation of NF-κB and mitogen-activated protein (MAP) kinases.[2,24] Cytoskeleton plays an important role in the regulation of nitric oxide synthases.[25] Actin cytoskeleton is known to associate with iNOS and eNOS.[26,27] Disruption of actin cytoskeleton enhances iNOS expression and NO production in vascular smooth muscles, epithelial cells and mesangial cells.[28–30] Disruption of the microtubule network inhibits LPS-induced NO production in murine peritoneal macrophages.[31] However, the role of actin cytoskeleton in the regulation of LPS-induced NO production in macrophages has not been studied so far. Understanding the role of actin cytoskeleton in LPS signaling is the main objective of this study. Here, we show that actin cytoskeleton is not required for LPS-induced NF-κB activation and signaling in murine macrophages; however, it is involved in the post-translational regulation of LPS-induced iNOS.

Materials and Methods

Cell culture

Elicited peritoneal macrophages were extracted from 6–8-week-old C57/BL6 mice. Brewer thioglycollate medium (HIMEDIA; 5 ml) was injected intraperitonealy into these mice. At 4 or 5 days after injection, macrophages were harvested by peritoneal lavage with RPMI 1640 medium containing 10% fetal bovine serum (Sigma). RAW 264.7 cells (a kind gift from Prof. Anjali Karande, IISc) were cultured using DMEM medium containing 10% fetal bovine serum (Sigma). These cells were maintained at 37°C with 5% carbon dioxide.

Cytochalasin D, latrunculin B and LPS treatment

In all experiments, 10 μM cytochalasin D (CytD; Sigma), 12 μM latrunculin B (LanB; Calbiochem) and 1 μg/ml LPS (derived from *Salmonella enterica* serovar Typhimurium; Sigma) were used unless stated otherwise. Cells were pretreated with CytD/LanB for 1 h and then cells were induced with LPS until the indicated time. CytD and LanB were maintained along with LPS until the end of the experiments. The same protocol was followed for inducing cells with CpG DNA (1 μg/ml; Invivogen).

Immunofluorescence microscopy

Macrophages were seeded on cover-slips in a 24-well plate (2–3 × 10^5 cells/well). After specific treatment, cells were fixed using 3.5% paraformaldehyde (Sigma) for 20 min. After washing three times with phosphate buffered saline (PBS), cells were incubated with p65 antibody (Santa Cruz) diluted in blocking buffer (0.1% saponin, 2% BSA and 2% goat serum in PBS) for 1 h. Cells were then washed twice with PBS and incubated for 1 h with the appropriate secondary antibody diluted in blocking buffer after which cells were again washed three times with PBS. After this, cells were incubated with propidium iodide (1 μg/ml) for 5 min to stain the nucleus and cover-slips were mounted on a glass slide. Samples were analyzed using confocal laser-scanning microscope (Zeiss LSM Meta).

Electrophoretic mobility-shift assay (EMSA)

Macrophages were seeded in a 6-well plate (10^6 cells/well) and treated as indicated in the caption to Figure 2B. The cells were then washed and scraped into PBS containing 2% fetal bovine serum. The cell pellet was resuspended in 400 μl hypotonic buffer (10 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.3 M sucrose, 0.5 mM DTT). The cells were lysed by addition of NP-40 to a final concentration of 0.5%. The nuclei were harvested by centrifugation and the nuclear pellet was resuspended in 30 μl hypertonic buffer (20 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 320 mM KCl, 0.2 mM EGTA, 0.5 mM DTT, 1× protease inhibitor cocktail). The suspension was centrifuged, the supernatant was collected and assayed for protein

content with the BCA Kit (Pierce). The oligonucleotide 5′-gatccaa**ggggactttcc**atg-3′ bearing the NF-κB binding site (bold) and a mutant oligonucleotide bearing a G→C mutation in the third base of the NF-κB binding site were [^{32}P]-end labeled using T4 polynucleotide kinase, [γ-^{32}P]-ATP and reaction buffer. Unincorporated nucleotides were removed by column chromatography over a Sephadex G-50 column. The labeled oligonucleotides were renatured with their corresponding complementary sequences to yield double-stranded probes. Binding reactions (10 μl) having 8 μg nuclear extract, binding buffer (10 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, 0.05% NP-40) and labeled oligonucleotides (40,000 cpm) were setup. The binding reaction was allowed to proceed for 20 min at 25—27°C. The DNA protein complexes were resolved over a 7% native polyacrylamide gel in 0.5× TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA pH 8.0) for 30 min at 150 V. The gel was then fixed (10% acetic acid, 10% methanol) and exposed to a phosphorimager cassette for 8—12 h. The phosphorimager screen was scanned with a phosphorimager (FLA-5 Fujifilm).

Luciferase reporter gene assay for NF-κB activation

For this experiment, RAW 264.7 cells were used, as the transfection efficiency of elicited peritoneal macrophages is very poor. Cells were seeded in 24-well plates (2–3 × 10^5 cells/well) and transfected with 0.5 μg of pNF-κB-TA-Luc vector (contains four tandem copies of the NF-κB consensus sequence) and 0.5 μg of pCMVβ-gal vector (both the vectors were gifts from Prof. Takashi Yokochi, Aichi Medical University, Japan). The transfected cells were treated as indicated in the caption to Figure 4. The cells were then lysed and the luciferase activity determined using the Promega luciferase assay system with a luminometer. β-Galactosidase activity was used to normalize transfection efficiencies.

Western-blot analysis

Macrophages were seeded in 35-mm plastic dishes (10^6 cells/dish) and pretreated with CytD/LanB for 1 h followed by LPS induction for 30 min (for IκB-α and p-p38) or 12 h (for iNOS). Cells were lysed in the lysis buffer (50 mM HEPES, 100 mM NaCl, protease inhibitor cocktail [Roche], 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% NP-40, 0.5% sodium deoxycholate) and boiled for 5 min at 100°C. Aliquots containing equal amounts of protein (50 μg/lane) were electrophoresed in SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was then probed with a specific antibody (anti-p-p38/p38 [Cell Signaling]; anti-Iκb-α [Santa Cruz]; anti-iNOS [Sigma]) and the respective HRP horse radish peroxidase (HRP) conjugated secondary antibodies (Bangalore Genei). Immune complexes were detected using an enhanced chemiluminescence reagent (PerkinElmer).

Reverse transcriptase-PCR

Total RNA from elicited peritoneal macrophages was isolated using TRI reagent (Sigma) after specific treatment. RNA (1 μg) was reverse transcribed using a reverse transcription system (Promega). cDNA thus generated was used to analyze iNOS or GAPDH expression by PCR using gene specific primers (Sigma). Primers used to amplify iNOS cDNA were 5′-aagtcaaatcctaccaaagtga-3′ and 5′-ccataatactggttgatgaact-3′. Primers used to amplify GAPDH cDNA were 5′-ggcaaattcaacggcacagt-3′ and 5′-agatggtgatgggcttccc-3′.

Determination of nitrite concentration

Griess reagent was used to measure NO as its end product, nitrite, as described previously.[32] Macrophages were seeded in 96-well plates (10^5 cells/well) and, after specific treatments, culture supernatants (50 μl) were mixed with 100 μl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride [both from Sigma]). Absorbance at 540 nm was measured in a microplate reader after 15 min incubation in the dark. The concentration of nitrite in the culture supernatant was determined with reference to a sodium nitrite standard curve.

RESULTS AND DISCUSSION

Actin polymerization can be inhibited by cytochalasin D (CytD) and latrunculin B (LanB).[33,34] These drugs provide a convenient and powerful method for perturbing the actin cytoskeleton. Hence, we used CytD and LanB to study the role of actin cytoskeleton in LPS signaling. LPS-induced actin polymerization cannot take place in the presence of these drugs as they are potent inhibitors of actin polymerization. To start with, we checked the maximum concentration of these drugs that can be used without any toxicity in elicited murine peritoneal macrophages. Using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, we found that the maximum concentrations of CytD and LanB that are non-toxic to elicited peritoneal macrophages were 10 μM and 12 μM, respectively (Fig.

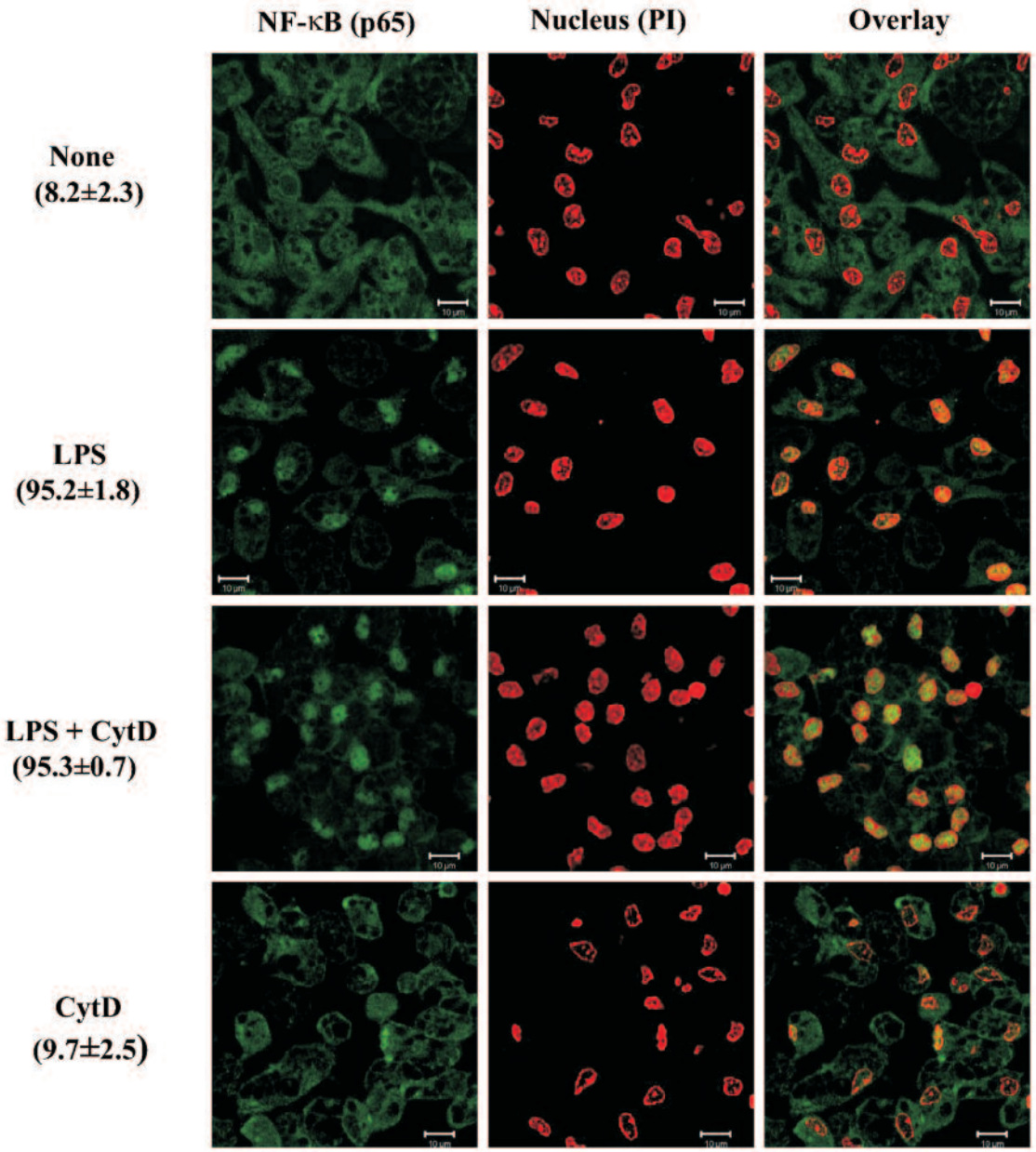

Fig. 1. Actin disruption by CytD does not affect LPS-induced p65 nuclear translocation. Cells were treated with CytD (10 μM) for 1 h followed by induction with LPS (1 μg/ml) for 1 h after which the cells were fixed and stained using p65 antibody and propidium iodide as described in Materials and Methods. Numbers in parentheses represent mean ± SE of percentage of cells showing nuclear localization of p65; more than 400 cells from different fields were examined in each case to calculate these values. The experiment was repeated three times and similar results were obtained. Scale bars correspond to 10 μm.

S1: see online supplementary material); these concentrations of CytD and LanB were used in all the experiments of this study (see Materials and Methods). The treated cells were more spherical in shape when compared to untreated cells (data not shown). Moreover, it has been reported that 2 μM CytD and 10 μM LanB cause almost complete disruption of actin cytoskeleton within 30 min of treatment.[35] Thus, 10 μM CytD and 12 μM LanB are sufficient to cause complete actin disruption without compromising the viability of the cells.

Effect of actin cytoskeleton disruption on LPS-induced p65 nuclear translocation

p65 (RelA) belongs to the family of NF-κB transcription factors. LPS treatment causes activation and nuclear translocation of p65 as a homodimer or heterodimer (forming different NF-κB complexes).[2] Thus, localization of p65 in the nucleus is an indication of NF-κB activation. We used immunofluorescence microscopy to investigate the LPS-induced NF-κB activation status and looked at

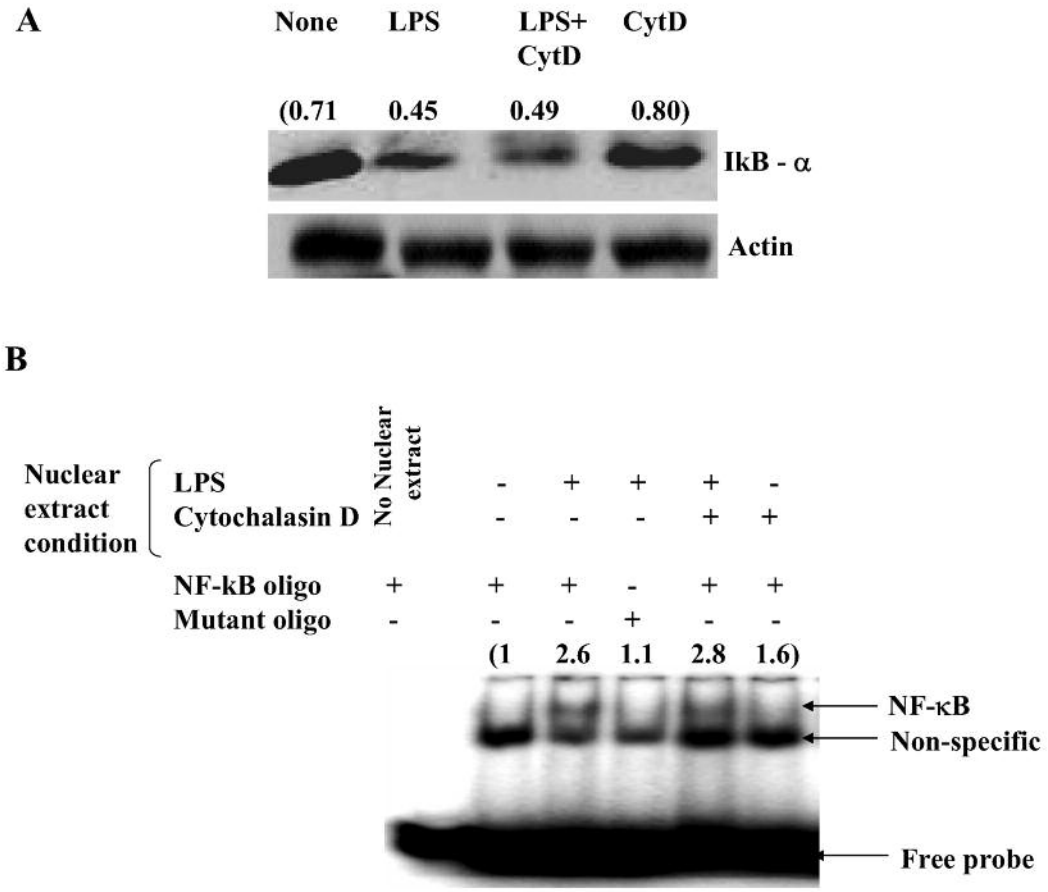

Fig. 2. (A) CytD treatment does not affect LPS-induced IκB-α degradation. Cells were treated with CytD (10 μM) for 1 h followed by LPS (1 μg/ml) induction for 30 min after which the cells were lysed and the lysate subjected to Western blotting using IκB-α antibody. Numbers in parentheses represent values obtained after densitometric analysis. (B) Electrophoretic mobility-shift assay. The cells were treated with CytD (10 μM) for 1 h followed by induction with LPS (1 μg/ml) for 1 h after which the nuclear extract was isolated and subjected to electrophoretic mobility-shift assay using a labeled oligonucleotide having a NF-κB binding site (NF-κB oligo). Another oligonucleotide having a mutated NF-κB binding site (mutant NF-κB oligo) was used to show that the thick non-specific band observed was not due to NF-κB binding. NF-κB specific mobility-shift was observed in nuclear extracts of cells treated with LPS or CytD+LPS. Numbers in parentheses represent values obtained after densitometric analysis. The density of the untreated sample was taken as one. (A) and (B) are representative of two independent experiments.

the localization of p65. Cells were treated with CytD/LanB for 1 h; the cells were then induced with LPS for 1 h after which the cells were fixed and processed for immunofluorescence microscopy. We did not observe any change in the LPS-induced p65 nuclear translocation in CytD/LanB-treated cells. Extension of CytD/LanB pretreatment to 6 h also did not affect p65 nuclear localization status (data not shown). Treatment with CytD/LanB alone did not cause any significant change in p65 localization when compared to untreated cells (Figs 1 and S2: see online supplementary material).

Degradation of IκB-α is also an indirect evidence of NF-κB activation. We next looked at IκB-α status. Cells were treated with CytD for 1 h followed by LPS induction for 30 min after which cells were lysed and the lysate was subjected to Western blotting. There was no significant difference in the level of IκB-α degradation between LPS and CytD+LPS-treated cells showing that CytD does not affect LPS-induced IκB-α degradation; extension of CytD pretreatment to 6 h yielded similar results (data not shown). The status of IκB-α in the cells treated with CytD alone was same as that of the untreated cells (Fig. 2A). This result shows that actin cytoskeleton disruption does not affect LPS-induced IκB-α degradation.

To confirm p65 nuclear localization, we performed an electrophoretic mobility-shift assay. The cell treatment conditions were the same as those followed in p65 nuclear localization studies. Nuclear extracts derived from the cells treated with LPS or CytD+LPS caused a mobility-shift specific to NF-κB, whereas nuclear extracts derived from untreated cells and the cells treated with CytD alone did not show a similar shift. A nuclear extract derived from LPS-treated cells did not cause a

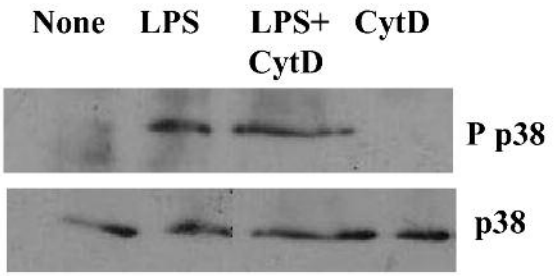

Fig. 3. CytD treatment does not affect LPS-induced p38 MAPK activation. Cells were treated with CytD (10 μM) for 1 h followed by LPS (1 μg/ml) induction for 30 min after which the cells were lysed and the lysate was subjected to Western blotting using p-p38 antibody. This is representative of two independent experiments.

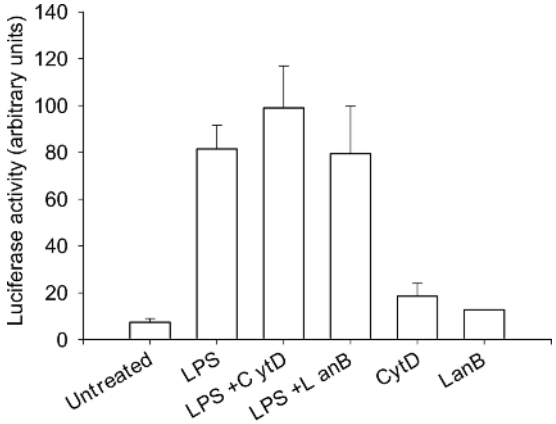

Fig. 4. Actin disruption does not affect the transcriptional activity of NF-κB translocated to the nucleus upon LPS induction as measured by the luciferase reporter gene assay. For this assay, RAW 264.7 cells were treated with CytD (10 μM)/LanB (12 μM) for 1 h followed by LPS (1 μg/ml) induction for 8 h after which the cells were lysed and subjected to luciferase assay. Values obtained from three experiments were pooled and mean values are plotted with the SE as error bars.

similar mobility-shift when incubated with a mutant oligonucleotide confirming that the mobility-shift we observed is specific to NF-κB (Fig. 2B). Taken together, these results show that disruption of actin cytoskeleton does not inhibit LPS-induced p65 nuclear translocation.

Effect of actin cytoskeleton disruption on p38 MAPK activation

LPS activates p38 mitogen activated protein kinase (MAPK)[36] and p38 controls the dynamics of actin.[37–39] We next investigated the LPS-induced activation of p38. The cell treatment conditions were as described for IκB-α degradation. We observed that CytD treatment does not affect LPS-induced p38 activation (Fig. 3). This suggests that disruption of actin cytoskeleton does not affect LPS-induced p38 MAPK activation.

Effect of actin cytoskeleton disruption on the ability of NF-κB as a transcription factor

The ability of the NF-κB family of proteins to act as transcription factors depends on their dimerization properties. For example, RelB forms a transcriptionally inactive complex with p65,[40] p50 homodimer is transcriptionally inactive and p65–p50 heterodimers are transcriptionally very active.[2] Thus, it is important to investigate the transcriptional activity of p65 that is localized in the nucleus of cells treated with CytD/LanB followed by LPS induction. For this investigation, we used the luciferase reporter gene assay. For this assay, murine macrophage-like cells (RAW 264.7) were used, as the transfection efficiency in murine peritoneal macrophages is poor. Cells were treated with CytD/LanB for 1 h followed by LPS induction for 8 h after which cells were lysed and subjected to luciferase assay. Cells treated with LPS alone showed about 10-fold increase in the luciferase activity when compared to untreated cells; treatment with CytD/LanB did not alter this LPS-induced luciferase activity. Cells treated with CytD/LanB alone did not show significant change in luciferase activity (Fig. 4). This result suggests that actin cytoskeleton disruption does not affect the transcriptional ability of LPS-induced NF-κB.

Effect of actin cytoskeleton disruption on LPS-induced iNOS expression

Next, we studied the expression of NF-κB target genes on LPS induction. We chose the iNOS gene, which can be induced by LPS treatment in macrophages. Both NF-κB and p38 MAPK are involved in LPS-induced iNOS expression.[22,36] The enzyme iNOS is responsible for the production of NO, which is one of the most versatile molecules in the immune system and an important contributor to tissue damage in endotoxemia.[23,41] Cells were treated with CytD/LanB for 1 h followed by LPS induction for 12 h after which total RNA/protein was isolated and subjected to reverse transcriptase-PCR/Western blot analysis. The LPS-induced iNOS mRNA and protein expression levels were unaltered even after actin disruption by CytD/LanB (Fig. 5A,B). Similar observation was made when the LPS concentration was reduced to 100 ng/ml (Fig. S3: see online supplementary material). Expression levels of iNOS mRNA and protein in the cells treated with CytD/LanB alone were similar to those in untreated cells. These results suggest that disruption of actin cytoskeleton does not affect LPS-induced expression of iNOS, one of the NF-κB target genes.

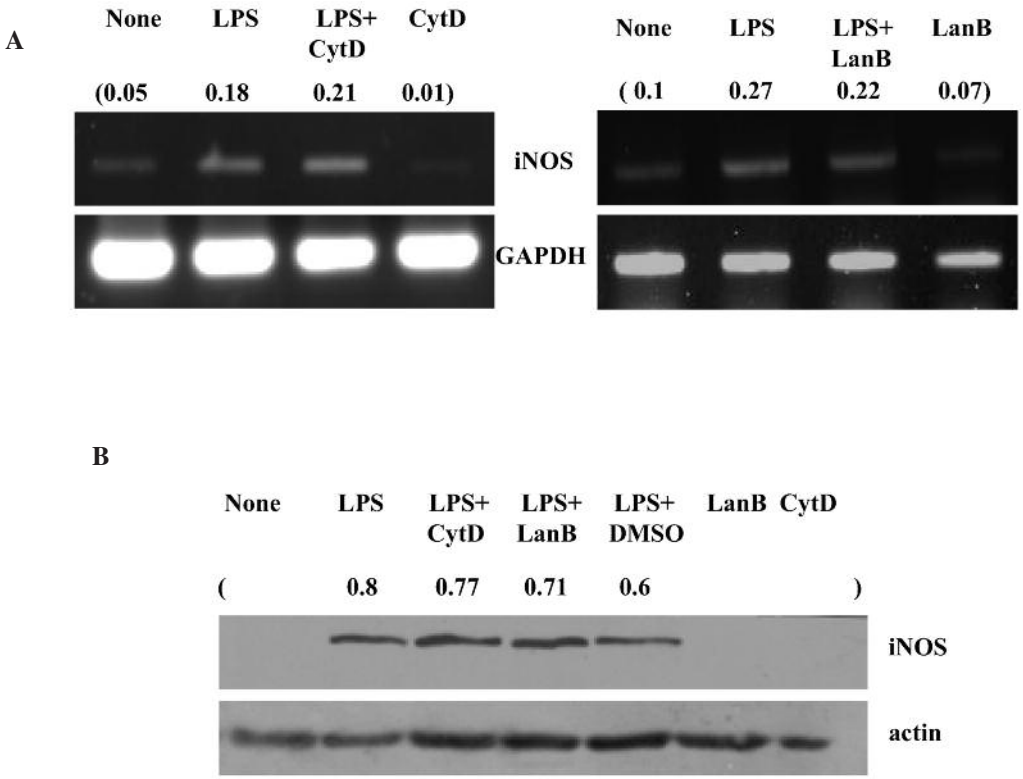

Fig. 5. Actin cytoskeleton disruption does not affect LPS-induced iNOS expression. Cells were treated with CytD (10 μM)/LanB (12 μM) for 1 h followed by LPS (1 μg/ml) induction for 12 h after which total RNA/protein was isolated and subjected to (A) RT-PCR and (B) Western blot. LPS-induced iNOS mRNA and protein expressions were unaltered by CytD and LanB treatment. Numbers in parentheses represent values obtained after densitometric analysis. (A) and (B) are representative of two and four independent experiments, respectively.

Effect of actin cytoskeleton disruption on LPS-induced NO production

Though LPS-induced iNOS expression was unaltered by actin cytoskeleton disruption, to our surprise, we observed a significant decrease in LPS-induced NO production in cells treated with CytD/LanB followed by LPS induction (Fig. 6A); this inhibition was not dependent on the LPS concentration used (Fig. 6B) but was dependent on the dose of CytD and LanB (Fig. 6C,D). Interestingly, CytD/LanB treatment could inhibit CpG DNA (TLR9 ligand)-induced NO production also, suggesting that this action is not specific to LPS (TLR4 ligand)-induced NO production (Fig. 6E). All these effects were also observed in RAW 264.7 cells (data not shown). Together, these results suggest a previously unappreciated role of actin cytoskeleton in the regulation of LPS-induced iNOS at the post-translational level in murine macrophages. Yet another interesting possibility is that CytD and LanB may prevent localization of iNOS with cytoskeleton which is needed for the optimal activity of iNOS.[25]

An important observation of this study is that disruption of actin cytoskeleton does not affect LPS-induced signaling. This suggests that LPS-induced actin polymerization has no role in its signaling via the NF-κB and p38 MAPK pathways. The next obvious question is 'what is the role of LPS-induced actin cytoskeleton re-organization?' Probably, LPS-induced actin cytoskeleton re-organization is involved in the regulation of LPS-induced proteins like iNOS at the post-translational level as suggested by our results (Fig. 6). The role of actin cytoskeleton in the post-translational regulation of eNOS has been reported recently.[26] CytD is reported to inhibit LPS-induced TNFα production in murine macrophages.[42] However, in that particular study, the authors did not investigate upstream signaling events such as NF-κB activation by LPS. Perhaps, actin cytoskeleton is also involved in the regulation TNF-α at the post-translational level.

It has been shown that actin cytoskeleton disruption causes NF-κB activation in human epithelial cells and human myelomonocytic cells.[18–20] However, we did not observe any signs of NF-κB activation in murine peritoneal macrophages treated with CytD or LanB alone. Probably, this discrepancy is due to the difference in the nature of the cells used in our experiments and the earlier reports. In fact, such cell-type specific effects of actin disruption are known; disruption of actin cytoskeleton in rat vascular smooth muscles causes increased nitrite production[28]

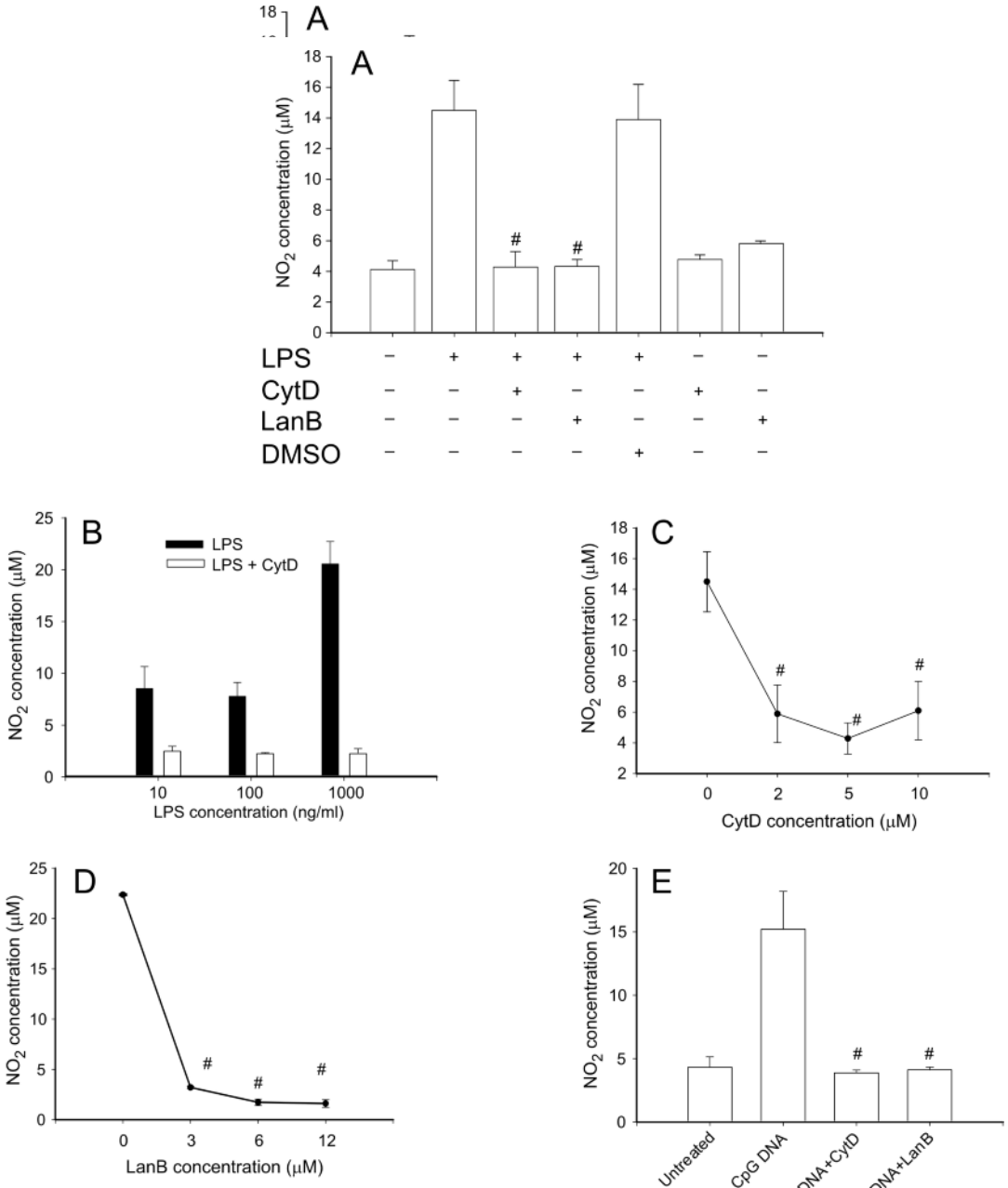

Fig. 6. Effect of actin cytoskeleton disruption on NO production. (A) CytD or LanB treatment inhibits LPS-induced NO production. (B) Inhibition of LPS-induced NO production by CytD does not depend on the concentration of LPS used. (C) CytD and (D) LanB inhibit LPS-induced NO production in a dose-dependent manner. (E) CytD and LanB inhibit CpG DNA-induced NO production. Cells were treated with CytD (10 μM)/LanB (12 μM) for 1 h [except in (B) and (C) where different doses as indicated were used] followed by LPS induction [in (A), (B), (C) and (D)] or CpG DNA (1 μg/ml) treatment [in (E)] for 12 h after which the culture supernatant was collected and subjected to Griess assay as described in Materials and Methods. The bars represent mean nitrite concentrations. Error bars represent SE. (A) is representative of three independent experiments and (B),(C), (D) and (E) are representative of two independent experiments done in triplicate samples. Statistical significance was defined as $^{\#}P < 0.05$ (Student's *t*-test).

whereas, in murine macrophages it causes reduction in nitrite production (Fig. 6). Moreover, LPS-induced actin re-organization and the role of actin cytoskeleton in mediating TNF-α production in rat alveolar epithelial cells are opposite to those in RAW 264.7 cells.[43] Thus, actin cytoskeleton appears to have differing roles in different cell types with respect to cell signaling.

CONCLUSIONS

Together, these results suggest that actin cytoskeleton has no role in the signaling of LPS resulting in the activation of p38 MAPK, NF-κB and the expression of iNOS gene. However, it may be involved in the post-translational regulation of LPS-induced iNOS. The exact

mode of the post-translational regulation of iNOS by actin cytoskeleton remains to be investigated.

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