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Folimycin (concanamycin A) inhibits LPS-induced nitric oxide production and reduces surface localization of TLR4 in murine macrophages

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Lipopolysaccharide (LPS) is a major cell wall component of Gram-negative bacteria and signals through a receptor complex which consists of TLR4, MD-2 and CD14. LPS signaling in macrophages induces the production of many pro-inflammatory molecules, including nitric oxide (NO). In this study, we have shown that folimycin, a macrolide antibiotic and a specific inhibitor of vacuolar ATPase (V-ATPase), inhibits LPS-induced NO production, but not TNF-α production, in murine elicited peritoneal macrophages. However, folimycin did not affect interferon-γ induced NO production. LPS-induced iNOS mRNA and protein expression and NF-κB activation were also inhibited by folimycin. Interestingly, folimycin-treated cells showed reduced surface expression of TLR4 molecules and dilated Golgi apparatus. These findings suggest that folimycin, by inhibiting V-ATPases, alters intra-Golgi pH, which in turn causes defective processing and reduced surface expression of TLR4 reducing the strength of LPS signaling in murine macrophages.

Keywords: Folimycin, LPS, Nitric oxide, TLR4

INTRODUCTION

Lipopolysaccharide (LPS) is a major cell wall component of Gram-negative bacteria and is one of the best studied microbial products. It induces activation of monocytes and macrophages. Activated macrophages produce several inflammatory cytokines including TNF-α, IL-6, 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] Significant progress has been made in the identification of LPS recognition molecules in mammalian cells. Core components include Toll-like receptor 4 (TLR4),[2,3] CD14[4] and MD-2.[5] CD14 binds to LPS and augments LPS responses. TLR4–MD-2 is thought to work downstream of this initial binding. As both TLR4 and MD-2 are indispensable for LPS signaling, mice lacking either TLR4 or MD-2 do not respond to LPS.[6,7]

NO exhibits a wide range of important functions *in vivo*. It acts as a vasodilator, a neurotransmitter and as a pro-inflammatory molecule. NO is synthesized by constitutively expressed NO synthases (eNOS and nNOS) and an inducible isoform of NO synthase (iNOS). NO production via iNOS is regulated in a complex manner by various stimuli.[8,9] Inhibition of iNOS-mediated NO production will be beneficial for the treatment of some inflammatory diseases.[10] Murine macrophages provide the best-studied example of the regulation of NO production. LPS-induced NO production 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.[11,12]

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Folimycin, also called concanamycin A, is a macrolide antibiotic isolated from *Streptomyces* spp. and is a specific inhibitor of vacuolar ATPase (V-ATPase). In this regard, folimycin is more potent than bafilomycins.[13] It disturbs the pH gradient between the acidic compartments and the cytosol, leading to impaired functioning of these organelles. It has been reported that TLR3, TLR7 and TLR9, whose signaling is pH dependent, fail to signal in cells treated with folimycin or other lysosomotropic agents.[14–16] Acidification of intracellular compartments is a prerequisite for several other essential processes like protein trafficking in the secretory pathway from the Golgi to the cell surface.[17–19] In addition, folimycin is known to inhibit protein trafficking to the cell surface.[20,21]

In this study, we show that folimycin can inhibit LPS-induced NO production in elicited murine peritoneal macrophages. Folimycin-treated cells showed decreased NF-κB translocation to the nucleus, decreased iNOS mRNA and protein expression and decreased NO production upon LPS stimulation. We also show that cells treated with folimycin have reduced expression of TLR4 on their surface.

MATERIALS AND METHODS

Cell culture

Elicited peritoneal macrophages were extracted from 6–8-week-old C57/BL6 mice. Brewer Thioglycollate Media (HIMEDIA) was injected intraperitoneally into these mice. After 4–5 days of injection, macrophages were harvested by peritoneal lavage with RPMI 1640 medium (Sigma). These cells were maintained at 37°C with 5% carbon dioxide in RPMI 1640 medium containing 10% fetal bovine serum (Sigma).

Reagents

Folimycin was obtained from Calbiochem. iNOS antibody, monoclonal anti-β-actin–peroxidase antibody and *Salmonella typhimurium* LPS were from Sigma. p65 and IκB-α antibodies were from Santa Cruz. TLR4 antibody was from Imgenix. GM130 antibody was from BD Biosciences. Recombinant murine interferon-γ (IFN-γ) was from Cytolab/Peprotech Asia. Fluorochrome conjugated and horseradish peroxidase (HRP) conjugated antibodies were obtained from Dianova and Bangalore Genie, respectively. ECL™ Western blotting reagents were from Amersham Biosciences. TNF-α ELISA kit was from eBioscience.

Treatment with folimycin and LPS

Elicited peritoneal macrophages were treated with specified concentration of folimycin for 4 h unless otherwise stated. After 4 h, LPS was added and incubated for various time periods. Folimycin was maintained in the medium until the end of the experiment unless otherwise stated. A LPS concentration of 1 μg/ml was used in all experiments.

Determination of nitrite concentration

NO was measured as its end product, nitrite, using the Griess reagent as described previously.[22] Aliquots (10^5 cells/well) were seeded in 96-well plates and specific treatment was given. After 12 h 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). Absorbance at 540 nm was measured in a microplate reader after 15 min. The concentration of nitrite in the culture supernatant was determined with reference to a sodium nitrite standard curve. Correction of the NO concentration was not performed because there was no significant difference in the cell number and viability in all experiments.

Determination of TNF-α concentration

Elicited peritoneal macrophages were pretreated with 25 nM folimycin for 4 h after which they were induced with 1 μg/ml of LPS. After 3 h or 12 h, supernatants were collected and TNF-α concentration was estimated using 'mouse TNF-alpha ELISA Ready-SET-Go!' from eBioscience. The ELISA was done according to manufacturer's instructions.

RT-PCR

Total RNA from elicited peritoneal macrophages was isolated using TRI reagent (Sigma) after specific treatment. RNA (1 μg) was reverse transcribed using reverse transcription system (Promega). cDNA thus generated was used to analyze iNOS, TLR4 and GAPDH expression by PCR (30 cycles) using gene specific primers (Sigma). Densitometric image analysis was done using Multi Gauge software and the density values of TLR4 were normalized to that of GAPDH. Primers used to amplify iNOS cDNA were 5′-aagtcaaatcctaccaaagtga-3′ and 5′-ccataatactggttgatgaact-3′. Primers used to amplify TLR4 cDNA were 5′-tggcatcatcttcattgtcc-3′ and 5′-gcttagcagccatgtgttcc-3′. Primers used to amplify GAPDH cDNA were 5′-ggcaaattcaacggcacagt-3′ and 5′-agatggtgatgggcttccc-3′.

Immunoblotting analysis for iNOS and IκB-α

Elicited peritoneal macrophages were seeded in 35-mm plastic dishes (10^6 cells/dish) and pretreated with 25 nM

folimycin for 4 h. After 4 h, 1 μg/ml of LPS was added and incubated for 30, 60 and 120 min (for IκB-α) 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% 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 iNOS or IκB-α antibody and respective HRP conjugated secondary antibodies. Immune complexes were detected using an enhanced chemiluminescence reagent.

Flow cytometry

Elicited peritoneal macrophages were seeded in 35-mm plastic dishes (10^6 cells/dish). After specified treatment, cells were harvested and stained with TLR4-specific antibody diluted in blocking buffer (2% BSA and 2%

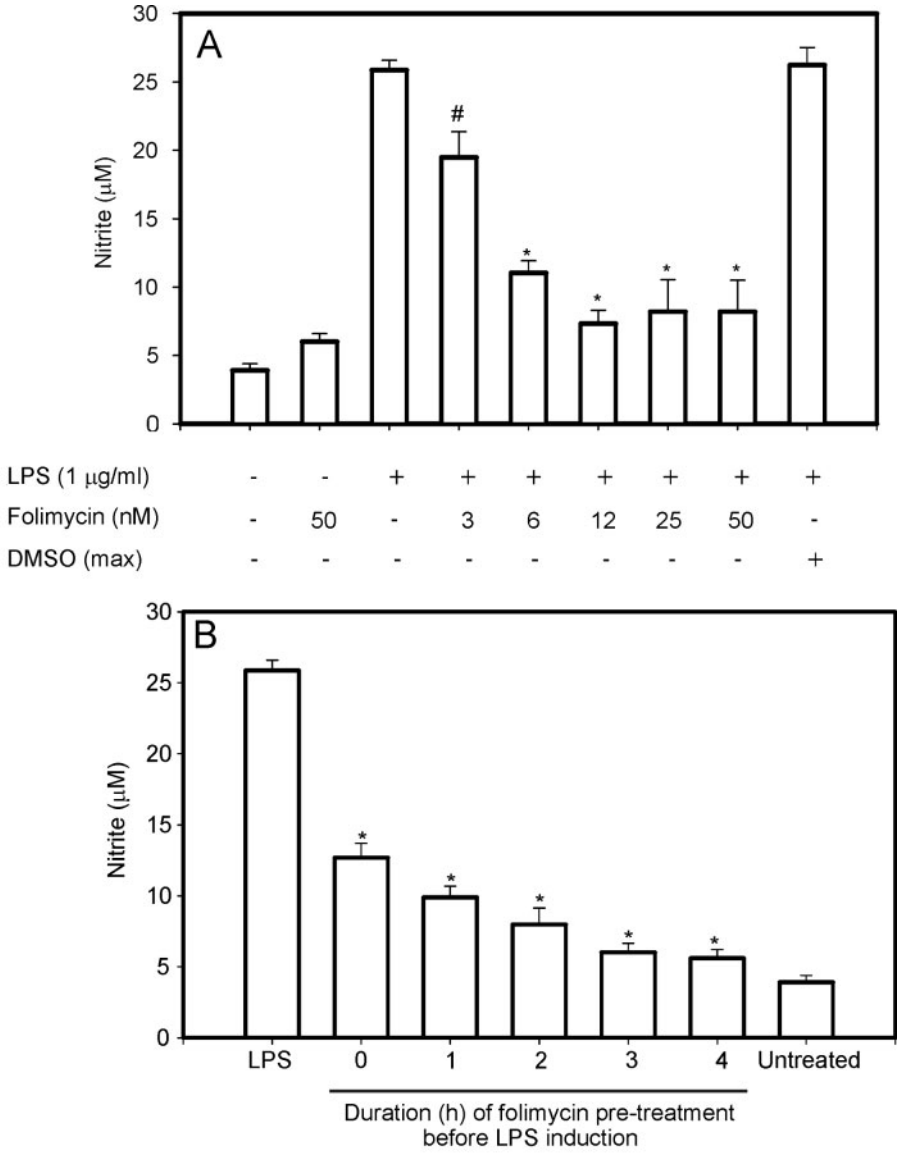

Fig. 1. (A) Folimycin inhibits LPS-induced nitric oxide production in a dose-dependent manner. Peritoneal macrophages were pretreated with indicated concentrations of folimycin for 4 h. After 4 h, 1 μg/ml of LPS was added and incubated for 12 h. DMSO (max): vector control for 50 nM folimycin. (B) Inhibition of LPS-induced nitric oxide production by folimycin is directly proportional to the duration of pretreatment. Peritoneal macrophages were pretreated for the indicated time periods with 25 nM folimycin after which 1 μg/ml of LPS was added and incubated for 12 h. Folimycin was maintained till the end of incubation in both the experiments. Statistical significance was defined as: $^{\#}P < 0.05$, $^{*}P < 0.01$. (Student's *t*-test). The graphs are representative of two independent experiments done in triplicate samples.

goat serum in PBS) for 1 h. Cells were washed twice with PBS and incubated for 1 h with specific secondary antibody diluted in blocking buffer. To investigate total TLR4, antibodies were diluted in blocking buffer containing 0.1% saponin. Incubations with antibodies were done at 4°C. After secondary antibody staining, cells were washed three times with PBS and resuspended in 300 µl PBS. Cells were then analyzed by flow-cytometer (BD FACScan) and the data were analyzed using WinMDI software. Appropriate isotype control antibodies were used in each experiment.

Confocal laser-scanning microscopy

Peritoneal macrophages (2–3 x 10^5) were seeded on cover-slips in a 24-well plate. After specific treatment, cells were fixed using 3.5% paraformaldehyde for 20 min. After washing three times with PBS, cells were incubated with specific antibody (GM130/TLR4/p65) 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 with appropriate secondary antibody diluted in blocking buffer. Then the cells were washed three times with PBS and the cover-slips were mounted on glass slides. Propidium iodide (1 µg/ml) was used to stain the nucleus. Samples were analyzed using confocal laser-scanning microscope (Zeiss LSM Meta). Adobe Photoshop 7 was used to adjust the contrast and brightness of images.

RESULTS

Folimycin inhibits LPS-induced NO production in elicited peritoneal macrophages

To understand the role of V-ATPases in LPS signaling, we used folimycin which is a specific and potent inhibitor of V-ATPases. Initially, we investigated the effect of folimycin on LPS-induced NO production in elicited peritoneal macrophages. As shown in Figure 1A, we

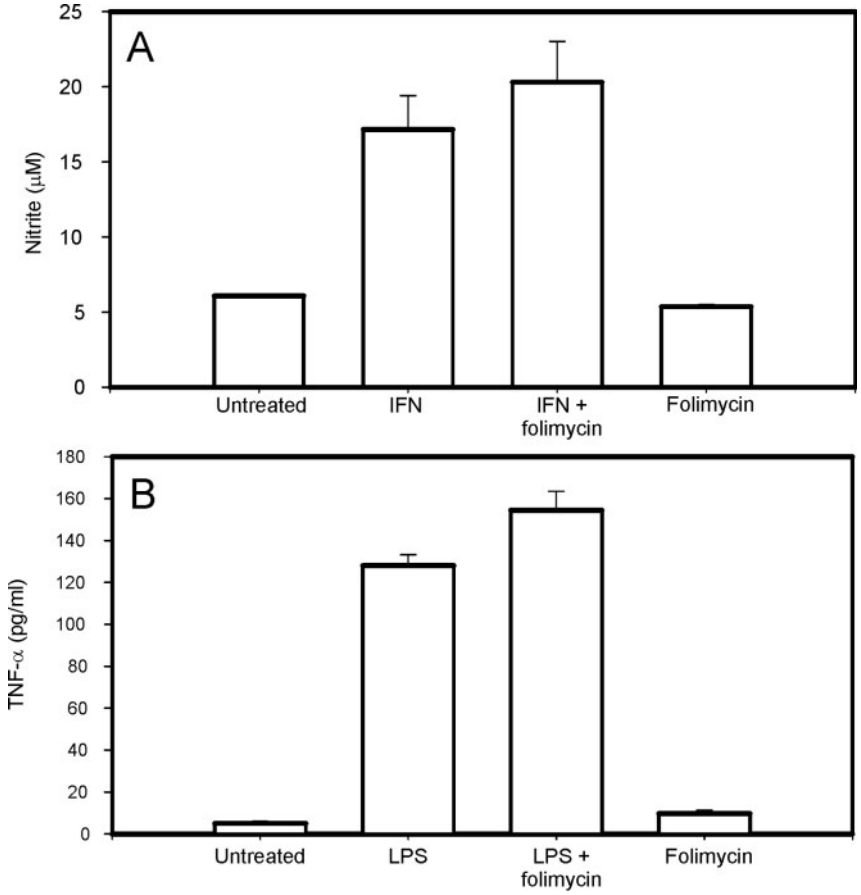

Fig. 2. (A) Folimycin does not inhibit IFN-γ induced nitric oxide production. Peritoneal macrophages were pretreated with 25 nM folimycin for 4 h after which 100 U/ml IFN-γ of was added and incubated for 12 h. (B) Folimycin does not inhibit LPS-induced TNF-α production. Peritoneal macrophages were pretreated with 25 nM folimycin for 4 h and cells were incubated with LPS for 3 h after which supernatant was collected and TNF-α was measured by ELISA. Folimycin was maintained till the end in both the experiments. Results are representative of two independent experiments.

observed that folimycin can inhibit LPS-induced NO production in a dose-dependent manner in the concentration range of 3–50 nM. Cells were pretreated with different concentrations of folimycin for 4 h. They were then induced with 1 μg/ml of LPS for 12 h and folimycin was maintained in the medium till the end of the incubation.

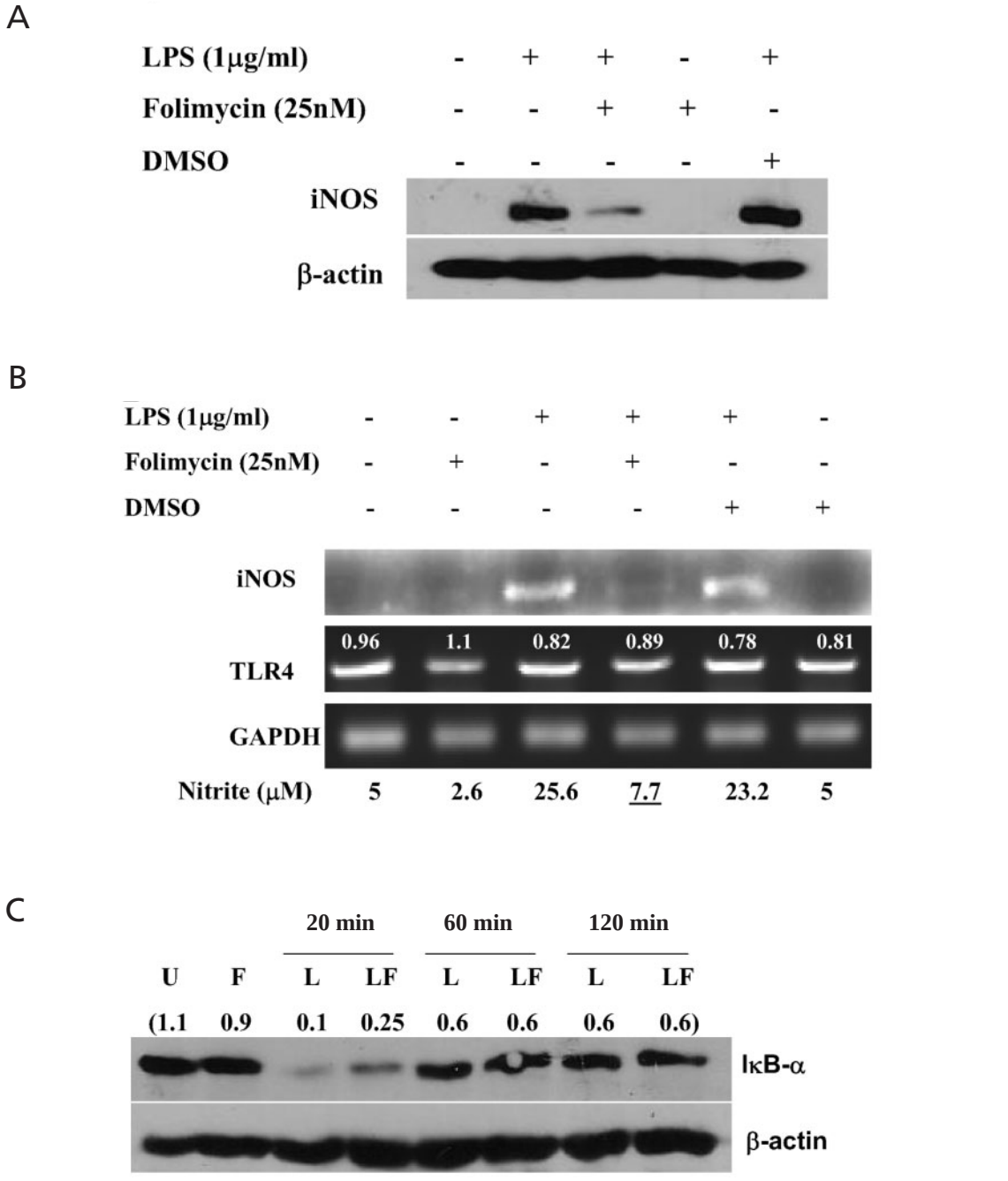

Fig. 3. (A) Folimycin inhibits LPS-induced iNOS protein expression. Peritoneal macrophages were either induced with LPS for 12 h or pretreated with 25 nM folimycin/DMSO for 4 h followed by induction with LPS for 12 h. After 12 h, total protein was extracted and subjected to Western blot analysis to check iNOS protein levels. (B) Folimycin inhibits LPS-induced iNOS mRNA expression. Experimental conditions were as for (A). After specific treatments, total RNA was extracted and reverse transcriptase PCR was done to check the expression level of iNOS mRNA. Culture supernatant was used to measure nitric oxide. Numbers inside the TLR4 expression panel indicate the values obtained after densitometric image analysis in which density values of TLR4 were normalized with those of GAPDH. (C) Folimycin inhibits LPS-induced degradation of IκB-α. Peritoneal macrophages were either induced with LPS for 20, 60 and 120 min or pretreated with folimycin for 4 h followed by LPS induction for 20, 60 and 120 min. Then, the total protein was extracted from the cell and subjected to Western blot analysis to check IκB-α levels. Numbers in parenthesis are the values obtained after densitometric image analysis in which density values of IκB-α were normalized to those of β-actin. U, untreated; L, LPS; F, folimycin. (A) is representative of 4 independent experiments and (B) and (C) are representative of two independent experiments.

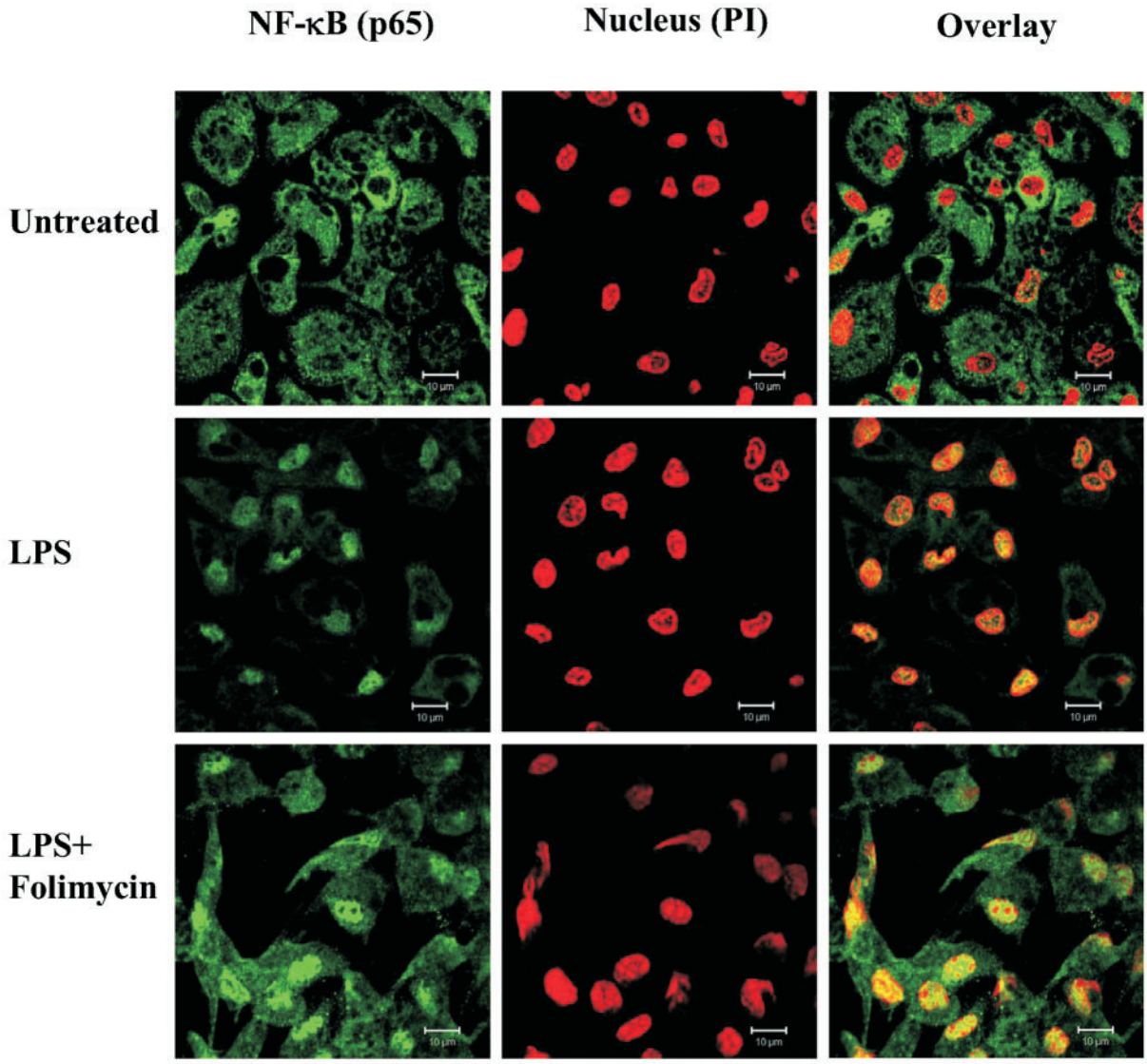

Fig. 4. Confocal laser-scanning microscopic images showing the localization of NF-κB (p65) with respect to the nucleus. Peritoneal macrophages were either induced with LPS for 30 min or pretreated with folimycin for 4 h followed by LPS induction for 30 min. After this, cells were fixed and stained for NF-κB (p65) using a specific antibody and for the nucleus using propidium iodide. Scale bar corresponds to 10 μm. Images shown are representative fields of two independent experiments.

DMSO in which folimycin was dissolved did not show any effect. This inhibition was also dependent on the duration of pretreatment with folimycin before LPS stimulation. Maximum inhibition was observed when pretreatment was given with 25 nM folimycin for 4 h before LPS stimulation (Fig. 1B). However, the inhibition by folimycin was not complete. Folimycin did not show any cytotoxicity at the concentrations used as indicated by MTT assay (data not shown). Folimycin did not inhibit IFN-γ induced NO production suggesting that the inhibition is specific to the LPS signaling pathway (Fig. 2A). This result also ruled out the possibility of direct interference with the NO assay by folimycin. The inhibitory action of folimycin on LPS-induced NO production was irreversible in agreement with a previous study (data not shown).[23] In contrast to the previous report, we did not observe any NO production when cells were treated with folimycin alone.[24] Surprisingly, folimycin did not show any effect on LPS-induced TNF-α production (Fig. 2B).

In the above experiments, NO was measured using the Griess reagent which actually measures nitrite, a stable end-product of NO oxidation. In order to know the NO level, which has very short half-life of few seconds, we used 4,5-diaminofluorescein diacetate and confirmed that folimycin inhibits LPS-induced NO production (data not shown).

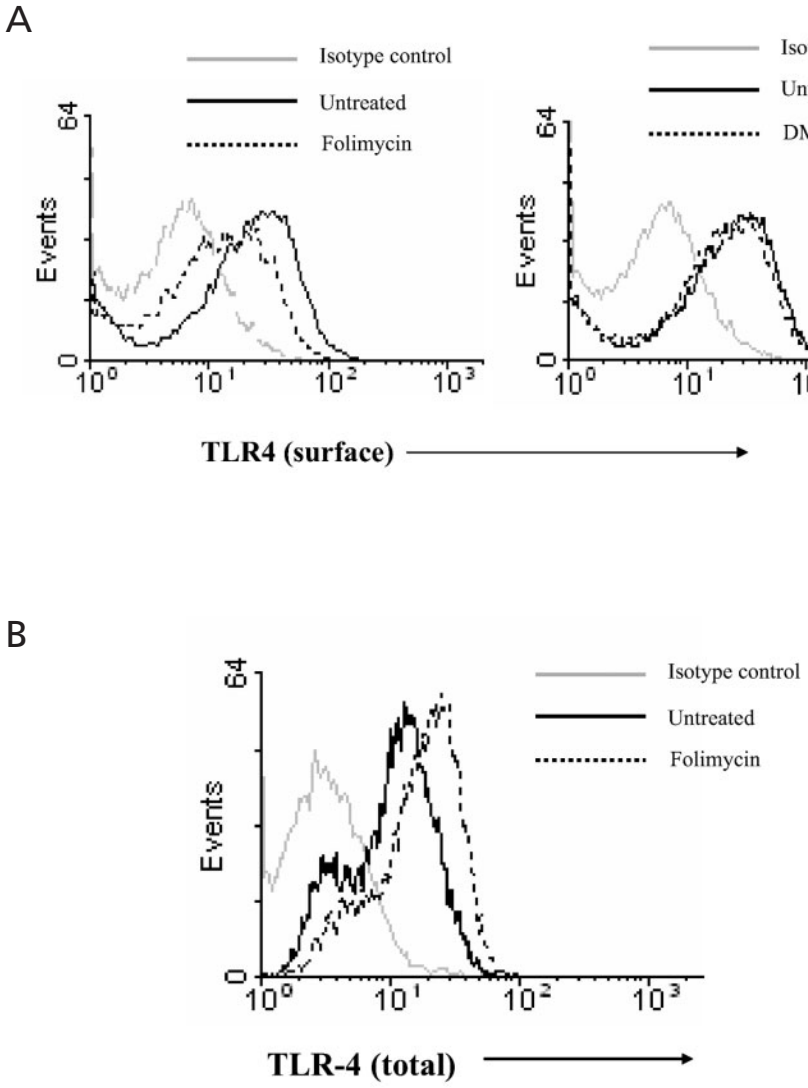

Fig. 5. (A) Folimycin inhibits surface expression of TLR4. Peritoneal macrophages were treated with 25 nM folimycin or DMSO for 4 h, stained for TLR4 using a specific antibody and analyzed by flow cytometer. (B) Folimycin increases total TLR4. Peritoneal macrophages were treated with 25 nM folimycin for 4 h and stained for TLR4 using a specific antibody dissolved in blocking buffer containing 0.1% saponin. Stained cells were analyzed by flow cytometer. (A) is representative of five independent experiments and (B) is representative of two independent experiments.

Folimycin inhibits LPS-induced expression of iNOS protein and iNOS mRNA

The next step was to dissect the LPS signaling pathway leading to NO production in order to determine where folimycin acts. As LPS-induced NO is synthesized by the iNOS enzyme, we looked at the iNOS protein and mRNA levels. Western blot analysis showed significantly reduced iNOS protein expression upon LPS induction in cells pretreated with folimycin (Fig. 3A). Reverse transcriptase-PCR analysis showed that LPS-induced iNOS mRNA expression was also significantly reduced in cells pretreated with folimycin (Fig. 3B). However, as evident in Figure 3A,B, folimycin did not cause complete inhibition of iNOS mRNA and protein expression. This is consistent with its effect on NO production (Fig. 1A,B). TLR4 mRNA expression was unaltered upon folimycin treatment (Fig. 3B). These results and the inability of folimycin to inhibit IFN-γ induced NO production suggest that the level of inhibition is at transcriptional or pre-transcriptional level.

Folimycin inhibits LPS-induced NF-κB activation

Activation of NF-κB is an important event in LPS signaling. LPS signaling leads to phosphorylation and subsequent degradation of IκB-α releasing NF-κB which translocates into the nucleus and activates its target genes such as iNOS and TNF-α.[12,25] In order to investi-

Table 1. Flow cytometric analysis of TLR4 surface expression in folimycin-treated cells

Experiment number	Mean fluorescence intensity	
	Untreated cells	Folimycin-treated cells
1	13.57	11.62
2	10.85	4.87
3	14.70	9.50
4	18.90	14.80
5	18.45	11.15
Mean ± SE	15.29 ± 1.52	10.39 ± 1.63

Statistical significance, $P < 0.01$ (Student's paired t-test).

gate whether folimycin inhibits NF-κB activation, we looked at the level of IκB-α which undergoes degradation upon LPS induction by Western blot analysis. Cells untreated with folimycin but induced with LPS for 20 min showed decreased IκB-α. However, in cells which were pretreated with folimycin and induced with LPS, there was less reduction in IκB-α levels indicating reduced activation of NF-κB. After 60 min and 120 min of LPS induction, there was no difference in the IκB-α levels between cells treated and untreated with folimycin (Fig. 3C). Direct demonstration of NF-κB localization after folimycin treatment was done using immunofluorescence microscopy. p65, a component of NF-κB, was found only in the cytoplasm in untreated cells. In LPS-induced cells, p65 was observed mainly in the nucleus as expected. In cells which were pretreated with folimycin and induced with LPS, p65 was observed both in the nucleus and the cytoplasm indicating reduced mobilization of p65 into the nucleus because of reduced activation of NF-κB (Fig. 4).

Folimycin reduces surface localization of TLR4

It is reported that folimycin and related compounds block the surface expression of virus envelope glycoproteins.[20,21,26,27] Based on this fact, we hypothesized that folimycin may be blocking the surface expression of LPS receptor molecules. To test this hypothesis, we analyzed the surface expression of TLR4 by flow cytometry. We observed about a 32% decrease in the surface TLR4 expression after folimycin treatment for 4 h when compared to untreated cells (Fig. 5A and Table 1). This inhibition was dependent on the duration of folimycin treatment. Maximum inhibition was observed in cells treated with folimycin for 4 h; 6 h and 8 h of treatment did not show any further decrease in TLR4 surface expression (data not shown). However, folimycin did not inhibit the surface expression of CD11b and MHC I (data not shown) which are not involved in LPS signaling suggesting that this particular action of folimycin is not a general phenomenon. LPS is known to affect the surface expression of TLR4.[28,29] However, we did not observe any change in the surface expression of TLR4 upon LPS treatment and the presence of LPS did not affect the inhibitory action of folimycin on surface expression of TLR4 (data not shown).

After confirming that there is inhibition of surface localization of TLR4, we looked at the total TLR4 level by staining the cells for TLR4 in the presence of saponin which permeabilizes the cells. There was a significant increase in the TLR4-positive population (Fig. 5B).

After showing that folimycin inhibits surface expression of TLR4, we looked at the intracellular distribution pattern of TLR4 in folimycin-treated cells. There was no change in the distribution of TLR4 with respect to Golgi apparatus (Fig. 6). As evident from Figure 6, TLR4 molecules were localized to the periphery of the cell. Figures 5A and 6, together, suggest that TLR4 molecules exit from Golgi apparatus but fail to reach the outer surface of the plasma membrane in folimycin-treated cells. Interestingly, we observed highly dilated Golgi apparatus in folimycin-treated cells which was consistent with previous reports.[30–32].

DISCUSSION

In this study, we have demonstrated two previously unknown effects of folimycin in murine macrophages; one is inhibition of LPS-induced NO production and another is reduction of surface localization of TLR4. The ability of folimycin to inhibit LPS-induced NO production but not TNF-α production was unexpected as NF-κB is involved in the regulation of both iNOS and TNF-α transcription. This discrepancy was also observed with lower LPS concentrations (100 ng/ml). Such discrepancy in the regulation of LPS-induced iNOS and TNF-α expression has been reported earlier.[33–35] It has been shown that when LPS pretreated murine peritoneal macrophages become refractory to subsequent LPS-induced NO production, the secondary LPS stimulated TNF-α production is markedly enhanced; when they become refractory to subsequent LPS-induced TNF-α production, the secondary LPS-stimulated NO production is markedly enhanced.[33] In addition, murine peritoneal macrophages treated with pertussis toxin markedly enhance LPS-induced TNF-α production but inhibit LPS-dependent NO production under the same conditions.[34] These results indicate that LPS-induced TNF-α and NO productions by macrophages are differentially regulated.

Treatment of peritoneal macrophages with folimycin also caused reduced degradation of IκB-α and reduced mobilization of p65 into the nucleus after LPS stimula-

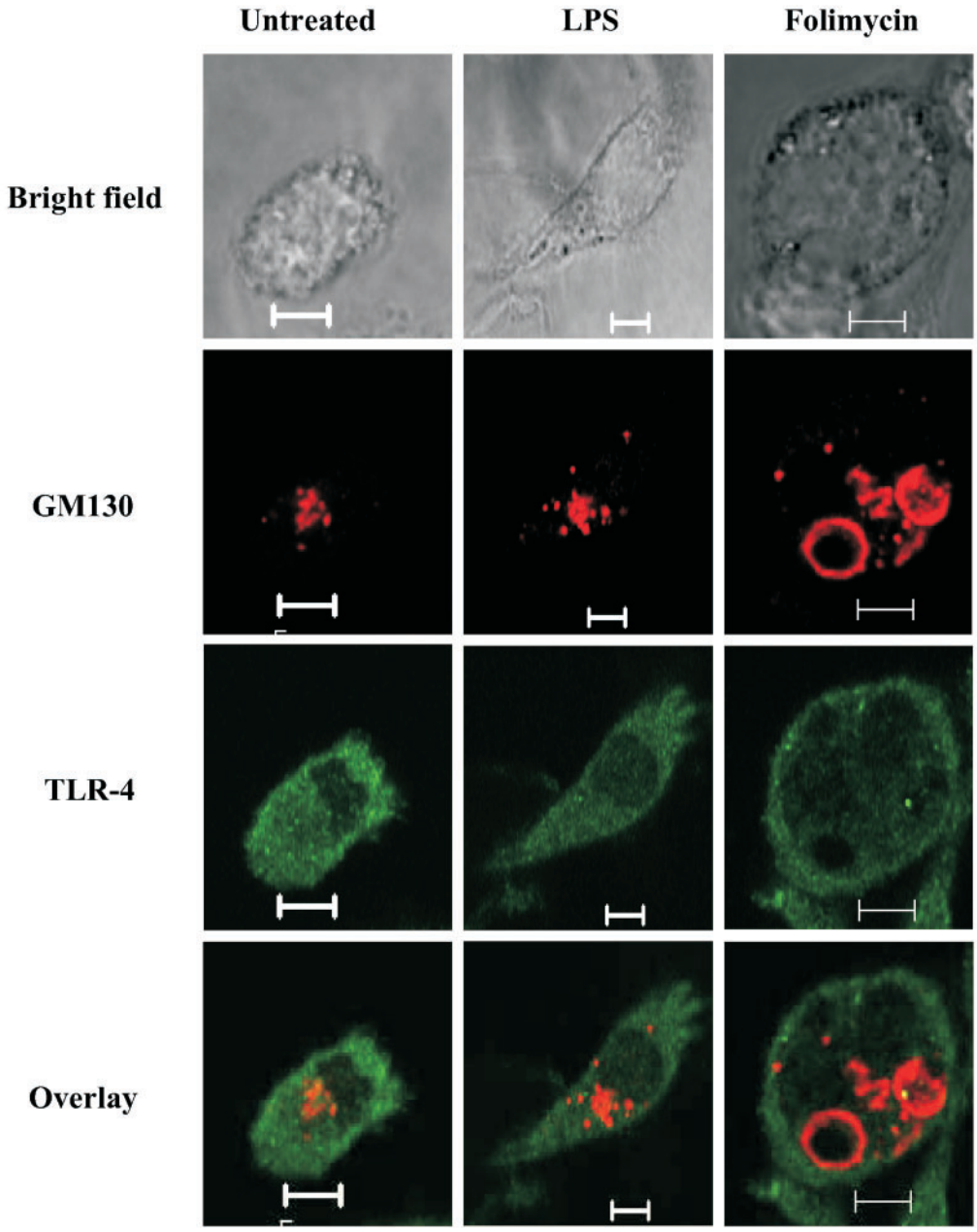

Fig. 6. Confocal laser-scanning microscopic images showing the localization of TLR4 with respect to the Golgi apparatus. Peritoneal macrophages were treated with LPS or folimycin for 4 h, fixed and stained for Golgi apparatus using GM130 antibody and for TLR4 using a specific antibody. Stained cells were observed with a confocal laser-scanning microscope. Folimycin-treated cells did not show any change in TLR4 distribution but showed dilated Golgi apparatus. Scale bar corresponds to 5 μm. Images are representative of two independent experiments.

tion indicating reduced activation of NF-κB upon LPS stimulation. Probably this reduction was sufficient to induce TNF-α but not enough to induce complete iNOS expression. In addition to this, there may be some other unknown mechanism(s) through which folimycin can inhibit LPS-induced iNOS expression but not TNF-α expression. Though TNF-α expression is known to be dependent on the activation of the NF-κB pathway, it is reported that unlike other LPS-inducible promoters that contain NF-κB binding sites, these sites from the TNF-α promoter are neither required nor sufficient for LPS induced activation.[36] Moreover, it has been shown in a desensitized human monocytic cell line that the expression of TNF-α was dramatically reduced upon subsequent LPS stimulation in spite of the mobilization of transcription factor NF-κB into the nucleus.[37]

Bafilomycin and folimycin which are specific inhibitors of V-ATPases are reported to induce NO production in RAW 264.7 cells which are murine macrophage-like cells.[24] However, in contrast to this report, we did not observe any NO production or iNOS mRNA expression upon folimycin treatment. This contrasting result may be because experiments in this study were carried out on freshly isolated primary culture of murine peritoneal macrophages.

TLR3, TLR7 and TLR9 signaling is reported to be pH dependent. These three molecules which are located in endosomes recognize specific nucleic acid patterns found in viral and bacterial pathogens and activate immune response. Chloroquine and bafilomycins which can increase the pH of endosomes can inhibit the signaling mediated through TLR3, TLR7 and TLR9 receptors.[14–16] TLR4 is located mainly on the cell membrane and, along with CD14 and MD-2, recognizes LPS molecules which come in contact with the cell. It is well established that TLR4 is essential for LPS signaling.[3] It was quite surprising to observe that folimycin can inhibit LPS-induced NO production as TLR4 is localized on the cell membrane unlike TLR3, TLR7 and TLR9 which are all present inside endosomes. Nonetheless, this finding indicated that the mechanism of inhibition of TLR4 signaling by folimycin is different from that of TLR3, TLR7 and TLR9 signaling.

Acidic pH in cellular compartments like the Golgi apparatus and endosomes is required for the efficient sorting and trafficking of proteins and lipids along the biosynthetic and endocytic pathways.[18,19] Acidification of these compartments is regulated and maintained by the action of V-ATPases in combination with various ion channels and transporters; thus, pharmacological agents which inhibit V-ATPases disturb normal biosynthetic and endocytic pathways.[38] Folimycin, SS33410 and bafilomycin are specific inhibitors of V-ATPases and are known to affect intracellular processing and trafficking of glycoproteins and recycling and externalization of some surface receptors.[20,21,26,27,30,39–42]. Our observation that folimycin inhibits surface localization of TLR4 is in accordance with these reports as TLR4 is also a glycoprotein. In fact, human TLR4 carries *N*-linked carbohydrates at nine potential glycosylation sites and these *N*-linked glycosylations are required for cell surface expression and binding of LPS. TLR4 mutants lacking multiple *N*-linked carbohydrates do not respond to LPS.[43] Moreover, MD-2, another essential component of LPS receptor complex, also contains two *N*-glycosylated sites and glycosylations at these sites are required to bind and respond to LPS.[43,44] CD14, another component of LPS receptor complex, is also a glycosylated protein.[45] Alteration in the pH of the Golgi apparatus by folimycin might affect the glycosylation of these proteins which constitute the LPS receptor complex and render them unavailable or non-responsive to LPS. Further investigation of the glycosylation patterns of TLR4, MD-2 and CD14 in folimycin-treated cells has to be done to prove this hypothesis.

The increase in total TLR4 molecules in folimycin-treated cells is probably because of intracellular accumulation of TLR4 molecules due to decreased recycling.

There was no observable difference in the intracellular distribution of TLR4 molecules in relation to the Golgi apparatus in folimycin-treated cells suggesting that TLR4 molecules, probably defective in their glycosylation, exit the Golgi apparatus but fail to reach the outer surface of the plasma membrane. However, we could observe dilated and swollen Golgi apparatus in folimycin-treated cells. In accordance with our results, dilated Golgi apparatus was observed in previous reports upon bafilomycin treatment in different cell lines.[30–32] This was also observed in colorectal cancer where intra-Golgi pH was altered.[46] Moreover, dilated Golgi apparatus has been observed along with defective glycosylation of glycoproteins.[17,31,46] This finding, along with the reports above, suggests that the function of the Golgi apparatus (probably the glycosylation process) is compromised in folimycin-treated cells. More experiments are required on the function of the Golgi apparatus to prove this point.

Being a vasodilator, nitric oxide contributes to vascular hyporeactivity, microvessel damage and organ dysfunction in sepsis.[1] High levels of iNOS activity have been reported in tissues of human sepsis patients,[47] and are essential for epithelial tight junction dysfunction in endotoxemic mice.[48] Moreover, inhibition of nitric oxide production by iNOS has been shown to help in the treatment of hypotension associated with sepsis.[49] These reports show the significant role played by nitric oxide in sepsis. However, it was observed that the nitric oxide synthase inhibitor 546C88 increased the mortality in patients with septic shock probably because of non-selective inhibition.[50] Thus, compounds which can selectively block NO production by iNOS or NOS II (but not by eNOS and nNOS) are promising candidates for the treatment of sepsis. Folimycin, if used along with TNF-α antagonists, serves as a potential candidate for the treatment of sepsis.

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