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Pretreatment of Macrophages with Paclitaxel Inhibits iNOS Expression

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ABSTRACT. We demonstrate that paclitaxel, an antitumor agent derived from yew tree, inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. Previously, paclitaxel has been known to induce iNOS gene expression in macrophages. However, in this report we described that the pretreatment of macrophages with paclitaxel (0.1 μM) for 8 h inhibited LPS-induced iNOS gene expression. Pretreatment of RAW 264.7 cells with paclitaxel significantly inhibited LPS-stimulated nitric oxide (NO) production. Western immunoblot of iNOS and RT-PCR analysis showed that the decrease of NO was due to the inhibition of iNOS gene expression in RAW 264.7 cells. Immunocytochemical staining of iNOS further confirmed that pretreatment of macrophages with paclitaxel inhibited macrophage activation. Electrophoretic mobility shift assay showed that paclitaxel inhibited NF-κ/Rel DNA binding. Collectively, these series of experiments indicate that paclitaxel inhibits iNOS gene expression by blocking NF-κB/Rel activation.

Keywords: Paclitaxel, Macrophages, iNOS, NF-κB/Rel.

INTRODUCTION

Lipopolysaccharide (LPS) is a potent immune system activator which induces local inflammation, antibody production in severe infectious diseases such as septic shock (Rietschel and Brade, 1992). Macrophages play a central role in a host's defense against bacterial infection and are major cellular targets for LPS action. Stimulation of murine macrophages by LPS results in the expression of an iNOS, which catalyzes the production of large amounts of nitric oxide (NO) from L-arginine and molecular oxygen (Palmer *et al.*, 1988). NO, in turn, participates in the inflammatory response of macrophages (Hibbs *et al.*, 1987). The promoter of the murine gene encoding iNOS contains two κB binding sites, located at 55 and 971 bp upstream of the TATA box, respectively (Lowenstein *et al.*, 1993). It has been reported that protein binding to the kB site is necessary to confer inducibility by LPS (Xie *et al.*, 1994).

Paclitaxel, isolated from the bark of the yew tree, is one of the more promising agents for treatment of breast cancer (Rowinsky, 1994) and is shown to block cells at the G_2/M junction of the cell cycle (Blagosklonny *et al.*, 1996). The primary mechanism of action of paclitaxel is attributed to its ability to bind to microtubules and to prevent their assembly. In addition to the blockage of mitosis, paclitaxel also triggers cellular responses that mimic those induced by a potent activator of the innate immune system, LPS, such as tyrosine phosphorylation of mitogen-activated protein kinases, translocation of NF-κB, and induction of gene expression (Perera *et al.*, 1996; Das and White, 1997). In murine macrophages, paclitaxel can induce the expression of a series of cytokines, such as IL-1α, IL-1β, TNF-α, and iNOS (Mantheny *et al.*, 1992; Kim and Paik, 2005).

The objective of this study was to investigate the effects of paclitaxel on LPS-induced iNOS gene expression. Since iNOS gene expression requires NF-κB/Rel activation, we further analyzed the effect of paclitaxel on the DNA binding activity of NF-κB/Rel.

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MATERIALS AND METHODS

Materials

Paclitaxel and LPS from *Salmonella thyposa* were purchased from Sigma (St. Louis, MO, USA). IFN-γ was purchased from R&D systems (Minneapolis, MN,

USA). Reagents used for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Anti-iNOS antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Cell culture

RAW 264.7 cells (murine macrophage line) were purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then cultured in the presence of 5% CO_2 at 37°C.

Western immunoblot analysis

Whole cell lysates (20 μg) were separated by 10% SDS-PAGE and then electro-transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were preincubated for 1 h at room temperature in Tris-buffered saline (TBS), pH 7.6 containing 0.05% Tween-20 and 3% bovine serum albumin. The nitrocellulose membranes were incubated with iNOS-specific antibody. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham).

RT-PCR

Total RNA was isolated using Tri Reagent (Molecular Researh Center, Cincinnati, OH, USA) as described previously (Chomczynski and Mackey, 1995). The forward and reverse primer sequences are: iNOS: 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3', 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3' and β-actin: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. Equal amounts of RNA were reverse-transcribed into cDNA using oligo(dT)15 primers. PCR was performed with cDNA and each primer. Samples were heated to 94°C for 5 min and cycled 30 times at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, after which an additional extension step at 72°C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3 : 1 gels (FMC Bioproducts, Rockland, ME) followed by staining in ethidium bromide. The iNOS and β-actin primers produce amplified products at 311 bp and 349 bp, respectively.

Nitrite quantitation

NO_2^- accumulation was used as an indicator of NO production in the medium as previously described (Green *et al.*, 1982). Cells were plated at 5×10^5 cells/ml in 96-well culture plates and treated with LPS and/or paclitaxel. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an O.D. reading at 550 nm.

Statistical analysis

The mean ± SD was determined for each treatment group in a given experiment. When significant differences occurred, treatment groups were compared to the vehicle control using a Dunnett's two-tailed *t* test (Dunnett, 1955).

RESULTS

Inhibition of iNOS gene expression by pre-treatment with paclitaxel in macrophages

To analyze the effect of pre-treatment with paclitaxel on iNOS gene expression, we treated RAW 264.7 cells with low dose of paclitaxel (0.1 μM) for 1, 2, 4, 8, and 24 h before the treatment with LPS (200 ng/ml) for 16 h. The expression level of iNOS gene was monitored by Western immunoblot analysis. As shown in Fig. 1A, iNOS protein production was inhibited by paclitaxel treatment in a pre-incubation time-dependent manner. Since 8 h

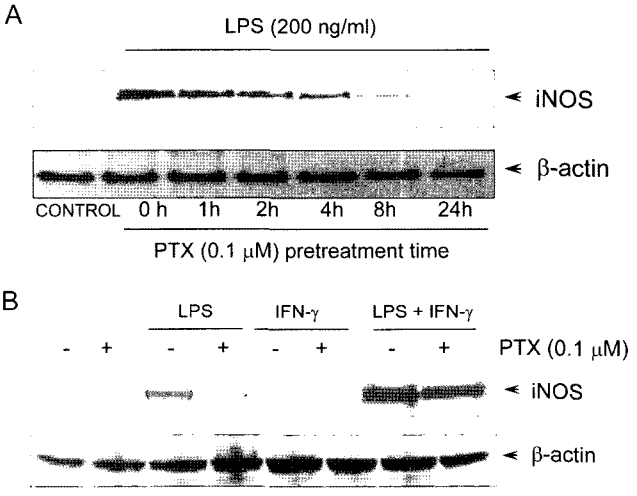

Fig. 1. *Inhibition of iNOS expression by paclitaxel in macrophages.* (A), RAW 264.7 cells were pretreated with paclitaxel (0.1 μM) for the indicated times before the treatment with LPS (200 ng/ml) for 16 h. (B), Cells were pretreated with paclitaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 16 h. Cell extracts were isolated and subjected to Western immunoblot assay using iNOS- and β-actin-specific antibody.

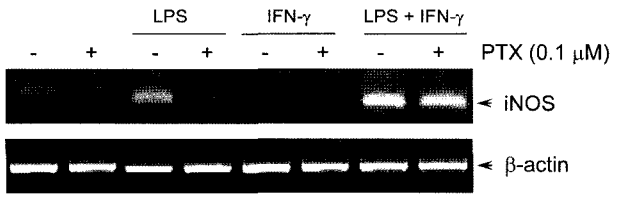

Fig. 2. Inhibition of iNOS gene expression by paclitaxel in macrophages. Cells were pretreated with paclitaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 16 h. Total RNA was isolated and analyzed for the magnitude of mRNA expression of iNOS using RT-PCR.

preincubation strongly inhibited LPS-induced iNOS expression, we chose 8 h pre-treatment and assessed the effect of paclitaxel pretreatment on LPS and/or IFN-γ-induced iNOS expression. As shown in Fig. 1B, paclitaxel significantly inhibited iNOS protein production by LPS. IFN-γ (100 ng/ml) did not increase the iNOS expression. However, co-treatment of LPS and IFN-γ synergistically increased the amount of iNOS, which was reduced partially by paclitaxel pretreatment (Fig. 1B).

To further analyze the mechanism by which paclitaxel inhibited iNOS protein production, we assessed the effect of pretreatment with paclitaxel on iNOS gene expression by RT-PCR. Consistent with the Western immuoblot results, the transcription of iNOS mRNA by LPS was inhibited by 8 h pretreatment with paclitaxel (0.1 μM) (Fig. 2). The result reflected that the decreased production of iNOS in macrophage was mediated by the inhibition of iNOS gene expression. Control β-actin was constitutively expressed and was not affected by the treatment of paclitaxel. These results indicate that paclitaxel decreases the gene expression of iNOS, which is involved in inflammation (Hibbs *et al.*, 1987).

Inhibition of nitrite production by pretreatment with paclitaxel in macrophages

RAW 264.7 cells were pretreated with paclitaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 24 h. When culture supernatants were isolated and analyzed for nitrite production, we found that pretreatement with paclitaxel (0.1 μM) significantly inhibited both LPS- and IFN-γ-induced nitrite generation (Fig. 3A). The synergism between LPS and IFN-γ on nitrite generation was inhibited slightly by the pretreatment with paclitaxel. The induction in nitrite generation by LPS was inhibited by paclitaxel in a dose-dependent manner (Fig. 3B).

Immunohistochemical staining of iNOS confirmed that the decrease of NO was due to the inhibition of iNOS production (Fig. 4). Immunohistochemical staining using an antibody specific for murine iNOS showed that immunoreactivity of iNOS on unstimulated cell was localized along the margin (arrows) of the cytoplasm. LPS treatment strongly stimulated the RAW 264.7 cells to express iNOS in the cytoplasm. The induction of iNOS in the cytoplasm was significantly inhibited by the pretreatment with paclitaxel (Fig. 4).

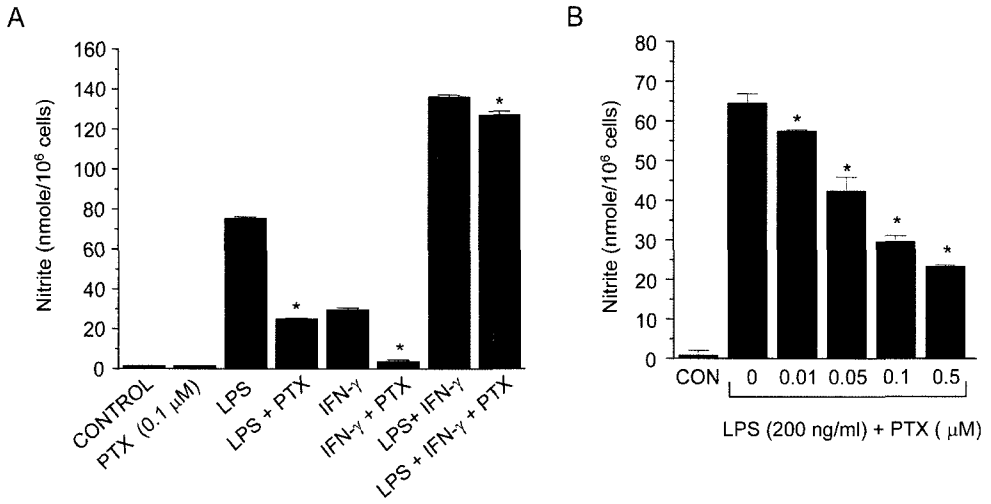

Fig. 3. Inhibition of nitrite production by paclitaxel in macrophages. (A), RAW 264.7 cells were pretreated with paclitaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 24 h. (B), Cells were pretreated with indicated concentrations of paclitaxel for 8 h before the treatment with LPS (200 ng/ml) for 24 h. Culture supernatants were subsequently isolated and analyzed for nitrite production. Each value shows the mean ± S.D. of triplicate determinations. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P<0.05$.

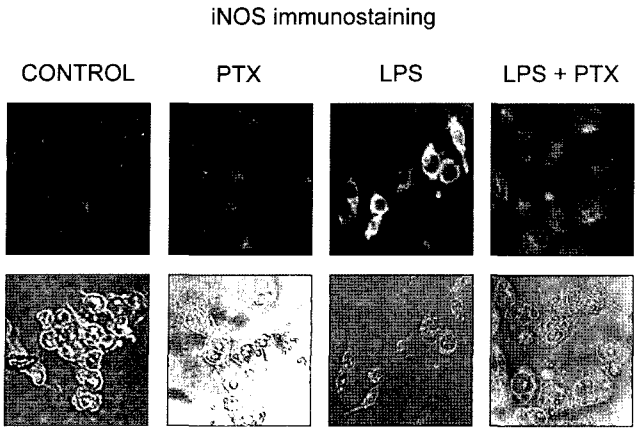

Fig. 4. Inhibition of iNOS expression by the pretreatement with paclitaxel in LPS-stimulated RAW 264.7 cells. Cells (5×10^5 cells/ml) were preincubated with paclitaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) for 16 h on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine iNOS. Immunoreactivity of iNOS was localized along the margin (arrows) of the cytoplasm.

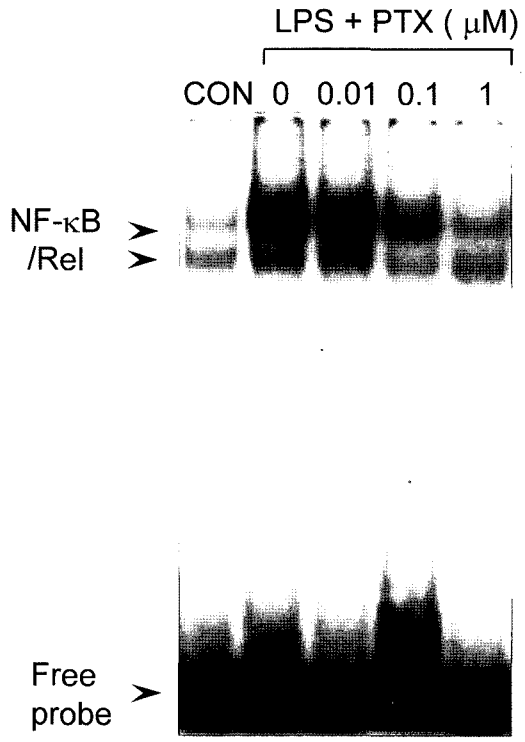

Fig. 5. Inhibition of NF-κB/Rel DNA binding by paclitaxel in LPS-stimulated RAW 264.7 cells. Cells (5×10^5 cells/ml) were pre-incubated with indicated concentrations of paclitaxel for 8 h before the treatment with LPS (200 ng/ml) for 2 h. Nuclear extracts were then isolated and analyzed for the activity of NF-κB/Rel.

Inhibition of NF-κB/Rel by pretreatment with paclitaxel in LPS-stimulated macrophages

To further investigate the molecular mechanism of paclitaxel-mediated inhibition of macrophage, we focused on the transcription factors whose binding sites are in the promoter of iNOS gene. Since it has been reported that protein binding at the κB binding site is necessary to confer inducibility by LPS of iNOS (Xie *et al.*, 1994), we assessed the effect of paclitaxel on NF-κB/Rel using EMSA. LPS treatment of RAW 264.7 cells induced a marked increase in NF-κB/Rel binding to its cognate site. And the induction of NF-κB/Rel binding was inhibited by paclitaxel pretreatment in a dose-related manner (Fig. 5). The specificity of the retarded bands was confirmed by the addition of an excess of ^{32}P-unlabeled double-stranded κB that competed for protein binding (data not shown). These results indicate that paclitaxel decreases DNA binding of NF-κB/Rel, which is important in the regulation of iNOS gene expression.

DISCUSSION

We demonstrate that paclitaxel treatment significantly attenuates LPS-induced NO production and iNOS transcription through the blocking of NF-κB/Rel pathway in the macrophage cell line RAW 264.7. The major finding of the present study is that paclitaxel significantly inhibits the iNOS expression in the RAW 264.7. Since paclitaxel inhibits NF-κB/Rel which is critically involved in the transcription of iNOS gene, the mechanism for the inhibition of iNOS may be related to the inhibition of transcription. However, we cannot exclude the possibility that paclitaxel promotes mRNA instability.

The effect of paclitaxel on iNOS immune cells is controversial. Ding *et al.* (1990) described that paclitaxel induces the secretion of tumor necrosis factor and down-regulation of tumor necrosis factor receptors in murine macrophages. Although the structure of paclitaxel is quite different from that of LPS, paclitaxel has been shown to possess many LPS-like activities, such as tyrosine phosphorylation of microtubule-associate protein kinases (Manthey *et al.*, 1992) and activation of NF-κB/Rel (Perrera *et al.*, 1996). Paclitaxel also activates iNOS gene expression in astrocytes (Cvetkovic *et al.*, 2004) and macrophages (Kim and Paik, 2005). However, our data showed pretreatment with paclitaxel for 8 h inhibited LPS-induced iNOS gene expression and NF-κB/Rel activation. This is the first report showing that paclitaxel inhibits iNOS gene expression in LPS-stimulated macrophage line RAW 264.7 cells. The mechanism how paclitaxel causes macrophages to hypo-responsive state to LPS, should be further studied.

Our study showed that NF-κB/Rel is positively regulated by LPS for iNOS gene expression, and paclitaxel pretreatment of RAW 264.7 cell significantly inhibited LPS-induced NF-κB/Rel activity. The NF-κB/Rel is pleiotropic regulator of many genes involved in immune and inflammatory responses, including iNOS (Xie *et al.*, 1994). NF-κB/Rel exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, IκB. Macrophage activation by certain external stimuli results in the phosphorylation of IκB, thus releasing the active DNA-binding form of NF-κB/Rel to translocate to the nucleus to bind κB motifs in the regulatory region of a variety of genes. EMSA studies showed strong induction by LPS of two separate κB binding complexes at 30 min. Paclitaxel inhibited activation of both of these κB binding complexes (Fig. 5).

In summary, these experiments demonstrate that paclitaxel inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. Based on our findings, the most likely mechanism that can account for this biological effect involves the inhibition of NF-κ/Rel.

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