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Nitric Oxide and Tumor Necrosis Factor-α Production by Oleanolic Acid via Nuclear Factor-κB Activation in Macrophages

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Oleanolic acid (OA), a pentacyclic triterpene acid, is reported to have antitumor activities; however, the mechanism underlying its antitumorigenic effects is poorly understood. To further determine the mechanism of OA, we investigated the effects of OA on the release of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) and on the level of inducible nitric oxide synthase (iNOS) and TNF-α gene expression in mouse macrophages. We found that OA elicited a dose-dependent increase in NO and TNF-α production. Reverse transcription–polymerase chain reaction showed that the increased NO and TNF-α secretion were due to an increase in iNOS mRNA and TNF-α mRNA, respectively. Since iNOS and TNF-α transcription have recently been shown to be under the control of the NF-κB transcription factor, the effects of OA on NF-κB activation were examined using a transient transfection assay and an electrophoretic mobility shift assay (EMSA). Transient expression assays with NF-κB binding sites linked to the luciferase gene revealed that the increased levels of iNOS mRNA and TNF-α mRNA induced by OA were mediated by the NF-κB transcription factor complex. Using DNA fragments containing the NF-κB binding sequence, OA was shown to activate the protein/DNA binding of NF-κB to its cognate site as measured by EMSA. These results demonstrate that OA stimulates NO and TNF-α release and is able to upregulate iNOS and TNF-α expression through NF-κB transactivation, which may be the mechanism whereby OA elicits its biological effects.

Key Words: **oleanolic acid; macrophages; iNOS; TNF-α; NF-κB.**

Macrophages play a significant role in host defense mechanisms. When activated they inhibit the growth of a wide variety of tumor cells and microorganisms. Nitric oxide (NO), a free-radical gas, is synthesized by inducible nitric oxide synthase (iNOS) (1, 2) and mediates diverse functions, including vasodilatation, neurotransmission, the inhibition of platelet aggregation, immunoresponses, and the inhibition of extracellular matrix production (3). NO has been identified as the major effector molecule involved in the destruction of tumor cells by activated macrophages (4, 5). Moreover, the involvement of NO during nonspecific host defense, macrophage-mediated killing, and the inhibition of the proliferation of microorganisms and tumor cells both *in vitro* and *in vivo* has been previously demonstrated (6, 7). Such NO-mediated tumoricidal activity is induced by DNA damage and leads to apoptotic cell death (8). The administration of NOS inhibitors to mice has been found to promote the growth of several transplantable tumors (7), and melanoma cells transfected with iNOS cDNA were found not to proliferate and metastasize well (9). Tumor necrosis factor-α (TNF-α) is produced by activated macrophages, fibroblasts, and many different types of cells. TNF-α has also been recognized and well characterized as an important host defense molecule that affects tumor cells (4, 10–12). In macrophages, nuclear factor κB (NF-κB) in cooperation with other transcription factors was found to coordinate the expression of genes encoding iNOS. Moreover, NF-κB plays a critical role in the activation of immune cells by upregulating the expression of many cytokines essential for immune response (13).

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, with relatively little knowledge of their modes of action. There is a growing interest in the elucidation of the biological roles of triterpenoid compounds, the major components of many traditional medicinal plants (14, 15), in terms of hepatoprotectory,

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analgesic, antitumor, anti-inflammatory and immunomodulatory effects. Oleanolic acid (OA; 3β-hydroxyolea-12-en-28-oic acid), is a triterpenoid, which is present in many kinds of medicinal plants, such as *Ligustrum lucidum* Ait. and *Glechoma hederacea* L. (15, 16), in the form of free acid or as aglycones of triterpenoid saponins (14). Treatment of animals with OA has been shown to protect against the acute hepatotoxicity produced by cadmium, carbon tetrachloride, and acetaminophen (17). In previous studies, we reported that the inhibition of the cytochrome P450 2E1 responsible for the metabolic activation of carbon tetrachloride is believed to be one of the hepatoprotective mechanisms of OA to carbon tetrachloride-induced hepatotoxicity (18).

It has been reported that OA produce a wide variety of antitumor activity, including decreases the incidence and multiplicity of azoxymethane-induced intestinal tumor (19), inhibition of angiogenesis which is important for the progressive growth of solid tumors (20), tumor promotion (21), and implanted tumor growth in mice (22). However, the mechanisms by which OA induces such cellular effects are not completely understood and the biochemical basis of these interesting inhibitory effects of OA on tumor promotion remains to be determined. To further characterize the mechanisms involved in OA-mediated antitumor effects, we tested the hypothesis that OA derives its antitumor effect by the release of NO and TNF-α from macrophages. We investigated the effects of OA on the release of NO and TNF-α, and on the level of iNOS and TNF-α gene expression in mouse macrophages.

MATERIALS AND METHODS

Chemicals. Chemicals and cell culture materials were obtained from the following sources: oleanolic acid, *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) and polymyxin B sulfate (Sigma Co.); MTT-based colorimetric assay kit (Roche Co.); LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin-streptomycin solution (Life Technologies, Inc.); pGL3-4KB-Luc and the luciferase assay system (Promega); pCMV-β-gal (Clontech); AmpliTaq DNA polymerase (Perkin–Elmer); other chemicals were of the highest commercial grade available.

Animals. Specific pathogen free-BALB/C mice (female, 5–7 weeks old) were obtained from KRIBB (South Korea). Mice were housed under normal laboratory conditions, i.e., at 21–24°C and 40–60% relative humidity under a 12-h light/dark cycle with free access to standard rodent food and water.

Preparation of peritoneal macrophages and cell cultures. Peritoneal macrophages were isolated from mice and cultured as described previously (23). RAW 264.7 cells, mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. OA was dissolved in dimethyl sulfoxide and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1% and this concentration did not show any effect on the assay systems.

Cell viability. Cell viability was assessed using a MTT-based colorimetric assay kit (Roche Co.), according to the manufacturer's instructions.

Nitrite assay. Peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured in 48-well plates. After incubating for 24 h, NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using Ellman's reagent as described previously (23).

Immunoassay of TNF-α. Peritoneal macrophages or RAW 264.7 cells were cultured at a density of 2×10^6 cells/ml for 6 h in 24-well plates. TNF-α production was quantified by sandwich immunoassays as described previously (23). Recombinant murine TNF-α was used as a standard.

Endotoxin assay. An E-Toxate test (*Limulus* amebocyte lysate, Sigma Chemical Co.) was used to assay OA for the presence of gram-negative bacterial endotoxin (LPS), according to the manufacturer's instructions.

RNA preparation and iNOS mRNA analysis by RT-PCR. RAW 264.7 cells were cultured with OA at a density of 1×10^6 cells/ml for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi (24). cDNA synthesis, semi-quantitative RT-PCR for iNOS, TNF-α, and β-actin mRNA, and analysis of results were performed as described previously (23). cDNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. A cycle number was used that fell within the exponential range of response for iNOS (754 bp, 35 cycles), TNF-α (692 bp, 35 cycles), and β-actin (153 bp, 17 cycles). PCRs were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Gel images were captured on a Gel Doc image analysis system (Kodak) and the yield of PCR products was normalized to β-actin after quantitative estimation using NIH Image software (Bethesda, MD). The relative expression levels were arbitrarily set at 1.0 in the control group.

Transfection and luciferase and β-galactosidase assays. RAW 264.7 cells (5×10^5 cells/ml) were plated in each well of a 12-well plate, and 12 h later transiently cotransfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to cells. After 18 h, cells were treated with CK or LPS for 12 h, and then lysed. Luciferase and β-galactosidase activity were determined as described previously (23). Luciferase activity was normalized using β-galactosidase activity and was expressed relative to the control activity.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as previously described (25). Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5′-GGGGACTTTCC-3′) (2) were end-labeled with [γ-^{32}P]dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly (dI–dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM Hepes, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml concentration each of aprotinin and leupeptin) for 10 min on ice. DNA binding was separated from the free probe using a 4.8% polyacrylamide gel in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

Statistical analysis. All experiments were repeated at least three times. Student's *t* test was used to assess the statistical significance of differences. A confidence level of <0.05 was considered significant.

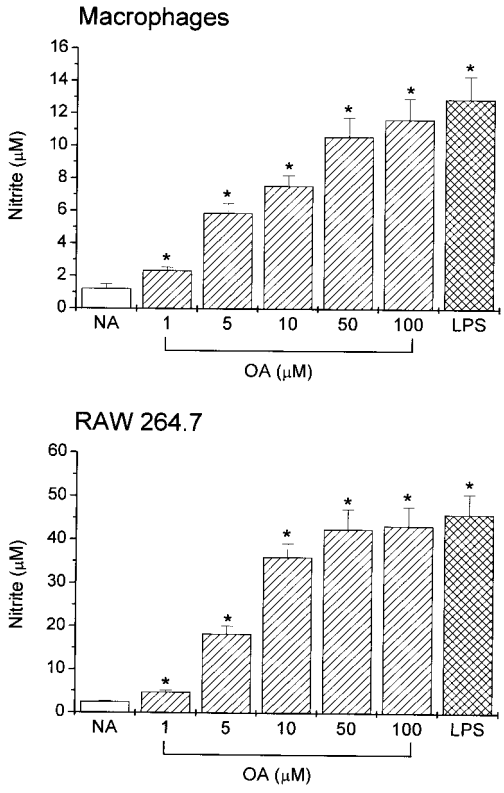


FIG. 1. Effects of OA on NO production. Murine peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured for 24 h in the presence of media alone, with the indicated concentrations of OA. NO production was determined by measuring the accumulation of nitrite in the incubation medium. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. $*P < 0.05$, significantly different from the control.

RESULTS AND DISCUSSION

OA is a triterpenoid compound that exists widely in food and some medicinal herbs (15). Since OA is known to have antitumor activity (19–22), we investigated the effects of OA on the release of NO and TNF-α, and its effects on the level of iNOS and TNF-α gene expression in mouse macrophages. Our findings indicate that in murine peritoneal macrophages and RAW 264.7, a murine macrophage cell line, OA stimulates NO and TNF-α release and is able to upregulate iNOS and TNF-α gene expression through NF-κB transactivation. OA-induced NO production was assessed after incubating for 24 h using the Griess reaction and the basal level of NO in untreated peritoneal macrophages was found to be less than 2 μM (Fig. 1). Moreover, OA showed a significant effect on nitrite production from 1 μM. The effect of OA gradually increased between concentrations 1 and 50 μM, and a plateau was reached at 50 μM, as shown in Fig. 1. Upon OA stimulation, NO synthesis by peritoneal macrophages increased in a dose-dependent manner. OA showed a cytotoxic action toward macrophages at concentrations over 200 μM (data not shown). In addition, the potent macrophage activator LPS (0.5 μg/ml) increased nitrite synthesis compared to the control. Consistent with these findings, OA also induced nitrite generation in a dose-dependent manner in RAW 264.7 cells (Fig. 1).

When macrophages were cultured in the presence of the OA, TNF-α was rapidly secreted. The concentration of TNF-α almost plateaued after 6 h of incubation, and remained constant between 6 and 24 h (data not shown). The maximum concentration of TNF-α in the culture medium was observed after 6 h of incubation (data not shown). Therefore, media that had been incubated for 6 h were used for the TNF-α assay in subsequent experiments. As found in the NO assay, upon OA stimulation, TNF-α secretions increased in a dose-dependent manner in peritoneal macrophages and RAW 264.7 cells (Fig. 2). These results suggest

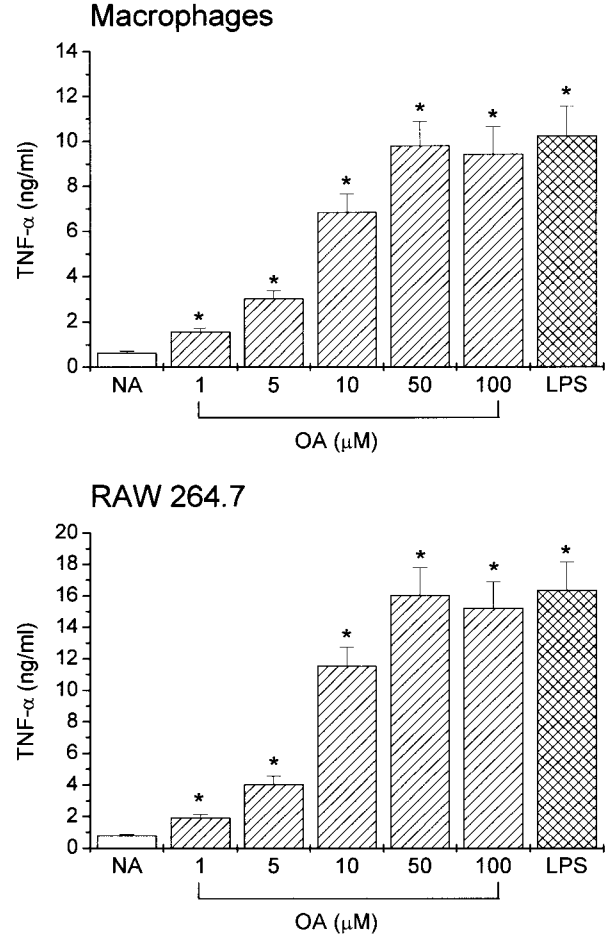


FIG. 2. Effect of OA on TNF-α secretion. Murine peritoneal macrophages (2×10^6 cells/ml) or RAW 264.7 cells (2×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of OA, or with LPS (0.5 μg/ml). The amount of TNF-α released into the culture medium was measured by immunoassay. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. $*P < 0.05$, significantly different from the control.

that the secretion of NO and TNF-α is regulated by the same mechanism, or that TNF-α, which is produced first, induces NO secretion via an autocrine or paracrine system. TNF-α is the first compound of the TNF-α and NO series to be secreted by macrophages (26). Thus, TNF-α is involved in the early phase of the cytokine cascade and induces NO production.

NO is involved in the killing and proliferative inhibition of microorganisms, the destruction of tumor cells by activated macrophages, and is a component of the nonspecific host defense (5, 7, 27, 28). Furthermore, it has demonstrated that murine macrophages stimulated by TNF-α (5) produce NO via the expression of the inducible NOS gene, and it is believed that the reactive nitrogen intermediates so induced play a significant role in tumoricidal and microbiocidal activities (5). TNF-α, an endogenous factor with tumor-selective cytotoxicity, and has been recognized as an important host defense molecule that affects tumor cells. Moreover, the induction of NO and TNF-α production and gene expression by activated macrophages can lead to cytostatic and cytotoxic effects on malignant cells (4, 6, 10–12, 23). Because of the pivotal role of NO and TNF-α in the antimicrobial and tumoricidal activities of macrophages, significant effort has been focused on developing therapeutic agents that regulate NO and TNF-α production (29). Based on these results and the relationship between nitrite and the cytolytic function of macrophages against a variety of tumors (7, 27), we suggest that the antitumor effect of OA might be mediated in part through the activation of NO and TNF-α secretion.

Macrophages can be induced to produce NO and TNF-α by LPS, endotoxins, or cytokines (27). To confirm, that the ability of OA to induce NO and TNF-α could not be attributed to LPS contamination, the OA was tested for the presence of contaminating LPS using the *Limulus* amoebocyte lysate test. The level of LPS in OA was below the detection limit, which was typically below 12.5 pg/ml (data not shown). Polymyxin B sulfate has been used previously as a LPS inhibitor in macrophage cultures (30), and although OA contained no detectable activity in the *Limulus* amoebocyte lysate assay, we checked for possible LPS contamination in OA, by adding polymyxin B (10 μg/ml) to cell cultures treated with OA (10 μM). As shown in Table 1, polymyxin B effectively inhibited the NO and TNF-α secretion induced by 0.5 μg/ml LPS, but had no effect on the OA. This result demonstrated that the production of NO and TNF-α by OA was unlikely to have resulted from LPS contamination in the OA.

As stated above, OA induced macrophage secretion of NO and TNF-α. To determine whether OA regulates NO and TNF-α secretion at the mRNA level, a reverse transcription-polymerase chain reaction (RT-PCR) assay was conducted. The intensity of the iNOS and TNF-α mRNA band increased 2 h after incubation and

TABLE 1

Effects of Polymyxin B on NO and TNF-α Secretion by OA and LPS

Treatment[a]	Nitrite (μM)[b]	TNF-α (ng/ml)[c]
Control	1.67 ± 0.28*,**	0.71 ± 0.84*,**
OA	36.11 ± 4.21	12.63 ± 1.41
OA + polymyxin B	33.93 ± 4.42	11.92 ± 1.32
LPS	41.74 ± 4.52	15.81 ± 1.94
LPS + polymyxin B	10.33 ± 1.23*,**	4.49 ± 0.06*,**

[a] RAW 264.7 cells (5×10^5 cells/ml for nitrite assay and 2×10^6 cells/ml for TNF-α immunoassay) cultured with OA (10 μM) or LPS (0.5 μg/ml), in the presence or absence of polymyxin B (10 μg/ml).

[b] Supernatants were harvested 24 h later and assayed for NO.

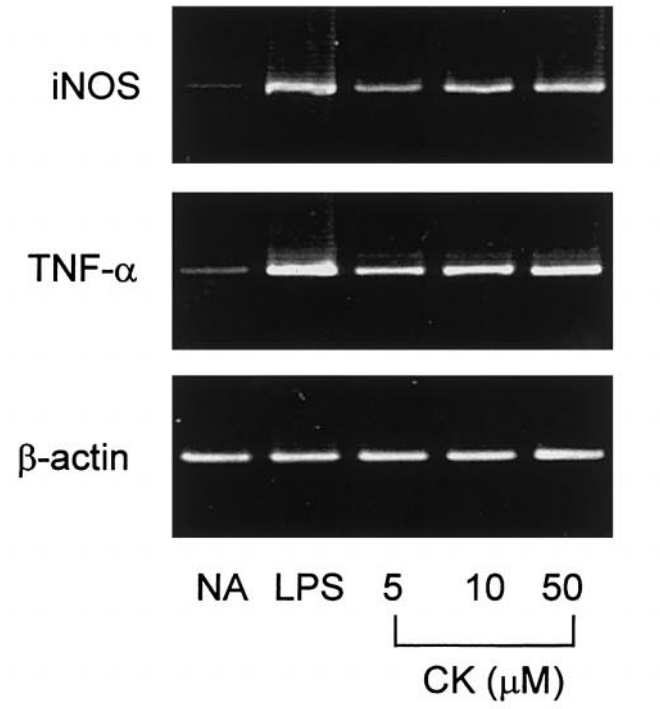
[c] Supernatants were harvested 6 h later and assayed for TNF-α. Values are expressed as means ± SD of three individual experiments, performed in triplicate.

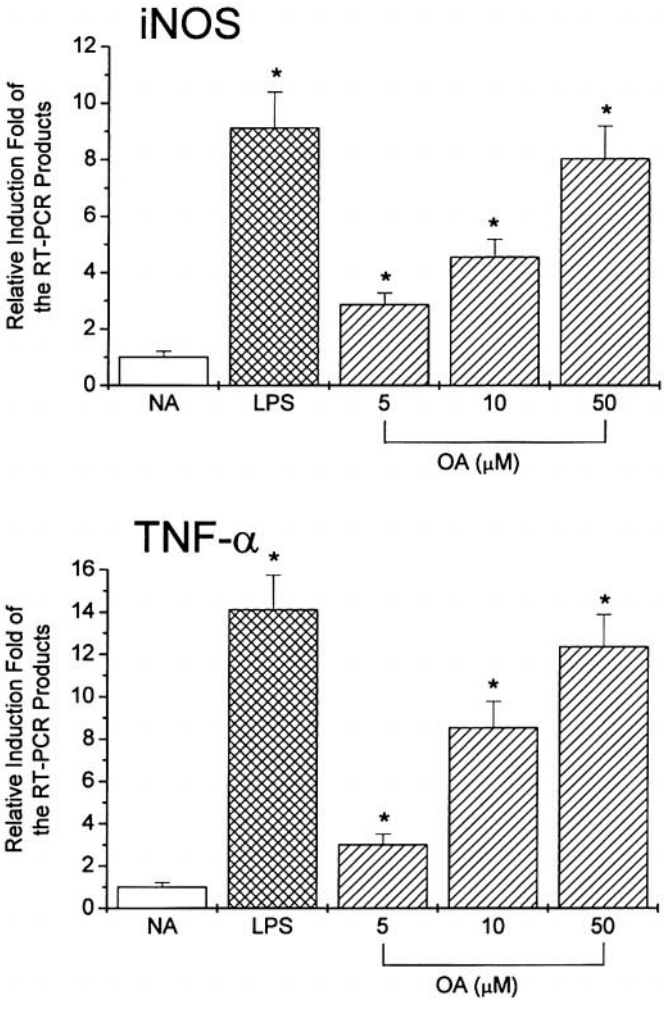
* $P < 0.05$, significantly different from LPS.

** $P < 0.05$, significantly different from OA.

a strong band was observed at 6 h (data not shown). Therefore, cells treated with OA for 6 h were used to assay iNOS and TNF-α gene expression in subsequent experiments. RAW 264.7 cells were cultured with OA for 6 h and the mRNA expression of iNOS was determined. LPS (0.5 μg/ml), an immunostimulatory agent, was used as a positive control. Consistent with the results obtained from the NO assay, iNOS mRNA levels were markedly increased by OA treatment (Fig. 3). This result indicates that OA upregulated, in a dose-dependent manner, NO accumulation in macrophages. Therefore, we believe that increased NO production by OA is regulated through transcriptional activation. Under the same treatment conditions, the TNF-α gene expression marker of macrophage activation was also examined, and similarly OA was found to significantly enhance the expression levels of the TNF-α gene (Fig. 3). This result is consisted with that obtained from the immunoassay of TNF-α in macrophages and indicates that the OA also upregulates TNF-α accumulation in a dose dependent manner.

NF-κB is a member of the Rel family, and is a common regulatory element in the promoter region of many cytokines. In activated macrophages, NF-κB in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding iNOS, TNF-α, and IL-1 (13). To further investigate the role of OA on iNOS and TNF-α gene expression, the effect of OA on NF-κB-dependent gene expression was assessed using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing 4 copies of the NF-κB binding sites and the luciferase activities were measured. LPS, an immunostimulatory agent, was used as a positive control. Nearly a 6-fold increase in the luciferase activity was observed compared to the unstimulated control cells, when cells were stimulated with





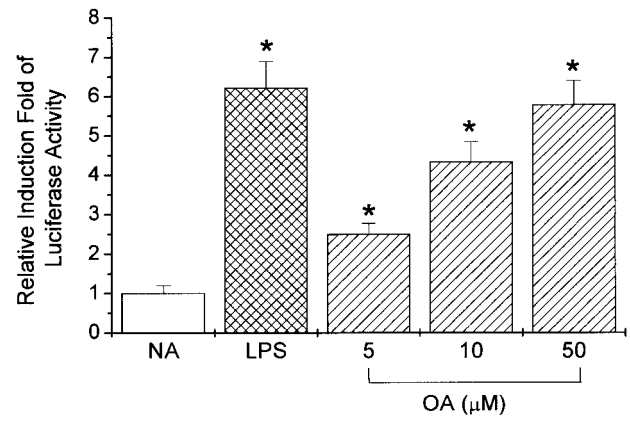


FIG. 4. Effects of OA on NF-κB-dependent luciferase gene expression. RAW 264.7 cells (5×10^5 cells/ml) were transiently cotransfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, cells were treated with the indicated concentrations of OA or LPS (0.5 μg/ml) for 12 h. Cells were then harvested, and luciferase and β-galactosidase activities determined. Luciferase activities were expressed relative to the control. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. $^*P < 0.05$, significantly different from the control.

LPS. Consistent with NO production and iNOS mRNA measurement, OA also significantly increased NF-κB-dependent luciferase activities in a dose dependent manner (Fig. 4). To further investigate the putative mechanism by which OA activates iNOS, the effects of OA on the activation of a family of transcription factors was monitored by gel shift assay. NF-κB binding activity was examined in the light of its critical role in the regulation of iNOS and TNF-α. EMSA demonstrated that OA induced a marked increase in NF-κB binding to its conserved site that could be visualized a distinct band (Fig. 5). These results indicate that the upregulation of the iNOS and TNF-α gene by OA is mediated by the transactivation of NF-κB. Although we demonstrated the upregulatory ability of OA on iNOS and TNF-α expression in macrophages, the mechanism by which OA stimulated iNOS and TNF-α expression in macrophages remains to be determined, such as the activation of Raf-1 and MAP kinases (31). Additional studies are needed to answer these questions and to elucidate the mechanisms involved.

FIG. 3. Effects of OA on iNOS and TNF-α mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of OA, or with LPS (0.5 μg/ml). Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. The ratio of the RT-PCR products of iNOS or TNF-α to β-actin was calculated. Induction-fold is represented as a mean ± SD of three separate experiments. $^*P < 0.05$, significantly different from the control.

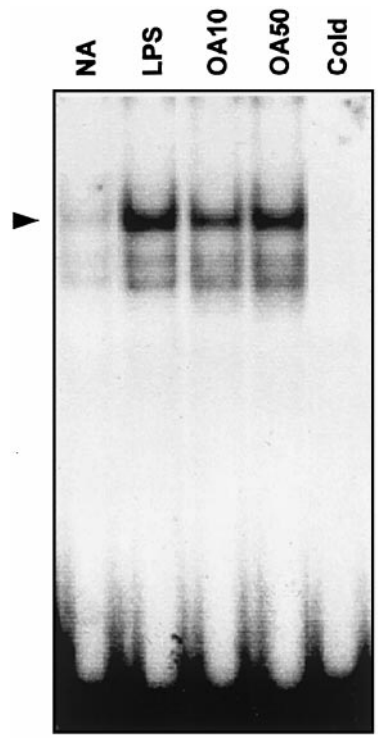


FIG. 5. Effects of OA on NF-κB-binding. RAW 264.7 cells were treated with LPS (0.5 μg/ml) or OA (10, 50 μM) for 1 h. Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described under Materials and Methods. The arrowhead indicates the NF-κB binding complex. Cold, 200-fold molar excess of nonlabeled NF-κB probe. One of three representative experiments is shown.

The cytotoxicity of tumor cells is dependent on the activation of macrophages, which is strongly correlated with the expression patterns of several cytokine mediators. Marcinkiewicz *et al.* (32) demonstrated that the increasing NO levels enhance the release of TNF-α and reactive oxygen species. This effect may be due to both an increased generation of the superoxide anion, and the preferential formation of peroxynitrite, which can be formed by direct reaction between NO and the superoxide radical; both have powerful cytotoxic properties (33). The decomposition product of peroxynitrite and the hydroxy radical is believed to be the most toxic oxygen molecule *in vivo* (34). NO and TNF-α were investigated in the current study to confirm the possibility that OA might be an immunostimulator and OA was found to elicit NO and TNF-α production. This result supports the possibility that NO and TNF-α induction by OA may contribute *in vivo* to its immunomodulatory and antitumoricidal activities. Biological response modifiers are widely used in cancer immunochemotherapy to potentiate therapeutic efficacy or to alleviate the toxicity of cytotoxic anticancer agents. It is interesting that OA can upregulate iNOS expression in macrophages. The potent antitumor activity of OA is remarkable, and we hope that it can be developed into an agent for cancer therapy. The use of OA has been recommended for skin cancer therapy in Japan (35). Cosmetic preparations containing OA are patented in Japan as topical skin cancer preventatives (35). Further studies on OA will be needed to prove its clinical usefulness in cancer therapy and its effectiveness in other diseases. To investigate the overall antitumor effect of OA, a study on the *in vivo* induction of gene expression and the production of immunomodulatory cytokines in mice is underway in our laboratory. However, the exact mechanism underlying OA induced NO production and release remains to be elucidated.

In summary, OA stimulates macrophage-derived NO and TNF-α production and is able to upregulate iNOS and TNF-α expression through NF-κB transactivation in murine macrophages. These actions might be providing a mechanistic basis for the antitumor properties of OA.

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