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Bacteriology

Expression of inducible nitric oxide synthase induced by lipid-associated membrane proteins of Ureaplasma urealyticum is regulated by nuclear factor κB-mediated mechanism in murine macrophages

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Abstract The aim was to investigate the molecular mechanisms responsible for the inducible nitric oxide synthase (iNOS) gene expression stimulated by lipid associated membrane proteins (LAMPs) of *Ureaplasma urealyticum* (*U. urealyticum*). Detection of NO, the expression of iNOS and the activation of nuclear factor κB (NF-κB) in direct response to *U. urealyticum* LAMPs in a murine macrophages, the effects of pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB and of cycloheximide (CHX), a protein synthase inhibitor were available. The results indicated that *U. urealyticum* LAMPs stimulated mouse macrophages to express iNOS and thus produce NO in dose- and time-dependent manner by activating NF-κB. The expression of iNOS, NO production and the activation of NF-κB were inhibited by *U. urealyticum* LAMPs combination with PDTC or CHX. In conclusion, our findings suggest that *U. urealyticum* may be an etiological factor to certain diseases due to its ability to stimulate the expression of iNOS, which is probably mediated through the activation of NF-κB.

Key words: *Ureaplasma urealyticum* Lipid-associated membrane proteins Nuclear factor kappa B Inducible nitric oxide synthase

U. urealyticum is the smallest prokaryotic organism capable of self-replication. The tiny microorganism could be isolated from urogenital, placentas and the respiratory tracts of preterm infants. Moreover, *U. urealyticum* infection may be involved in non-specific urethritis (NSU), prostatitis, postpartum fever, infertility, pelvic inflammatory disease, neonatal pneumonia and even chronic lung disease (CLD) [1]. It is known that *U. urealyticum* lacks cell wall structure and contains abundant membrane proteins, but its pathogenicity is still unknown clearly. At present, it was reported that mycoplasmal lipid-associated membrane proteins (LAMPs) are exposed on the cell surface of mycoplasma, including *mycoplasma penetrans*, *mycoplasma salivarium*, *mycoplasma pirum*, *mycoplasma pneumoniae*, *and mycoplasma genitalium* [2, 3] and constantly interact with various elements in the environment, thus affecting the immune system.

In order to investigate the potential pathogenicity of *U. urealyticum*, we examined whether *U. ureaplasma* LAMPs could induce the RAW 264.7 cells to express iNOS and produce NO via the activation of NF-κB. In addition, we evaluated the effects of pyrrolidine dithiocarbamate (PDTC) or the protein synthase inhibitor cycloheximide

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(CHX) on the activation of NF-κB, the expression of iNOS and NO production.

MATERIALS AND METHODS

Cell culture and stimulation

The RAW 264.7 murine macrophages cells (obtained from ATCC) were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum, 50 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 ℃ in 5% CO_2 humidified air. The cells were detached and plated in 24-well plates at a density of 2×10^6 cells in fresh media and stimulated with 0.5 μg/ml to 4 μg/ml of LAMPs or in combination with 25 μM pyrrolidine dithiocarbamate (PDTC). To examine if LAMPs could induce NO production directly, the macrophages were incubated with 3 μg/ml LAMPs in combination with the protein synthase inhibitor cycloheximide (CHX) at a concentration of 1 μg/ml for 24 h.

Nitrite Assay

All supernatants were collected after stimulation and stored at -70 ℃ for NO analysis. The accumulation of NO_2^- was determined as an indicator of NO production in the medium. Nitrite was quantified by a spectrophotometric assay based on the Griess reaction. Briefly, The 50 μl supernate were mixed with an equal volume of Griess reagents Ⅰ and Ⅱ (Beyotime, Jiangsu, China) at room temperature. The absorbance was measured at 540 nm. The nitrite production was calculated from a standard curve sodium nitrite ($NaNO_2$).

Preparation of LAMPs from *U. urealyticum*

U. urealyticum serotype standard strain 4 (ATCC) was cultured in urea plasma broth medium to the start of stationary phage, and then pelleted by centrifugation. The preparation of LAMPs and the aqueous phage (used as a control) was performed as described previously by Wang[4]. The protein concentration of LAMPs was measured by using the bicinchoninic acid kit (Pierce, Rockford, IL). The preparations were preincubated for 2 h with 100 μg/ml polymyxin B sulfate prior to cell stimulation in order to eliminate endotoxins that had probably contaminated the solutions during preparation.

RT-PCR

Total RNAs were isolated from the different treated cells with Trizol reagent (Invitrogen, USA). The first strand cDNA was synthesized from 1 μg of total RNA using AMV reverse transcriptase and random 9-mers (TaKaRa, Dalian, China). The cDNA products were used for subsequent amplification by PCR, which was composed of 35 cycles of denaturation (94 ℃ for 30 s), annealing (56 ℃ for 30 s), and extension (72 ℃ for 1 min), using a thermocycler (Eppendorf, Germany) and oligonucleotide primers. The parallel expression of β-actin mRNA was tested under the same PCR conditions as an internal standard. The sequences of primers were as follows: iNOS, 5′-TTG GAG CGA GTT GTG GAT TGT-3′, 5′-GCC CTT TGT GCT GGG AGT C-3′ (652bp); β-actin, 5′-GTG GGC CGC TCT AGG CAC CAA-3′, 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ (540 bp). PCR products were electrophoresed in 1.2% agarose gel and stained with ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, PCR bands on the photograph of the gel were scanned using an ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and normalized the iNOS signal relative to the corresponding β-actin mRNA signal from the same sample. Data were expressed as the iNOS/β-actin ratio.

Western blotting analysis

To assess iNOS protein expression, total cell protein was extracted as follows: cells were lysed with cell lysis buffer [50 mM Tris-Cl (pH8.0), 150 mmol/ml NaCl, 0.02% NaN_3, 100 μg/ml PMSF, 1 μg/mL Aprotinin, 1% TX-100]. To evaluate NF-κB translocation, nuclear extracts were prepared as described previously [5]. Both of protein concentrations were determined by the bicincho-

ninic acid kit. For Western blotting analysis, 10 μg of protein per sample was separated by 8 % (for iNOS) or 10 % (for NF-κB) SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Amersham Biosciences UK). The membranes were incubated with the polyclonal rabbit antibody against mouse iNOS (Santa Cruz Biotechnology, USA) or NF-κB P65 (Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized by incubating with horseradish peroxidase-conjugates goat anti-rabbit IgG and enhanced chemiluminescence reagents (Amersham Biosciences UK). The protein bands in each line were photographed, and densitometrically quantified using the ImageQuant software.

Immunolocalization of NF-κB

Cells were plated on glass coverslips and incubated with 3 μg/ml LAMP or 10 μl of aqueous for 2 h for evaluating NF-κB. After treatment, the cells were fixed with cold methanol and acetone. Intracellular NF-κB proteins were visualized by immunocytochemistry or indirect immunofluorescence using polyclonal rabbit anti-NF-κB p65 followed by biotinylated goat anti-rabbit IgG or fluorescein isothiocyanate-labeled goat anti-rabbit IgG (SABC, Beijing, China). Slides were viewed and photographed by using Nikon microscope (ECLIPSE TE2000-5, Japan).

Statistical analysis

The mean ±S. D. was determined for each treatment group in each experiment. The data were analyzed by a one-way ANOVA test followed by an independent sample *t* test using SPSS10. 0. A *P* value of less than 0. 05 was considered significant.

RESULTS

NO production and iNOS expression after treatment with U. urealyticum LAMPs

LAMPs prepared from *U. urealyticum* stimulated the RAW 264. 7 murine macrophages production of NO in a dose- and time-dependent manner (Fig. 1). After treatment with different concentrations of LAMPs, the mouse macrophages produced the variant amounts of NO. When the concentration of LAMPs was added from 0. 5 μg/ml to 4 μg/ml, the production of NO was markedly increased. The induced NO production could be detected in the conditioned media after 4 h of stimulation with LAMPs and reached peak levels at 32nd hour. To confirm that NO was produced by iNOS, the LAMPs-induced levels of iNOS expression were examined by using RT-PCR and Western blotting. The RAW 264. 7 cells were induced to express iNOS mRNA after treatment with LAMPs for 24 h, and iNOS mRNA levels decreased in the presence of 25 μM PDTC or 1 μg/ml CHX (Fig. 2). The levels of iNOS proteins showed similar patterns in the LAMPs-stimulated cells, as determined by Western blotting.

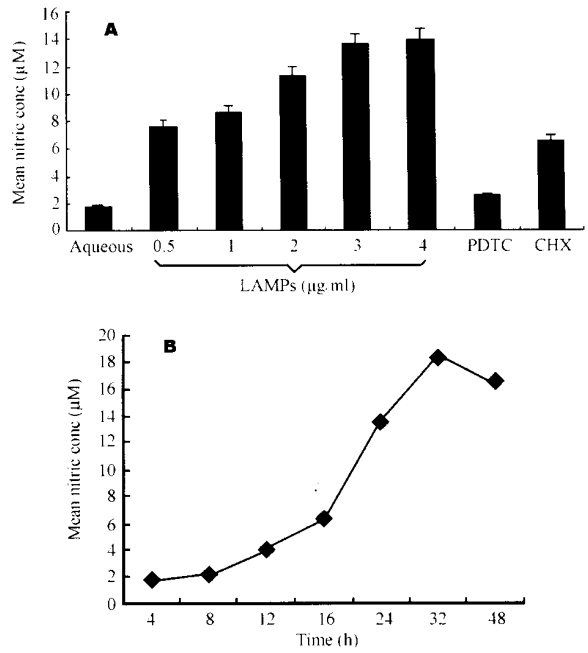

Fig 1. The effect of different concentrations (A) and different time (B) of LAMPs on NO productions in mouse macrophages.

Effects of PDTC or CHX on LAMPs-induced NO production and iNOS expression

To assess the effect of PDTC or CHX on LAMPs-stimulated NO production in RAW 264. 7 cells, the concentration of produced nitrite was determined by the Griess method. We found that PDTC or CHX significantly inhibited the production of

NO in mouse macrophages stimulated by LAMPs (Fig. 1A). The effects of PDTC or CHX on the expression of iNOS mRNA and protein showed similar patterns in LAMPs-stimulated cells, as determined by RT-PCR and Western blotting (Table 1).

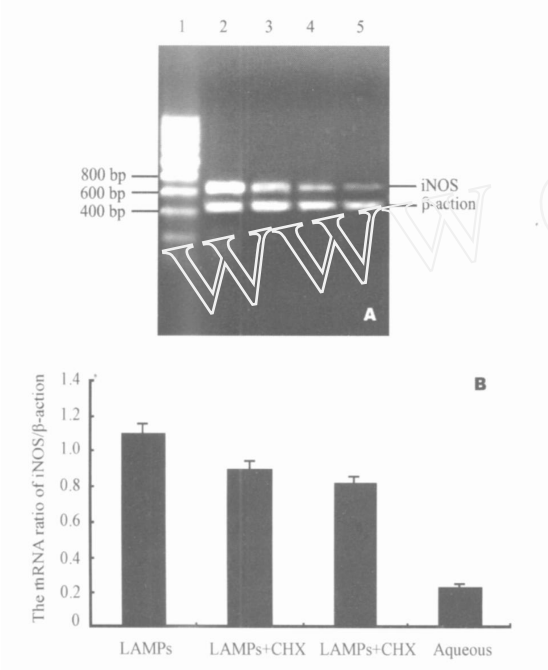

Fig 2. The effect of different treatment manner on iNOS mRNA expression.

A: Westing blotting. Lane 1: 200 bp marker; Lane 2: LAMPs; Lane 3: LAMPs + CHX; Lane 4: LAMPs + PDTC; Lane 5: Aqueous phase. B: The gray scale of iNOS/β-actin.

Table 1. Mean optical density value of iNOS and NF-κB proteins (n = 4, $\bar{x} \pm s$).

	iNOS	NF-κB
LAMPs	91.56 ±0.29	91.09 ±0.84
LAMPs + CHX*	80.45 ±0.54	74.66 ±0.27
LAMPs + PDTC*	75.68 ±0.18	59.55 ±0.46

$P < 0.01$. *group vs LAMPs group.

Activation of NF-κB by LAMPs

We determined the effect of LAMPs on NF-κB translocation in RAW 264.7 cells using immunocytochemistry, indirect immunofluorescence, and Western blotting. After treatment of macrophages with 3 μg/ml of LAMPs for 2 h, NF-κB p65 was observed to translocate from cytoplasm into nuclear whereas NF-κB p65 wasn't located in nuclear of cells with 10 μl of aqueous phase for 2 h (Fig. 3A). After treatment of macrophages with 10 μl of aqueous phase for 2 h, NF-κB p65 only appeared in the cytoplasm, whereas NF-κB p65 was located in the cytoplasm and nuclear of cells treated with 3 μg/ml of LAMPs for 2 h (Fig. 3B). In nuclear extracts, NF-κB activation was detected by Western blotting and found that LAMPs stimulated NF-κB translocation from cytoplasm into nuclear, the translocation was suppressed by PDTC and CHX (Table 1).

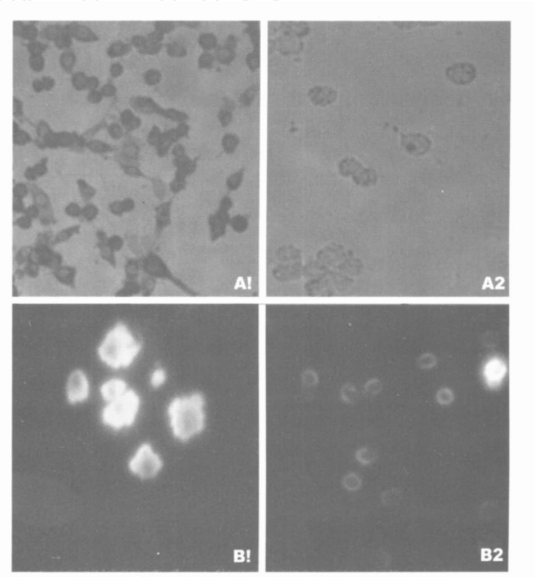

Fig 3. NF-κB p65 translocation was detected by immunocytochemistry (A) and indirect immunofluorescence (B) (×400).

A1, B1: LAMPs; A2, B2: Aqueous phase.

DISCUSSION

Mycoplasma are wall-less Gram-positive bacteria and lack lipopolysaccharide, peptidoglycan and lipoteichoic acid. However, some components of mycoplasma can activate macrophages to synthesize or secrete a series of inflammatory proteins associated with infections. For example, heat-killed *U. urealyticum* antigen and LAMPs derived from *M. penetrans*, an AIDS-associated mycoplasma, can stimulate macophages to produce NO [6, 7] via iNOS. Mycoplasmal lipid-associated membrane proteins (LAMPs) are exposed on the cell surface, highly antigenic, and are likely to be an important immunogenic target for hosts' responses in mycoplasmal infections. It is necessary that we in-

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vestigate the potential pathogencity of *U. urealyticum* LAMPs and the molecular mechanisms responsible for iNOS expression.

NF-κB is known as a widespread rapid-response transcription factor, because it regulates genes involved in inflammation, including cytokines, cell adhesion molecules, and enzymes (e.g., iNOS, COX-2) [8]. In unstimulated cells, NF-κB is present as an inactive heterodimer of the p50/p56 submits bound to the NF-κB inhibitory protein. Stimulation of macrophages with LAMPs leads to activation of NF-κB, allowing the translocation of NF-κB into nucleus, binding to DNA, and leading to activate the genes. Our data demonstrate that *U. urealyticum* LAMPs is a potent activator of NF-κB, as evidenced by the rapid and intense NF-κB activation in macrophages. This suggests that NF-κB activation may regulate a large number of genes transcription including iNOS.

The induction of iNOS is triggered and regulated by a series of signaling pathways including NF-κB transcription factor. Recent studies have demonstrated that there are NF-κB binding sites in the promoter region of iNOS gene [9]. The activated NF-κB can induce many defense-related genes transcriptionally. The family of NF-κB protein was required for the enhanced iNOS gene expression when macrophages were exposed to LPS and other signals. When NF-κB was activated, we detected the expression of iNOS by RT-PCR and Western blotting. The results reveal that LAMPs can markedly induce in macrophages the expression of iNOS mRNA and iNOS protein and generate large amounts of NO in time- and dose-dependent manner. NO is generated from L-arginine by three different NO synthases. The neural (nNOS) and the endothelial (eNOS) isoforms are constitutively expressed in selected tissues. The third, inducible and Ca^{2+}-independent NO synthase (iNOS), is expressed only following transcriptional activation of its gene, which occurs in acute and chronic inflammation.

Inhibiting high-output NO production by blocking iNOS expression or its activity may be a useful strategy for treatment of inflammatory disorders. We showed that both pyrrolidine dithiocarbamate (PDTC) and cycloheximide (CHX) supressed the activation of NF-κB induced by LAMPs and could significantly down-regulate iNOS expression and NO production. Since PDTC, a relatively specific inhibitor of the activation of NF-κB in macrophages, blocks the iNOS induction, it appears that NF-κB is involved in the induction of iNOS. The present study demonstrates that PDTC, an NF-κB inhibitor and antioxidant, has been shown to inhibit NF-κB, via interference with reactive oxygen metabolism, chelation of divalent metal ions, and changes in intracellular thiol levels [10, 11]. Whereas the reason cycloheximide inhibites NF-κB isn't still clear, and is probably that cycloheximide, as a protein synthase inhibitor, blocks the protein synthase to release endogenous cytokines [6].

In conclusion, the result implies that *U. urealyticum* may be an important pathogenic factor for host cells due to stimulation of the expression of iNOS and NO production, which is mediated through the activation of NF-κB.

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