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Docetaxel에 의한 p65의 핵내 이동 억제에 관한 연구

- p65의 핵내 이동에 관한 연구 -

조선대학교대학원

의학과

이 정 희

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지도교수 임 진 수

이 논문을 의학 박사학위신청 논문으로 제출함

2007년 4월 일

조선대학교 대학원

의 학 과

이 정 희

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위원장 조선대학교 교수 장 인 엽 (인)

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위원 조선대학교 교수 전 제 열 (인)

위원 조선대학교 교수 임 진 수 (인)

2007년 6월 일

조선대학교 대학원

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ABSTRACT

Inhibition of p65 nuclear translocation by docetaxel

p65 nuclear translocation

Lee Jung-Hee
Advisor : Prof. Im Jin-Su, M.D., Ph.D.
Department of Medicine
Graduate School of Chosun University

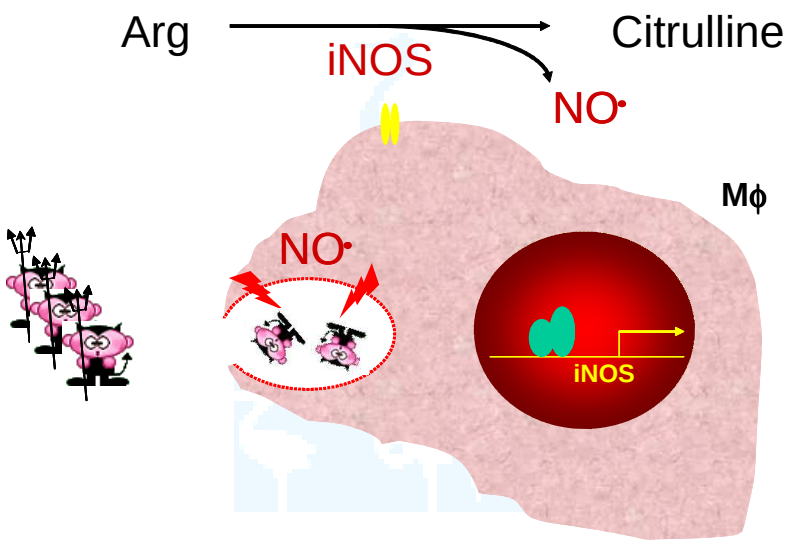
주목나무에서 추출한 항암제인 docetaxel이 큰 포식세포에서 lipopolysaccharide (LPS)에 의한 iNOS 유전자의 발현에 미치는 영향에 대해 연구하였다. 본 연구를 통하여 docetaxel (0.1 μM)을 8시간 전처리하면 LPS에 의해 유도되는 inducible nitric oxide synthase (iNOS) 유전자의 발현을 억제함을 확인하였다. Docetaxel은 큰 포식세포에서 일산화질소의 생성을 유의성 있게 억제하였으며, Western blot과 reverse transcriptase-polymerase chain reaction (RT-PCR)을 통하여 iNOS 유전자 발현을 억제시킴으로써 일산화질소의 생성을 억제함을 확인하였다. iNOS에 대한 면역형광염색실험결과, iNOS의 발현이 docetaxel에 의해 억제됨을 재확인하였다. Electrophoretic mobility shift assay를 수행한 결과 docetaxel이 nuclear factor-κB/Rel (NF-κB/Rel)의 DNA 결합 활성을 억제함을 확인하였다. 또한 NF-κB/Rel를 구성하는 중요한 단백질인 p65에 대한 면역형광염색을 통하여 docetaxel이 p65의 핵내 이동을 억제함을 확인하였다. 이러한 결과들로부터 docetaxel이 NF-κB/Rel 의 활성을 억제함으로써 iNOS 유전자의 발현을 억제함을 확인하였다.

I. INTRODUCTION

Docetaxel, 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 G2/M junction of the cell cycle (Blagosklonny et al., 1996). The primary mechanism of action of docetaxel is attributed to its ability to bind to microtubules and to prevent their assembly. In addition to the blockage of mitosis, docetaxel 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-kB, and induction of gene expression (Perera et al., 1996; Das and White, 1997). In murine macrophages, docetaxel can induce the expression of a series of cytokines, such as IL-1a, IL-1b, TNF-a, and iNOS (Mantheny et al., 1992; Kim and Paik, 2005).

Bacterial LPS is a potent activator of the immune system and induces local inflammation, antibody production, and septic shock in severe infections (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 iNOS, which catalyzes the production of large amounts of 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).

Nitric Oxide (NO·)



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 κB site is necessary to confer inducibility by LPS (Xie *et al.*, 1994).

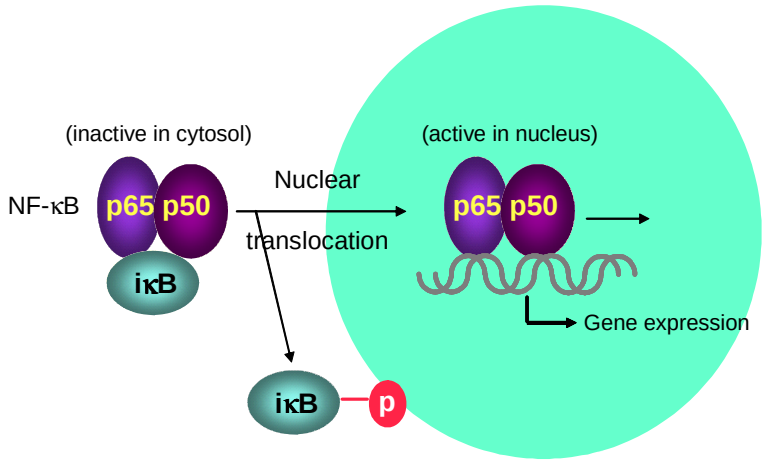
The NF-κB/Rel is a pleiotropic regulator of many genes involved in immune and inflammatory responses, including iNOS (Xie *et al*., 1994). NF-κB/Rel exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, IκB. Macrophage activation by certain external stimuli results 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.

NF-κB-inducible genes involved in immune responses

Class of gene	NF-κB-dependent gene
Cytokines/growth factors	IL-1α and β
	IL-2, -3, -6, -8, -12
	TNF-α
	IFN-β
	G-CSF, M-CSF, GM-CSF
Cytokine receptors	IL-2Rα
Stress proteins	SAA
	Complement factors B, C3 and C4
	α1-acid glycoprotein
Leukocyte adhesion molecules	ICAM-1, VCAM-1, MAdCAM-1, E-selectin
Immunoregulatory molecules	Igκ
	MHC class I and II
	TCRα and β
	β2-microglobulin
	Ii
	TAP1

p65

a component of NF-κB transcription factor important in the expression of inflammatory genes related to autoimmune diseases



In the present studies, the effect of docetaxel on the production of NO, an important indicator of inflammation was investigated. To further investigate the mechanism by which docetaxel inhibits the expression of iNOS gene, the effects of docetaxel on the activation of NF-κB/Rel nuclear translocation was analyzed. The present studies demonstrate that docetaxel inhibits iNOS gene expression through the inhibition of NF-κB/Rel nuclear translocation.

II. MATERIALS AND METHODS

A. Materials

Docetaxel was purchased from CalBiochem (San Diego, CA). LPS from *Salmonella thyposa* was purchased from Sigma (St. Louis, MO). Reagents used for cell culture were purchased from Gibco BRL (Grand Island, NY). Anti-iNOS and anti-p65 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

B. Cell culture

RAW 264.7 cells (murine macrophage line) were purchased from American Type Culture Collection (Bethesda, MD). Cells were grown in RPMI 1640 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^{o}C.

C. Isolation of peritoneal macrophages

Peritoneal cells were harvested by sterile peritoneal lavage with 10 ml phosphage buffted saline, massaged 1 min, washed, resuspended in culture medium, and plated at 5 x 10^5 cells/ml. Nonadherent cells were removed by repeated washing after a 2-h incubation at 37ºC. Adherent macrophages then cultured in the presence of 5% CO_2 at 37^{o}C.

D. Nitrite quantitation

Nitrite accumulation was used as an indicator of NO production in the medium as previously described (Green *et al.*, 1982). Cells were plated at 5 x 10^5cells /ml in 96-well culture plates and treated with docetaxel for 24 hr. 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.

E. Western immunoblot analysis

Cells were plated at 5 x 10^5cells /ml in 96-well culture plates and treated with docetaxel for 24 hr. Cells were pelleted, lysed with lysis buffter, and quantitated the protein amount. 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 hr 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 antibodies. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham).

F. RT-PCR

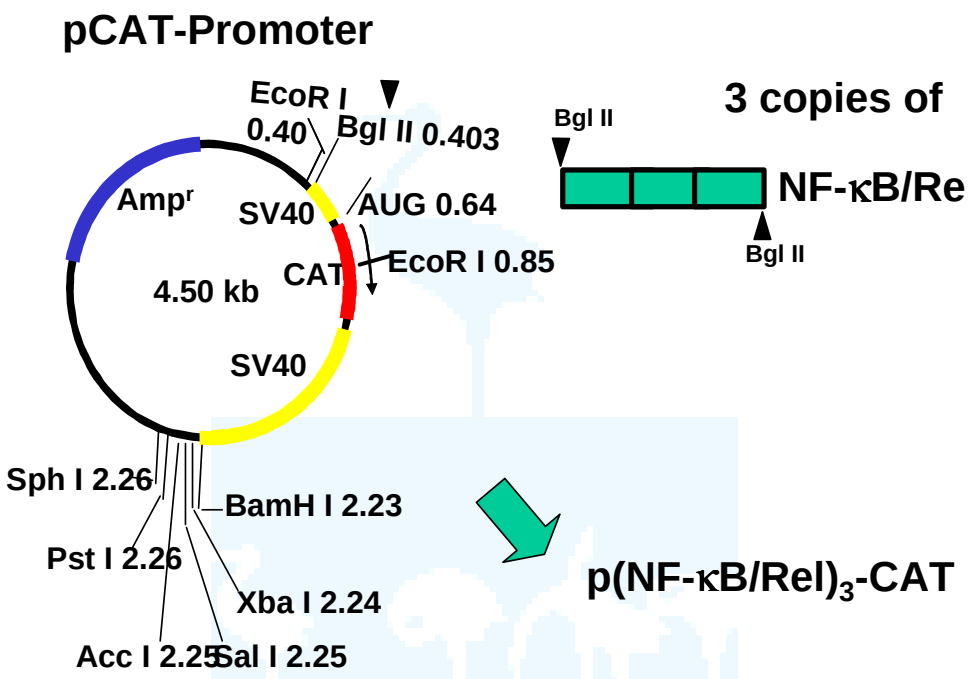
Cells were plated at 5 x 10^5cells /ml in 6-well culture plates and treated with docetaxel for 6 hr. Total RNA was isolated using Tri Reagent (Molecular Researh Center, Cincinnati, OH, USA) as described previously (Chomczynski and Mackey, 1995). The forward and reverse primer sequences are: iNOS (5'-CTG 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 ^oC for 5 min and cycled 30 times at 94 ^oC for 1 min, 55 ^oC for 1 min 30 seconds, and 72 ^oC for 1 min, after which an additional extension step at 72 ^oC 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 produced amplified products at 311 bp and 349 bp, respectively.

G. Transient transfection of RAW 264.7 cells

Vector constructions were performed as previously described (Jeon *et al.*, 1998). RAW 264.7 cells were transfected using the DEAE-dextran method (Xie *et al.*, 1993b), diluted to 5 X 10^5 cells per 1 ml of complete media, plated on 24 well plates, and then incubated in the presence of 5% CO_2 at 37 ^oC for

24 hr. The transfectants were treated with LPS and docetaxel. Eighteen hours later the cells were lysed with lysis buffer (250 μl). The lysates were centrifuged (12,000 x g for 10 min at 4 ^{o}C), and the supernatant was assayed for the expression of CAT enzyme using CAT ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

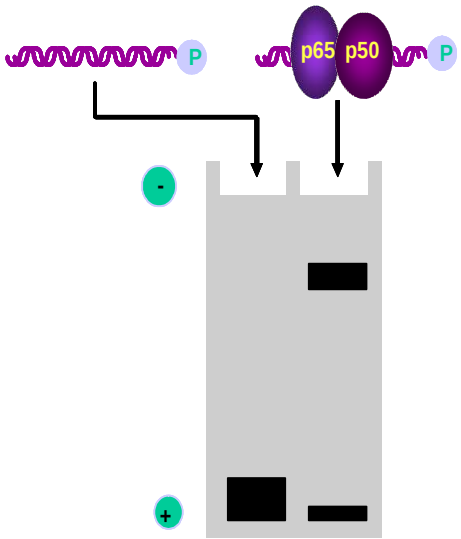
Vector construction



H. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed as previously described (Jeon *et al.*, 1996). Cells were plated at 5 x 10^5cells /ml and treated with docetaxel for 2 hr. Nuclear extracts were prepared as previously described (Xie *et al.*, 1993a). Treated and untreated RAW 264.7 cells were harvested, centrifuged, washed with phosphate bufferd saline, lysed with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, pH 7.5), and nuclei were pelleted by centrifugation at 3,000 x g for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin). Following lysis, the samples were centrifuged at 14,500 x g for 15 min, and supernatant was retained for use in the DNA binding assay. The oligonucleotide suquences for NF-κB/Rel (Pierce *et al.,* 1988) was: 5'-GAT CTC AGA GGG GAC TTT CCG AGA GA-3. The double-stranded oligonucleotides were end-labeled with [^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with poly (dI-dC) and the [^{32}P]-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin) for 10 min. DNA binding activity was separated from free probe using a 4% polyacrylamide gel in 0.5X TBE buffer. Following electrophoresis, the gel was dried and subjected to autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)



When cells were activated, p65 and p50 protein translocated into the nucleus. Incubation of radioisotope labelled oligonucleotide with nuclear extract isolated from macrophages stimulated with LPS and/or IFN-γ induced strong binding showing the upper part of the gel, while unbound oligonucleotide move fast down to the lower part of the gel. The density of the upper band represents the NF-κB DNA binding activity.

I. 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).

III. RESULTS

A. Effect of docetaxel on nitrite production in macrophages.

To investigate the effects of docetaxel on NO production, the accumulation of nitrite, the stable end product of NO, was measured in the culture media using Griess reagent. Peritoneal cells were plated at 5 x 10^5cells /ml in 96-well culture plates and treated with docetaxel in the presence of LPS for 24 hr. 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. Potent macrophage activator LPS (200 ng/ml) alone increased the production of nitrite for 9-fold over basal levels in peritoneal macrophages (Table1). This induction in nitrite generation by LPS was inhibited by docetaxel in a dose-dependent manner. RAW 264.7 cells were pretreated with docetaxel (0.1 μM) for 8 hr before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml)for 24 hr. When culture supernatants were isolated and analyzed for nitrite production, pretreatement with docetaxel (0.1 μM) significantly inhibited both LPS- and IFN-γ-induced nitrite generation (Fig. 1). The combination of LPS and IFN-γ synergistically induced nitrited generation by RAW 264.7 cells. And the synergism between LPS and IFN-γ on nitrite generation was inhibited significantly by the pretreatment with docetaxel (Fig. 1). The induction in nitrite generation by LPS was inhibited by docetaxel in a dose-dependent

manner (Fig. 2).

These results demonstrate that docetaxel significantly inhibited NO production in both mouse peritoneal macrophages and mouse macrophage cell line, RAW 264.7 cells.

Table1. Inhibition of nitrite production in macrophages by docetaxel

	Treatment	Nitrite (nmole/10^6 cell)
Peritoneal cells	Control	3.5 ± 1.1
	LPS (200 ng/ml)	32.4 ± 2.3
	LPS + DTX (0.01 μM)	34.2 ± 4.7
	LPS + DTX (0.05 μM)	24.3 ± 5.2
	LPS + DTX (0.1 μM)	18.7 ± 4.8
	LPS + DTX (0.5 μM)	10.3 ± 2.2

Each value shows the mean ± S.D. of triplicate determinations.

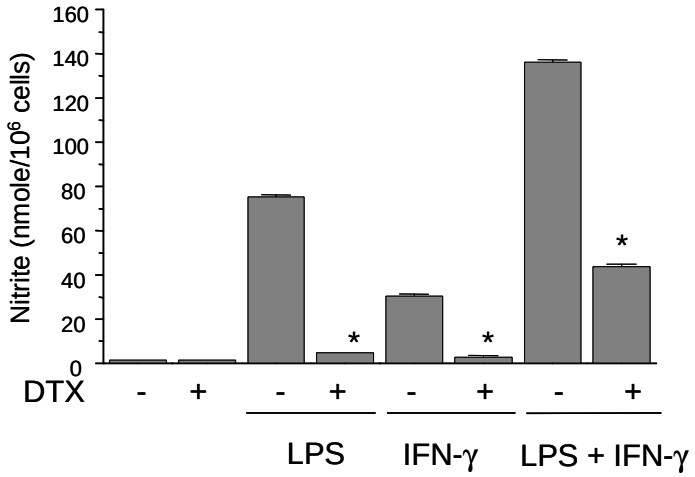


Figure 1. Inhibition of nitrite production by docetaxel in macrophages. RAW 264.7 cells were pretreated with docetaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 24 h. Culture supernatants were subsequently isolated and analyzed for nitrite production. Each 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 $P < 0.05$.

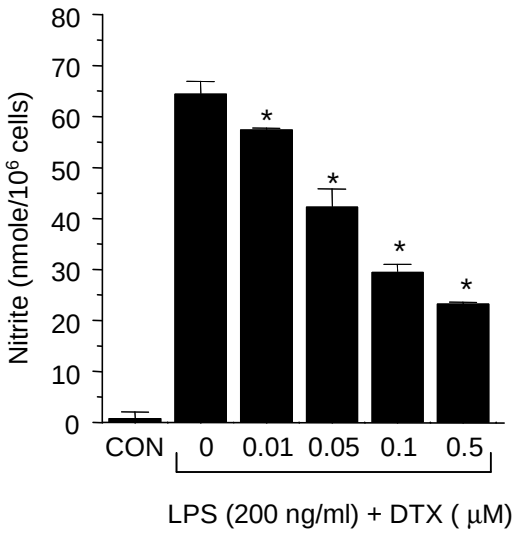


Figure 2. Inhibition of nitrite production by docetaxel in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with docetaxel for 8 h before the treatment with LPS (200 ng/ml) for 24 h. Culture supernatants were subsequently isolated and analyzed for nitrite production. Each value shows the 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 $P < 0.05$.

B. Effect of docetaxel on iNOS gene expression in macrophages.

Immunohisto-chemical staining of iNOS showed that the decrease of NO was due to the inhibition of iNOS production (Fig. 3). No effect on cell viability was observed in any of the treatment groups, and the viability was always exceeded 90% as determined by trypan blue staining (data not shown).

To analyze the effect of pre-treatment with docetaxel on iNOS gene expression, we treated RAW 264.7 cells with low dose of docetaxel (0.1 μM) for 1, 2, 4, 8, 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. 4, iNOS protein production was inhibited by docetaxel treatment in a pre-incubation time-dependent manner. Since 8 h preincubation strongly inhibited LPS-induced iNOS expression, we chose 8 h pre-treatment and assessed the effect of docetaxel pretreatment on LPS and/or IFN-γ induced iNOS expression. As shown in Fig. 5, docetaxel 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 docetaxel pretreatment (Fig. 5).

To further analyze the mechanismby which docetaxel inhibited iNOS protein production, we assessed the effect of pretreatment with docetaxel 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 docetaxel (0.1 μM) (Fig. 6). 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 docetaxel. These results indicate that docetaxel decreases the gene expression of iNOS, which is involved in inflammation (Hibbs *et al.,* 1987*).*

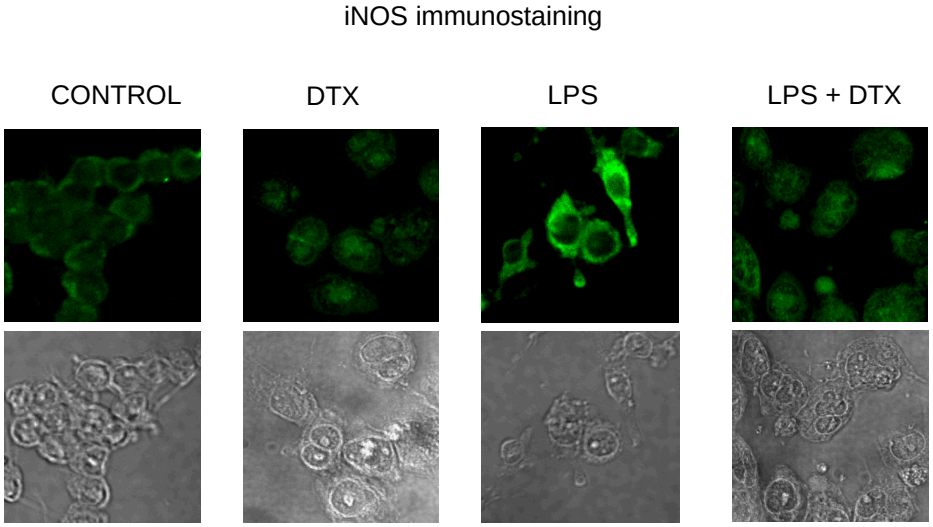


Figure 3. Inhibition of macrophage activation by docetaxel in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (5x 10^5 cells/ml) were incubated with docetaxel (0.1 μM) in the presence of LPS (200 ng/ml) for 24 hr on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine iNOS. Immunoreactivity of iNOS was localized along the margin of the cytoplasm of in control.

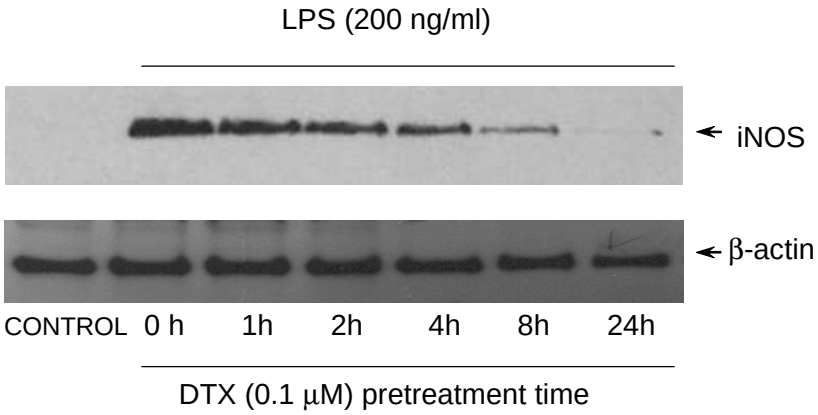


Figure 4. Inhibition of iNOS production by docetaxel in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with docetaxel (0.1 μM) for the indicated times before the treatment with LPS (200 ng/ml) for 16 hr. Cell extracts were isolated and subjected to Western immunoblot assay using iNOS- and β-actin-specific antibody.

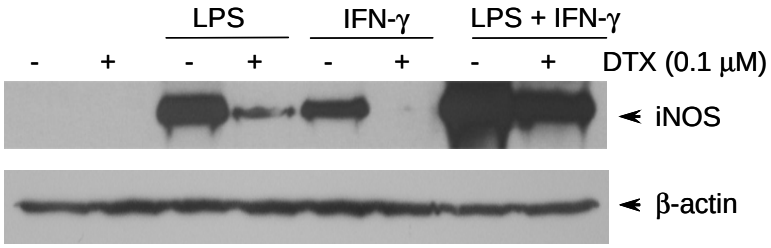


Figure 5. Inhibition of iNOS production by docetaxel in RAW 264.7 cells. RAW 264.7 cells were pretreated with docetaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 16 h. Cell extracts were isolated and subjected to Western immunoblot assay using iNOS- and β-actin-specific antibody.

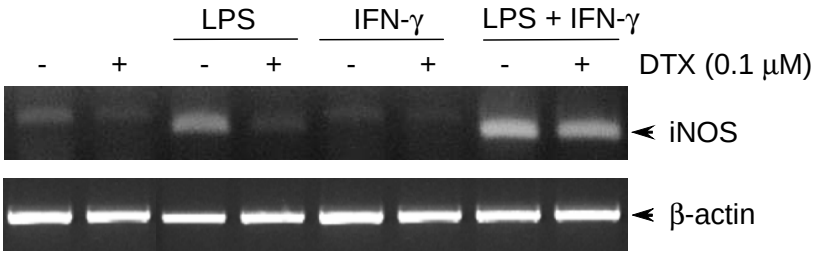


Figure 6. Inhibition of iNOS gene expression by docetaxel in macrophages. Cells were pretreated with docetaxel (0.1 μM) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN-γ (100 ng/ml) for 16 h. Total RNA was isolated and analyzed for the magnitude of mRNA expression of iNOS using RT-PCR.

C. Inhibition of NF-κB/Rel in response to docetaxel in LPS-stimulated RAW 264.7 cells.

To further investigate the molecular mechanism of docetaxel-mediated inhibition of macrophage, I 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), I assessed the effect of docetaxel on NF-κB/Rel using a transient transfection assay. When RAW 264.7 cells were transiently transfected with p(NF-κB/Rel)3-CAT, the CAT gene expressions were found to be induced by LPS and IFN-γ (Fig. 7). Basal levels of CAT expression in unstimulated RAW 264.7 cells were below 4 pg/ml ± 1.1. On LPS-and IFN-γ-stimulation, CAT expression by RAW 264.7 cells increased significantly. Pretreatment of docetaxel significantly inhibited bothe LPS-and IFN-γ-induced CAT expression. The synergism between LPS-and IFN-γ on CAT expression was inhibited partially by docetaxel (Fig. 7). Docetaxel treatment inhibited LPS-induced CAT expression in a dose-dependent manner (Fig. 8). RAW 264.7 cells expressed very strong basal octamer activity, and the activity was not influenced by either LPS or docetaxel (Fig. 9).

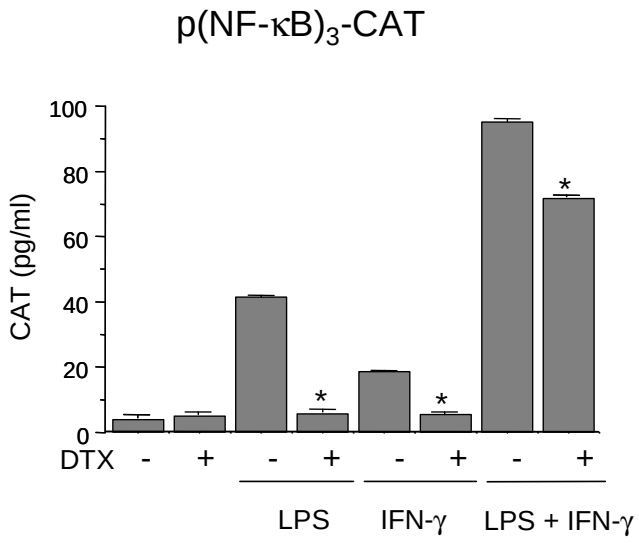


Figure 7. Inhibition of p65 transcriptional activation by docetaxel in RAW 264.7 cells. RAW 264.7 cells were transfected with $p(\kappa B)_3$-CAT by DEAE dextran method. Twenty-four hours after transfection, cells were treated with docetaxel in the presence of LPS and/or IFN-γ for 18 hr. Cell extracts were then prepared and analyzed for the expression of CAT using CAT ELISA kit.

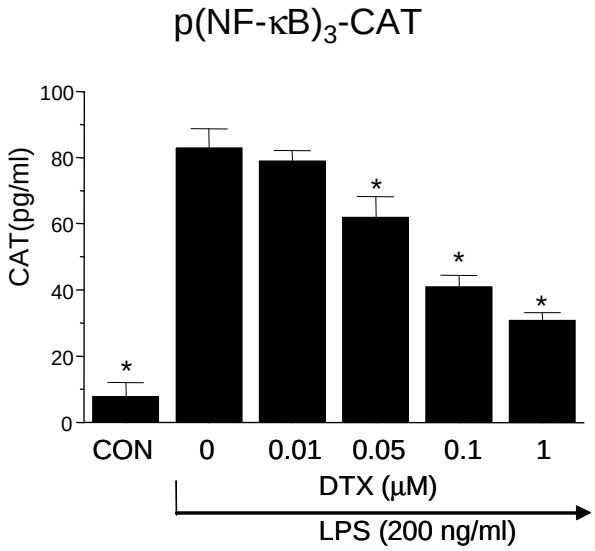


Figure 8. Inhibition of p65 transcriptional activation by docetaxel in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were transfected with $p(\kappa B)_3$-CAT by DEAE dextran method. Twenty-four hours after transfection, cells were treated with the indicated concentrations of docetaxel in the presence of LPS (200 ng/ml) for 18 hr. Cell extracts were then prepared and analyzed for the expression of CAT using CAT ELISA kit.

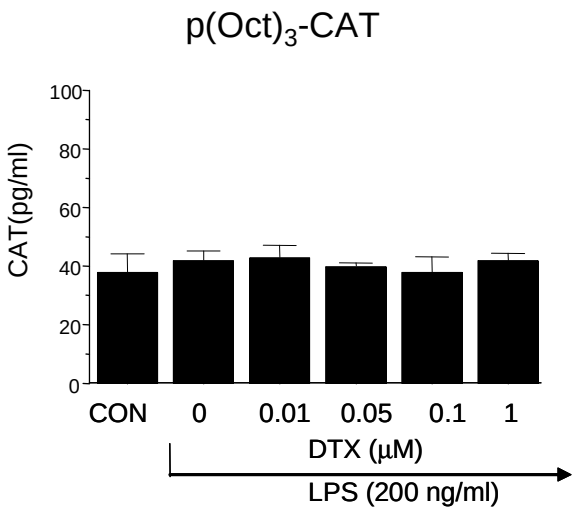


Figure 9. Effect of docetaxel on octamer transcriptional activation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were transfected with p(Oct)3-CAT by DEAE dextran method. Twenty-four hours after transfection, cells were treated with the indicated concentrations of docetaxel in the presence of LPS (200 ng/ml) for 18 hr. Cell extracts were then prepared and analyzed for the expression of CAT using CAT ELISA kit.

D. Inhibition of NF-κB/Rel nuclear translocation by docetaxel in LPS-stimulated RAW 264.7 cells.

To further investigate whether docetaxel inhibits the nuclear translocation of p65, which is a component of NF-κB/Rel and has a transcriptional activation activity, the nuclear translocation of p65 was analyzed using immunohistochemical staining. LPS-stimulated RAW 264.7 cells showed marked p65 staining in the nuclei, while unstimulated cells showed weaker nuclear NF-κB/Rel expression, but stronger staining in the cytoplasm. docetaxel treatment inhibited LPS-induced nuclear translocation of p65 (Fig. 10).

The DNA binding of the NF-κB/Rel transcription factor is preceded by the nulear translocation of NF-κB/Rel. The effect of docetaxel on the NF-κB/Rel whose binding motif is in the promoter of iNOS gene was further investigated using EMSA. LPS and/or IFN-γ treatment of RAW 264.7 cells induced marked increase in NF-κB/Rel binding to its cognate site. And the induction of NF-κB/Rel binding was significantly inhibited by docetaxel (Fig. 11). NF-κB/Rel binding induced by LPS was inhibited by docetaxel in a dose-related manner (Fig. 12). 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 (Fig. 13A). The NF-κB/Rel binding complex was identified by gel supershift assay (Fig. 13B). The band was supershifted dramatically when the nuclear extract was preinducbated with antibodies against p50 and p65. Oct1 DNA binding activity was not affected by either LPS or docetaxel (Fig. 14).

These results indicate that docetaxel decreases the nuclear translocation

and DNA binding of NF-κB/Rel, which is important in the regulation of iNOS gene expression.

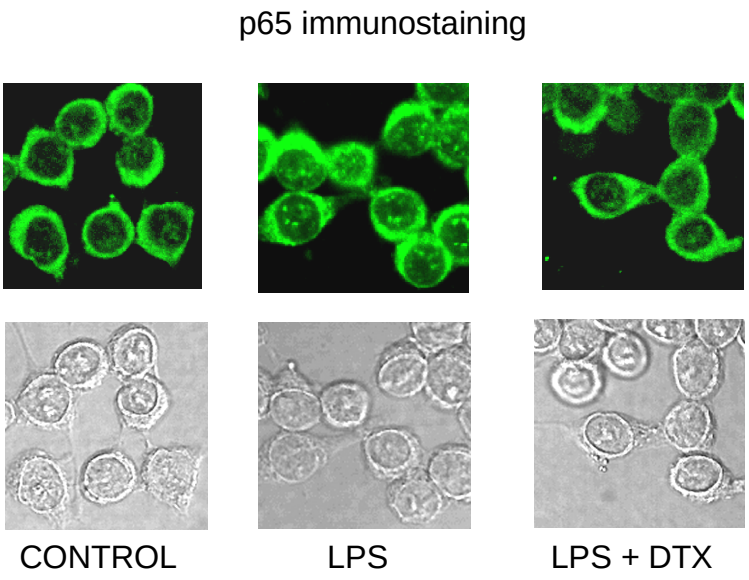


Figure 10. Inhibition of p65 nuclear translocation by docetaxel in LPS-stimulated RAW 264.7 cells. Cells (5x 10^5 cells/ml) were incubated with docetaxel (0.1 μM) in the presence of LPS (200 ng/ml) for 2 hr on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine p65.

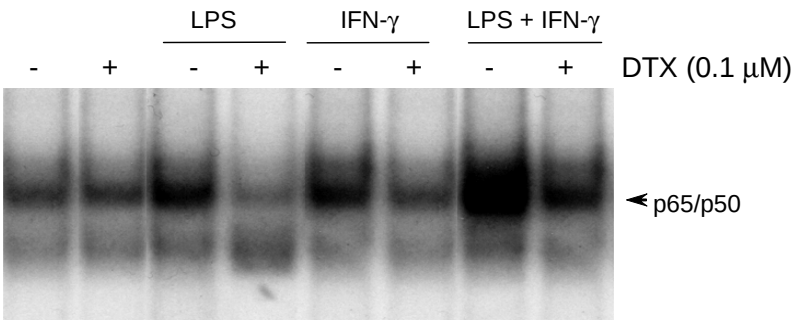


Figure 11. Inhibition of p65/p50 DNA binding by docetaxel in RAW 264.7 cells. Cells (5x 10^5 cells/ml) were incubated with docetaxel (10, 50, 100, and 200 ng/ml) in the presence of LPS (200 ng/ml) for 2 hr. Nuclear extracts (5 μg/ml) were then isolated and analyzed for the activity of NF-κB/Rel. Reaction products were electrophoresed, and the gels were dried and autoradiographed.

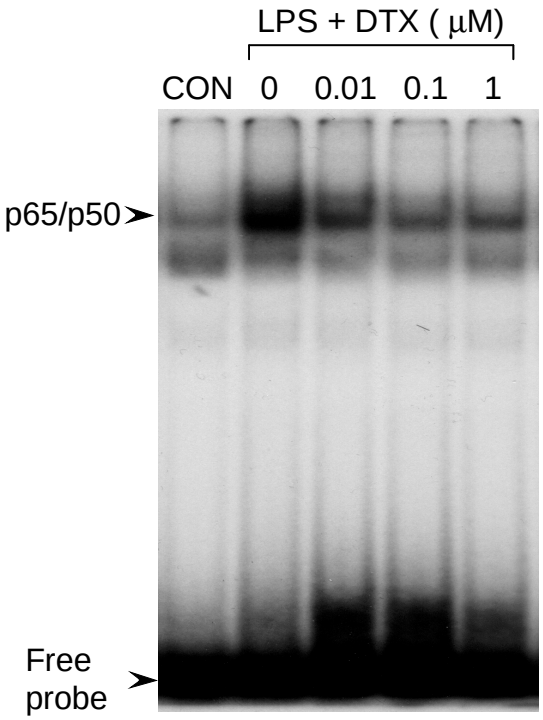


Figure 12. Inhibition of p65/p50 DNA binding by docetaxel in LPS-stimulated RAW 264.7 cells. Cells (5x 10^5 cells/ml) were incubated with docetaxel in the presence of LPS (200 ng/ml) for 2 hr. Nuclear extracts (5 mg/ml) were then isolated and analyzed for the activity of NF-κB/Rel. Reaction products were electrophoresed, and the gels were dried and autoradiographed.

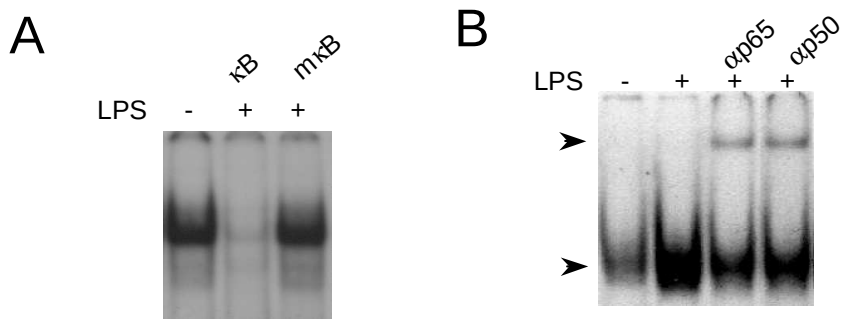


Figure 13. Specificity of NF-κB/Rel DNA binding in LPS-stimulated RAW 264.7 cells. Cells (5x 10^5 cells/ml) were incubated with LPS (200 ng/ml) for 2 hr. Nuclear extracts (5 μg/ml) were then isolated and analyzed for the activity of NF-κB/Rel. In competition studies, 1 pmole of unlabeled κB or mutant κB was added to the reaction mixture (A). For supershift assays, nuclear extracts (5 μg) were incubated with poly (dI-dC), antibodies specific for p65 or p50, and ^{32}P-labeled κB probe for 25 min (B). Reaction products were electrophoresed, and the gels were dried and autoradiographed.

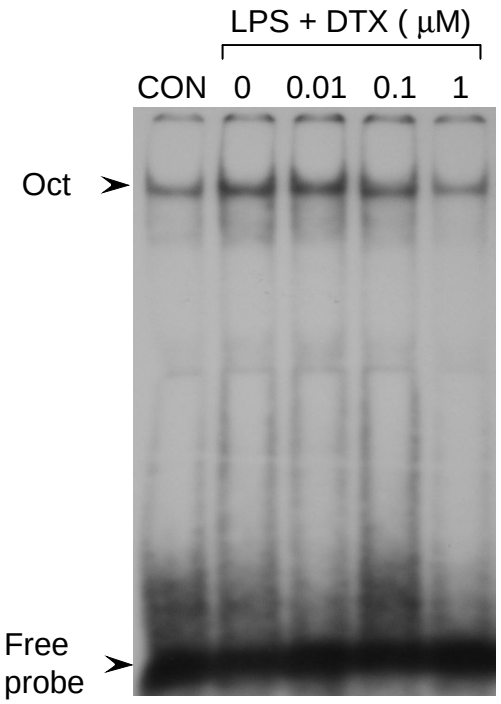


Figure 14. Inhibition of Oct DNA binding by docetaxel in LPS-stimulated RAW 264.7 cells. Cells (5x 10^5 cells/ml) were incubated with docetaxel in the presence of LPS (200 ng/ml) for 2 hr. Nuclear extracts (5 mg/ml) were then isolated and analyzed for the activity of Oct Reaction products were electrophoresed, and the gels were dried and autoradiographed.

IV. DISCUSSION

This study demonstrate that docetaxel treatment significantly attenuates LPS-induced NO production and iNOS transcription through the blocking of p65 nuclear translocation in the macrophage line RAW 264.7. 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 iNOS, which catalyzes the production of large amounts of 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 κB site is necessary to confer inducibility by LPS (Xie *et al.*, 1994).

This study showed that NF-κB/Rel was positively regulated by LPS for iNOS gene expression, and docetaxel treatment of RAW 264.7 cell significantly inhibited LPS-induced NF-κB/Rel activity. The NF-κB/Rel is a 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 2 hr. docetaxel inhibited activation

of both of these κB binding complexes; however, the magnitude of inhibition seemed greater for the protein complex represented by the top of the two bands. The upper band appears to be composed of p50/p65 heterodimers, whereas the lower band appears to consist of p50 homodimers. It has been shown that p50 proteins have DNA binding activity and p65 proteins have transactivation domains in their C termini and thus are able to activate transcription of target genes (Schmitz and Baeuerle, 1991). This finding suggests that docetaxel may inhibit the formation of p50/p65 heterodimers based on the EMSA studies.

The DNA binding of the NF-κB/Rel transcription factor is preceded by the nulear translocation of NF-κB/Rel. To further investigate whether docetaxel inhibits the nuclear translocation of p65, which is a component of NF-κB/Rel and has a transcriptional activation activity, the nuclear translocation of p65 was analyzed using immunohistochemical staining. LPS-stimulated RAW 264.7 cells showed marked p65 staining in the nuclei, while unstimulated cells showed weaker nuclear NF-κB/Rel expression, but stronger staining in the cytoplasm. docetaxel treatment inhibited LPS-induced nuclear translocation of p65 (Fig. 10). These results indicate that docetaxel decreases the nuclear translocation and DNA binding of NF-κB/Rel, which is important in the regulation of iNOS gene expression.

In summary, these experiments demonstrate that docetaxel, isolated from the bark of the yew tree, inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. Based on these findings, the most likely mechanism that can account for this biological effect involves the inhibition of p65 nuclear translocation. At least two significant points are brought out

by these studies. First, these experiments further confirm the critical role of NF-κ/Rel in the regulation of iNOS. Second, due to the critical role that NO release plays in mediating inflammatory responses, the inhibitory effects of docetaxel on iNOS suggest that docetaxel may represent a useful anti-inflammatory agent.

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영문초록

Inhibition of p65 nuclear translocation by docetaxel

p65 nuclear translocation

Lee Jung-Hee
Advisor : Prof. Im Jin-Su, M.D., Ph.D.
Department of Medicine
Graduate School of Chosun University

In this study, docetaxel, an antitumor agent derived from yew tree, inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. In this report we described that the pretreatment of macrophages with docetaxel (0.1 μM) for 8 h inhibited LPS-induced iNOS gene expression. Pretreatment of RAW 264.7 cells with docetaxel 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 docetaxel inhibited macrophage activation. Electrophoretic mobility shift assay showed that docetaxel inhibited NF-κB/Rel DNA binding. Immunocytochemical staining of p65, a component of NF-κB/Rel, showed that docetaxel inhibited nuclear translocation of p65 protein. Collectively, these series of experiments indicate that docetaxel inhibits iNOS gene expression by blocking NF-κB/Rel activation.

저작물 이용 허락서

학 과	의학과	학 번	20057459	과 정	박사
성 명	한글: 이 정희 한문 :李 正熙 영문 : Jung Hee Lee				
주 소	전남 화순군 화순읍 광덕리 163-1				
연락처	E-MAIL :				
논문제목	한글 : Docetaxel에 의한 p65의 핵내 이동 억제에 관한 연구 영문 : Inhibition of p65 nuclear translocation by docetaxel				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 조건부 동의( ) 반대( )

2007년 8 월 일

저작자: 이 정 희 (서명 또는 인)

조선대학교 총장 귀하