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Induction of Mac-2BP by nerve growth factor is regulated by the PI3K/Akt/NF-κB-dependent pathway in the HEK293 cell line

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Mac-2BP is a ligand of the galectin family that has been suggested to affect tumor proliferation and metastasis formation. We assessed Mac-2BP expression at the transcriptional and translational levels to evaluate nerve growth factor (NGF)-induced Mac-2BP expression. A time kinetic analysis using reverse transcription-polymerase chain reaction showed that NGF-induced Mac-2BP transcript levels were 4-5 times higher than in controls. Mac-2BP enzyme-linked immunosorbent assay and immunofluorescence staining showed a 2-3-fold increase in intracellular and secreted Mac-2BP as a result of NGF stimulation. This increase was regulated by Akt activation and NF-κB binding. p65 and p50-NF-κB are major transcriptional factors in the Mac-2BP promoter region, and were shown to be regulated in accordance with the Akt activation states. Collectively, these results suggest that NGF induces Mac-2BP expression via the PI3K/Akt/NF-κB pathway. [BMB reports 2008; 41(11): 784-789]

INTRODUCTION

Mac-2BP is a large oligomeric glycoprotein that has been identified in the fluid from human breast cancer cells (1,2). Supranormal serum levels of Mac-2BP have been found in patients with breast, lung, pancreatic, and gastric tumors, Non-Hodgkin's lymphoma, asthma, and human immunodeficiency virus infection (2-9). The reported evidence implicates Mac-2BP as a diagnostic and/or prognostic marker of poor outcome in several tumor types. Mac-2BP, like other members of its family, is involved in the host response to tumors and infections (10). Moreover, several reports have shown that the protein may function in cell adhesion processes. In particular, it has been demonstrated that Mac-2BP, as a ligand of galectin-1, -3, and -7, may promote homotypic cell-to-cell contacts (11,12), or may regulate cell adhesion by binding to cellular matrix proteins including β1-integrin, collagens, and fibronectins (13). Therefore, Mac-2BP may favor the establishment of new tumor colonies via enhancement of adhesive interactions between tumor cells and the extracellular matrix (14,15).

Neurotrophins are a family of structurally related secretory proteins that are involved in the survival, development, and death of specific populations of neuronal and non-neuronal cells (16). Nerve growth factor (NGF) is the best-characterized member of this family. NGF interacts with two classes of membrane receptors: the TrkA proto-oncogene product $p140^{TrkA}$, which exhibits intrinsic tyrosine kinase activity, and a secondary receptor, $p75^{NTR}$, which is a member of the tumor necrosis factor (TNF) receptor family (17).

In the current study, we have determined that NGF induces Mac-2BP expression at the transcriptional level, and is dependent on an Akt/NF-κB-dependent pathway.

RESULTS

Nerve growth factor induces Mac-2BP expression

To evaluate the role of NGF in Mac-2BP expression, we utilized reverse transcription-polymerase chain reaction (RT-PCR). NGF was applied to serum-starved HEK293 cells; Mac-2BP transcripts peaked within 6 h and then slowly diminished over time. Mac-2BP transcripts were increased steadily as the result of NGF treatment (Fig. 1A). Mac-2BP ELISA showed a 2-3-fold increase in the secreted Mac-2BP in culture supernatants of HEK293 or SNU-638 cell line treated with NGF (Fig. 1B). An increase of Mac-2BP was verified via immunofluorescence staining with an anti-Mac-2BP monoclonal antibody. The increase in Mac-2BP was verified by immunofluorescence staining with an anti-Mac-2BP monoclonal antibody. We inhibited glycosylation by pretreatment with 10 μg/ml tunicamycin for 30 min prior to NGF treatment, and staining of Mac-2BP. As Mac-2BP secretion was prohibited by the inhibition of glycosylation, intracellular Mac-2BP was more readily detectable in

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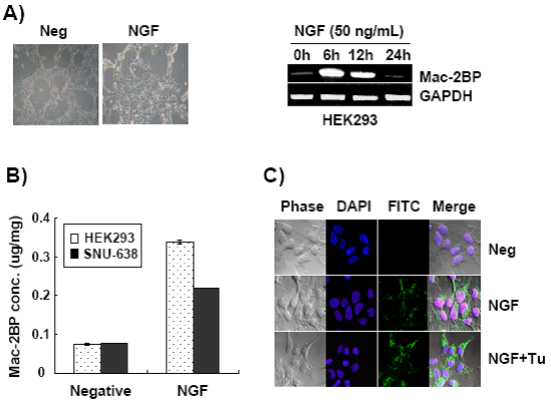

Fig. 1. Mac-2BP expression induced by NGF. (A) After the cells were incubated with NGF for 6-24 h, cell morphology was assessed under a microscope (left panel). Total RNAs were prepared from the stimulated cells, and RT-PCR was conducted with Mac-2BP or GAPDH primers. (B) The cells treated for 24 h with NGF were lysed, and Mac-2BP ELISA was conducted with the protein extracts that had been prepared. (C) HEK293 cells were serum-starved for 18 h followed by 24 h of NGF and/or tunicamycin treatment. For immunofluorescence analysis, the cells were fixed and stained with Mac-2BP monoclonal antibody and cellular localization and expression of Mac-2BP were observed with a confocal microscope.

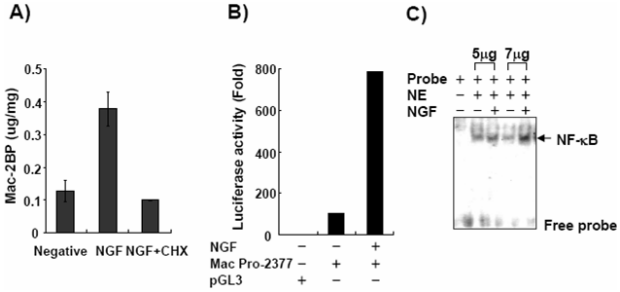

Fig. 2. Regulation of Mac-2BP expression by NGF at the transcriptional level. (A) Cells were pre-treated with 2 μg/ml of CHX for 40 min prior to NGF treatment. After an additional 24 h of incubation, the cells were lysed and Mac-2BP ELISA was conducted using cytosolic soluble proteins. (B) The -2377 bp of the human Mac-2BP promoter region was constructed and transfected into HEK293 cells using Lipofectamine reagent, and then subjected to NGF treatment for 24 h. The cells were lysed, and the relative luciferase activities expressed as the ratio of the pGL3 reporter activity to that of the pRL control plasmid. Values are expressed as means ± SD of three independent measurements. (C) Nuclear extracts were prepared from the HEK293 cells stimulated with 50 ng/ml of NGF for 24 hr, and the DNA binding activity of NF-κB was determined with a consensus dsDNA oligomer probe via an EMSA assay. The arrow indicates NF-κB complex.

these cells than in the NGF-treated cells (Fig. 1C).

NGF-induced Mac-2BP expression is regulated at the transcriptional level

To determine whether the increase in Mac-2BP was derived from novel protein synthesis, we induced the inhibition of protein synthesis by a 40 min pre-incubation with cycloheximide prior to the NGF treatment. NGF-induced upregulation of Mac-2BP was decreased to the levels seen in the untreated control cells (Fig. 2A). Moreover, in an effort to assess the regulation steps of Mac-2BP expression, we cloned -2377 bp of the Mac-2BP promoter flanking regions within the pGL3 basic luciferase reporter vector system (pGL3-Mac Pro-2377), and transiently introduced it into HEK293 cells. The relative luciferase activities of the Mac-2BP promoter were clearly increased by NGF (Fig. 2B)

To test whether NF-κB might be involved in the expression of Mac-2BP, serum-starved HEK293 was treated for 24 h with NGF, and the nuclear extracts were prepared. The DNA binding activity of NF-κB was clearly enhanced to a greater degree than was observed with the control extracts. This indicates that NGF increases the expression of Mac-2BP at the transcriptional level via NF-κB activation (Fig. 2C).

NGF-induced Mac-2BP expression is dependent on the PI3K/Akt/NF-κB pathway

To evaluate the signal transduction of Mac-2BP expression by NGF, we assessed the activation of MAP kinase or Akt. As shown in Fig. 3A, phosphorylated-p140^{TrkA} tyrosine kinase receptor was detected within 1 h, and the results of time kinetic analysis showed that phospho-p44/42 MAP kinase (Thr202/Tyr204) and phospho-Akt (Ser 473) were detected between 3 h and 6 h of incubation with NGF (Fig. 3B). To evaluate the relationships between Akt, NF-κB, and Mac-2BP expression, we pre-incubated HEK293 cells with PI3 kinase inhibitors prior to NGF treatment. Mac-2BP expression was analyzed at the transcriptional and translational levels using RT-PCR and Mac-2BP enzyme-linked immunosorbent assay (ELISA) (Figs. 3C-E) indicating that the inhibition of PI3 kinase induced a reduction in the Mac-2BP transcripts and its protein via the inhibition of Akt, although this did not completely suppress the expression. These results indicate that the expression of Mac-2BP as a consequence of NGF signaling is dependent on Akt activation.

DNA binding activity of NF-κB is crucial for Mac-2BP expression

There are two putative binding sites of NF-κB on the promoter region of Mac-2BP (NCBI number; U91729). To characterize the Mac-2BP promoter region, we conducted a competition assay using cold probes and a supershift analysis with anti-p50 or p65 antibodies. The results of the competition assay showed that the NGF-treated cells exhibited profound binding of the consensus or upstream and downstream NF-κB probes as compared to the untreated control cells. A downstream NF-κB site also evidenced stronger binding than the upstream NF-κB site (Fig. 4A).

To investigate the effects of PI3K inhibitors on NF-κB activation, we evaluated the localization of the NF-κB subunits. As shown in Fig. 4B, p65 and the phosphorylated form of p65 were polarized in the nuclear region. The NGF signal also in-

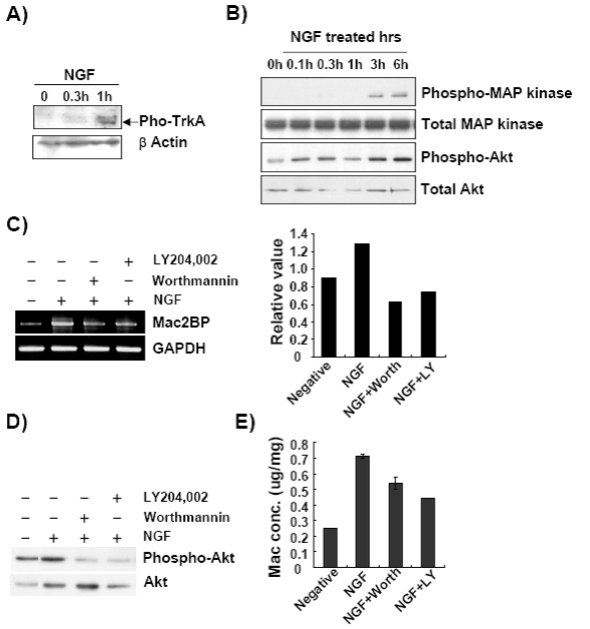

Fig. 3. Critical role of NGF signaling via a PI3K/Akt-dependent pathway in Mac-2BP expression. (A) After NGF treatment for the indicated amounts of time, the HEK293 cells were lysed and soluble cytosolic proteins were immunoblotted with phospho-p140^{TrkA} antibody. (B) HEK293 cells were harvested at the indicated time points and the cytosolic extracts were prepared. Total proteins of p42/44 MAP kinase, Akt, or phosphorylated forms of these proteins were measured using Western blot analysis. (C) Starved HEK293 cells were pre-treated with PI3K inhibitors for 30 min prior to NGF treatment. RT-PCR was conducted with Mac-2BP-specific primer. The relative values of the PCR products were calculated using a densitometer (MAC-2BP/GAPDH). (D) PI3K inhibited cells were lysed and the total Akt or phosphorylated Akt was determined using Western blotting. (E) Mac-2BP expression was assessed by Mac-2BP ELISA.

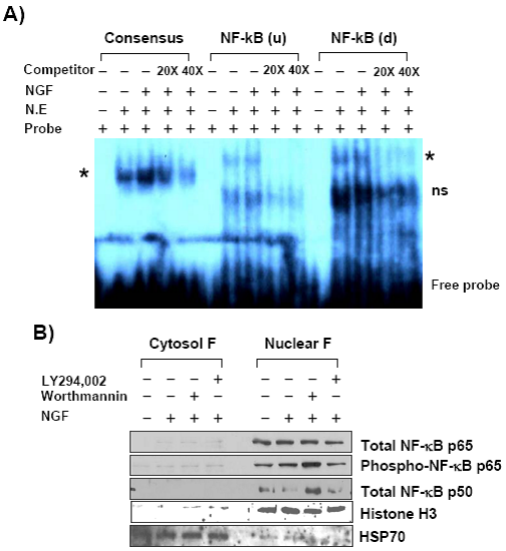

Fig. 4. Critical effect of NF-κB binding on Mac-2BP expression. (A) EMSAs were conducted in order to assess NF-κB binding activity using commercial consensus or upstream (u) and downstream (d) putative NF-κB sequences of the Mac-2BP promoter. Competition assays were conducted with a molar ratio of 20X or 40X cold probes. (B) Starved HEK293 cells were pre-treated with PI3K inhibitors for 30 min, and were incubated with NGF for 24 h. The cells were prepared as nuclear and cytosolic proteins. Western blotting was conducted with the indicated antibodies.

creased the activation of the p65 NF-κB subunit, whereas it was suppressed by LY294,002. Wortmannin increased phosphorylated p65 or p50 levels in the nuclear regions of the cells indicating that wortmannin may regulate NF-κB activation occurring via other pathways. Taken together, the results indicate that NGF may increase Mac-2BP expression by the induction of NF-κB binding on its promoter region, and is also dependent on the PI3K/Akt/NF-κB pathway.

DISCUSSION

We have recently determined that Mac-2BP may prove useful as a diagnostic and prognostic marker for gastric cancers (8). In the current study, to evaluate the molecular basis of Mac-2BP expression in HEK293, we assessed the effects of NGF on Mac-2BP expression and dissected the signal pathway involving the binding of NGF to its receptor.

As shown in Fig. 1, NGF induced Mac-2BP expression in human embryonic kidney 293 cells. The Mac-2BP transcript level achieved a peak at 6 h and then decreased slightly until 24 h. NGF-induced Mac-2BP was also detected in a quantitative Mac-2BP ELISA assay (Fig. 1B). NGF was shown to play roles in the induction and secretion of Mac-2BP, and immunofluorescence staining with Mac-2BP monoclonal antibody evidenced the up-regulation of Mac-2BP as a result of NGF treatment. Because Mac-2BP is known to be a highly glycosylated protein, we pre-treated samples with 10 μg/ml tunicamycin for the easy detection of Mac-2BP. Therefore, the sample containing NGF plus tunicamycin allowed for easier observation of Mac-2BP as compared to the negative or NGF-treated samples (Fig. 1C).

We also demonstrated that the induction of Mac-2BP resulting from NGF treatment is regulated at the transcriptional level, as shown in Fig. 2. Experiments in protein synthesis inhibition and reporter assays demonstrated the regulation of Mac-2BP expression upon transcription by NGF, and the DNA binding activity of NF-κB was shown to be involved in the transcription of Mac-2BP. To evaluate the signal cascades of Mac-2BP expression, we analyzed the activated states of the p140^{TrkA} receptor, p42/44 MAP kinase, and Akt (Fig. 3). The NGF signal induced an increase in the phosphorylated form of p140^{TrkA} within 1 h of NGF stimulation, and the activation of p42/44 MAP kinase and Akt were also increased as a consequence of NGF treatment. This result also suggests that Mac-2BP is subject to control by NGF as a result of PI3K/Akt/NF-κB signaling. In neuronal systems, the binding of NGF to p140^{TrkA} or p75^{NTR}

activates several signaling pathways, including the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase pathways. This also results in the activation of several transcriptional factors (18, 19) including NF-κB (20). NGF activates NF-κB and AP-1 in sympathetic neurons, and NGF-inducible NF-κB is required for neuronal survival (21).

We report here that two NF-κB binding sites on the Mac-2BP promoter region are critical DNA-bound components for the expression of Mac-2BP, and that the activity is regulated by the PI3K/Akt pathway. The Mac-2BP transcripts or its secreted products were regulated via the inhibition of PI3K inhibitors including wortmannin and LY294,002, and its regulation was potentially involved in NF-κB transactivation (Figs. 2C, 3C-E). The identified sequence begins at the upstream position -1622/-1611 and the downstream position -1580/-1589. NF-κB p50 usually binds to both positions containing the consensus sequence and promoter binding sites; however, p65-NF-κB displayed unique binding on the upstream site. Collectively, these results suggest that p65-NF-κB binding is critical for Mac-2BP expression, because p50-NF-κB lacks a transactivation domain.

The regulation of NF-κB activity by NGF and by Akt remains a matter of some controversy, and the discrepancies among previous studies may be related in part to cell type specificity. NGF-induced dissociation of the cytosolic p65/IκBα complex via phosphorylation of IκBα at a tyrosine residue 42 has been described (22). This mechanism results in nuclear translocation of p65-NF-κB without significant degradation of IκBα. In addition, other cytokine signaling studies have shown that the transcriptional activity of NF-κB is regulated independently of IκB. For example, inhibition of IL-1 stimulated PI3K activity by pretreatment with LY294,002 or wortmannin also causes a dramatic loss of NF-κB-dependent gene expression (23). Despite the dramatic decrease in NF-κB-induced gene expression, LY294,002 and wortmannin have no effects on interleukin-1 (IL-1) -stimulated degradation of IκBα or the nuclear translocation or DNA binding of NF-κB itself in HepG2 cells (23).

Consistent with the evidence provided in the aforementioned studies, our results also indicate that Mac-2BP is indeed regulated via Akt expression or activation, and that NF-κB transactivation potential is crucial to the PI3/Akt-dependent pathway. However, the nuclear localization of p65 is not inconsistent with our prediction that NF-κB p65 and p50 proteins are localized at the nuclear region even in unstimulated cells. In addition, wortmannin-pretreated cells, unlike LY294,002, evidenced higher levels of nuclear phospho-p65-NF-κB than observed in the NGF-treated cells (Fig. 4B). At this time, we do not have a clear explanation for the differences in our results. However, these findings reminded us that wortmannin could regulate NF-κB activation or transactivation occurring via multiple pathways. Our results are similar to other reports that inhibitors of phophatidylcholine-specific phospholipase C and protein kinase C block IL-1 and TNF-α-induced, NF-κB-dependent gene expression without affecting cytokine-induced IκB degradation or the nuclear translocation or DNA binding of NF-κB (23,24).

Although the precise mechanism by which the regulation of NF-κB by Mac-2BP occurs remains to be determined, the results of our study help to elucidate the manner in which NGF and other signaling molecules regulate Mac-2BP. In summary, we have uncovered new evidence indicating that Mac-2BP is regulated by NGF at the transcriptional level, and that this occurs in a PI3/Akt/NF-κB-dependent fashion.

MATERIALS AND METHODS

Cell culture and treatment

HEK293 cells were cultured in MEM-alpha (Gibco-BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Road Logan, Utah) and antibiotics in a humidified 5% CO_2 incubator at 37°C. Signaling blockers were administered including 100 nM wortmannin and 10 μM LY294, 002 (Sigma-Aldrich, St, Louis, MO) for 30 min, followed by stimulation with 50 ng/ml of recombinant human β-NGF (R&D Systems, Minneapolis, MN) for the indicated amounts of time.

Antibodies

Anti-Mac-2BP was purchased from Alexis (San Diego, CA); anti-p50 and total p42/p44 MAP kinase were obtained from Upstate (Charlottesville, VA); anti-total Akt and phospho-Akt (Ser 473), phospho-p42/p44 MAP kinase (Thr202/Tyr204), and total p65 and phopho-p65 were purchased from Cell Signaling Technologies (Danvers, MA); anti-histone H3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-heat shock protein 70 was obtained from Stressgen Biotechnologies (Ann Arbor, MI).

RT-PCR

Total RNA of the cells was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) using the manufacturer's instructions. The first-strand cDNAs were synthesized with a ProSTART First-Strand RT-PCR kit ST (Stratagene, La Jolla, CA). The following primer pair for PCR was used: Mac-2BP sense; 5'-ACACGGTCATCCTGACTGC-3', Mac-2BP antisense; 5'-ACAGGGACAGGTTGAACTGC-3'.

Mac-2BP ELISA

The cells were suspended in a cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 1 mM PMSF and incubated for 40 min on ice. The quantities of Mac-2BP in the cytosol of the cells were measured via ELISA using an s90k/Mac-2BP ELISA kit (Bender Med Systems GmbH, Vienna, Austria), in accordance with the instructions provided by the manufacturer.

Western blot analysis

Proteins were separated by 12-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to Hybond-P PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The nuclear and cytosolic fractions were separated using a commercially available kit according to the

protocol of the manufacturer (Pierce Biotechnology, Rockford, IL). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris-HCl, pH7.6, 150 mM NaCl, 0.1% Tween-20), and were incubated for 2 h with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature. After extensive washing, the protein bands were visualized with an Immobilon^{TM} Western Chemiluminiscent HRP substrate (Millipore, Billerica, MA).

Immunofluorescence confocal microscopy

Two million HEK293 cells were grown on 3-glycopropyl trimethoxysilane (Sigma-Aldrich) coated coverslips under starvation conditions for 18 h. NGF (50 ng/ml) and NGF plus tunicamycin (10 μg/ml) were administered for 24 h. The cells were then washed with PBS, fixed, and permeabilized in BD Cytofix /Cytoperm^{TM} (BD Biosciences Pharmingen, San Diego, CA) for 20 min. After washing, the cells were blocked for 10 min in 1% bovine serum albumin (BSA)/PBS at room temperature, washed, and stained overnight with an anti-Mac-2BP monoclonal antibody (Alexis, San Diego, CA) at 4^{o}C, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti- mouse IgG and 4',6-diamidino-2-phenylindole (DAPI; Calbiochem, San Diego, CA) staining. Cell-containing coverslips were mounted onto glass slides using Dakocytomation mounting medium (Produktionsvej 42, Glostrup, Denmark) and visualized with a LSM510META Zeiss confocal microscope (Carl Zeiss, Jena, Germany) at a magnification of 40X. The confocal images were captured by the Zeiss LSM Image Browser program.

Luciferase reporter assay

The -2377 bp DNA fragment (nucleotide positions -2377 to +61) including the 5' flanking region was inserted into the pGL3-basic luciferase vector (Promega, Madison, WI). The inserted sequences were verified via DNA sequence analysis (Genotech, Daejeon, Korea). For transient transfection, the cells were plated at a density of 5×10^5 cells /12 wells on day 1. One day later, 0.5 μg of pGL3-Mac pro-2377 vector and 0.2 μg of Renillar vector as an internal control were co-transfected with Lipofectamine Plus^{TM} Reagent (Invitrogen). After 3 h of incubation, the transfection media was changed with 1% fetal bovine serum (FBS)/MEM-α, and the next day, NGF was administered for an additional 24 h. The cells were lysed and a luciferase assay was conducted using the Dual-Luciferase^{R} reporter assay system, in accordance with the instructions provided by the manufacturer (Promega). Luciferase activity was determined using a Microlumat Plus luminometer (Berthold Technologies, Bad Wildbad, Germany) by measuring light emission for 10 s.

Preparation of nuclear extracts and electro mobility shift assay (EMSA)

Nuclear extract (5-7 μg) of nuclear extracts were incubated with ^{32}P-labeled probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM MgCl2, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) in the presence or absence of competitor or antibodies for 20 min at room temperature. The mixtures were then run on 6% polyacrylamide gels in 0.5 × TBE, and were dried and analyzed via autoradiography. The following probes that had been constructed through the annealing of single-stranded oligonucleotides were utilized for the EMSAs: upstream NF-κB site (NF-κBu -1622/-1611) sense '-GGCCTCTGGGTTTTCCATTT-3', antisense, 5'-GGAGA CCCAAAAGGTAAAAT-3'; downstream NF-κB site (NF-κBd-1580/-1589) sense, 5'-GGATGGCTTAGGACTTTCCC-3', antisense, 5'-TACCGAATCCTGAAAGGGCC-3'. The NF-κB consensus oligonucleotides were purchased from Promega.

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