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Aurintricarboxylic Acid Protects against Cell Death Caused by Lipopolysaccharide in Macrophages by Decreasing Inducible Nitric-Oxide Synthase Induction via IκB Kinase, Extracellular Signal-Regulated Kinase, and p38 Mitogen-Activated Protein Kinase Inhibition

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ABSTRACT

To elucidate the mechanisms involved in cell protection by aurintricarboxylic acid (ATA), an endonuclease inhibitor, high nitric oxide (NO)-induced macrophage apoptosis was studied. In RAW 264.7 macrophages, a high level of NO production accompanied by cell apoptosis was apparent with lipopolysaccharide (LPS) treatment. Direct NO donor sodium nitroprusside (SNP) also dramatically induced cell death, with an EC_{50} of 1 mM. Coincubation of ATA (1–500 μM) in LPS-stimulated RAW 264.7 cells resulted in a striking reduction of NO production and cell apoptosis, whereas only a partial cell protection was achieved in response to SNP. This suggests that abrogation of inducible nitric-oxide synthase (iNOS)-dependent NO production might contribute to ATA protection of LPS-treated cells. Immunoblotting and reverse transcription-polymerase chain reaction analysis revealed that ATA down-regulated iNOS protein through transcriptional inhibition of iNOS gene expression but was unrelated to iNOS protein stability. ATA not only inhibited nuclear factor-κB (NF-κB) activation through impairment of the targeting and degradation of IκBs but also reduced LPS-induced activator protein-1 (AP-1) activation. These actions of ATA were not caused by the influence on LPS binding to macrophage membrane. Kinase assays indicated that ATA inhibited IκB kinase (IKK), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) activity both in vivo and in vitro, suggesting a direct interaction between ATA and these signaling molecules. Taken together, these results provide novel action targets of ATA and indicate that ATA protection of macrophages from LPS-mediated cell death is primarily the result of its inhibition of NO production, which closely relates to the inactivation of NF-κB and AP-1 and inhibition of IKK, ERK and p38 MAPK.

Apoptosis is an essential process of the development and tissue homeostasis of most multicellular organisms, and the deregulation of apoptosis has been implicated in the pathogenesis of many disease states. One of the hallmarks of apoptosis is the orderly cleavage of genomic DNA of nucleosomal or oligonucleosomal lengths. To date, a variety of endonucleases responsible for chromatin degradation have been identified. Although some evidence indicates that endonuclease(s) leading to oligonucleosomal DNA fragmentation is common and an essential event in apoptosis, endonuclease-mediated DNA fragmentation may not play a central role in apoptosis for some death inducers. For instance, it was reported that endonucleolytic DNA degradation is neither required nor sufficient for K^+ withdrawal-induced apoptosis of cultured cerebellar granule neurons (Schulz et al., 1998) and heat shock-induced apoptosis of the U937 leukemic cell line (Shrivastava et al., 2000).

Aurintricarboxylic acid (ATA), a negatively charged triphenylmethane derivative (473 Da), has been demonstrated to prevent apoptosis in a variety of cell models. It was used as an antiapoptotic drug to counteract ischemic or cytotoxic

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ABBREVIATIONS: ATA, aurintricarboxylic acid; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor-κB; NO, nitric oxide; LPS, lipopolysaccharide; iNOS, inducible nitric-oxide synthase; IKK, IκB kinase; ERK, extracellular signal-regulated kinase; AP-1, activator protein-1; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; EMSA, electrophoretic mobility shift assay; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; RT, reverse transcription; PCR, polymerase chain reaction; SNP, sodium nitroprusside; NOS, nitric-oxide synthase; GST, glutathione *S*-transferase; IFN-γ, interferon-γ.

injury to neurons (Rosenbaum et al., 1998; Vincent and Maiese, 1999; Heiduschka and Thanos, 2000). As such, the use of ATA as a therapeutic agent for conditions such as ischemic stroke and Alzheimer's disease has been proposed (Rosenbaum et al., 1998). ATA also possesses the ability to inhibit apoptosis of non-neuronal cells, such as hemopoietic cells (Rui et al., 1998; Shrivastava et al., 2000), endothelial cells (Escargueil-Blanc et al., 1997), oligodendrocytes (Vollgraf et al., 1999), and lutein cells (Viergutz et al., 2000).

The pharmacological action of ATA (10–100 μM) as an inhibitor of apoptosis in serum- and growth factor-deprived neuronal cultures was first thought to reside in its inhibition of cellular endonucleases (Martin et al., 1988; Batistatou and Greene, 1991). After these observations, ATA was also demonstrated to act in an endonuclease-independent manner to interact with several cellular targets, which might also contribute to its antiapoptotic effects. These include the topoisomerases (Benchokroun et al., 1995), the interferon-α and *N*-methyl-D-aspartate receptors (Zeevald et al., 1993), and different important signaling cascades. For example, it is a potent activator of the mitogen-activated protein kinase (MAPK) cascade in PC-12 (Okada and Koizumi, 1995) and Nb2 lymphoma (Rui et al., 1998) cells. It can activate the erbB4 receptor, a member of the epidermal growth factor receptor family, in SH-SY5Y neuroblastoma cells (Okada and Koizumi, 1997). Its activation on both MAPK and erbB4 activation share characteristics with growth factors that can rescue cells from programmed cell death caused by serum starvation. In addition, ATA affects Nb2 lymphocytes through a selective activation of the Janus tyrosine kinase 2-STAT5 pathway, which promotes cell viability and proliferation (Rui et al., 1998). A recent in vitro study showed that ATA inhibited DNA–NF-κB binding at 30 μM (Sharma et al., 2000). All these findings suggest that ATA does not act exclusively as an endonuclease inhibitor but might exert its antiapoptotic effect via its action on signal transduction pathways that promote cell survival. These endonuclease-independent mechanisms that contribute to ATA antiapoptotic effects might be cell type-specific. How ATA activates these signaling pathways, as mentioned above, remains unclear.

Apoptotic pathways depend totally on the insult and cell types, and the biochemical elucidation on target enzyme and signaling pathways will provide insight into the molecular mechanism responsible for the antiapoptotic action of ATA. As such, it is worthwhile to examine the action of ATA on excess nitric oxide (NO)-induced apoptosis, which is a principal cytotoxic mediator implicated in many inflammatory conditions. There is considerable evidence for the apoptotic effects of NO on macrophages exposed to endotoxin lipopolysaccharide (LPS) (Albina and Reichner, 1998; Hortelano et al., 1999). The use of inhibitors of inducible nitric-oxide synthase (iNOS), overexpression of arginase, and scavengers for NO generated by these activated cells have been demonstrated to block cell injury and death (Misko et al., 1998; Gotoh and Mori, 1999). Besides reduction in NO generation, therapies aimed at inhibiting NO-dependent cell apoptosis may contribute to improving the outcome of sepsis, which remains a clinical conundrum. Thus, in this study, we address the beneficial effect of ATA in macrophages that undergo apoptosis caused by the large amounts of NO produced through iNOS induction by LPS. Unexpectedly, we found that ATA could prevent LPS-induced apoptosis through inhibition of iNOS expression at the transcriptional level. The inhibition of IκB kinase (IKK), extracellular signal-regulated kinase (ERK), and p38 MAPK may contribute to the blockade of NF-κB and AP-1 activation, which are two major transcription factors essential for iNOS gene expression.

Experimental Procedures

Materials

Oligonucleotides specific for NF-κB, AP-1, and STATs binding were synthesized on a DNA synthesizer (PS250; Cruachem Ltd., Glasgow, UK), using the cyanoethyl phosphoroamidate method, and purified by gel filtration. The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of NF-κB, AP-1, and STAT are as follows: NF-κB, 5′-GATCAGTTGAGGGGACTTTCCCAGGC-3′; AP-1, 5′GATCCGCTTGATGACTCAGCCGGAA-3′; and STAT, 5′-ATCGTTCATTTCCCGTAAATCCCTA-3′. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). [α-^{32}P]ATP (3000 Ci/mmol), horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies, and the enhanced chemiluminescence detection agent were purchased from Amersham Biosciences (Piscataway, NJ). Rabbit polyclonal anti-iNOS and anti–β-tubulin antibodies were purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies specific for p65 NF-κB, p50 NF-κB, IKKα, IKKβ, IκBα, IκBβ, ERK, p38 MAPK, c-fos, c-jun, STAT-1, and protein A/G agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to the phosphorylated p38 MAPK and ERK were purchased from New England Biolabs (Beverly, MA). The plasmid of pGEX-IκBα (amino acid 5–55) was provided by Dr. Frank S. Lee (University of Pennsylvania Medical Center, PA). Annexin V-FLUOS was purchased from Roche Applied Science (Indianapolis, IN). All materials for SDS-PAGE were obtained from Bio-Rad (Hercules, CA). Phenol-extracted LPS (L8274) from *Escherichia coli*, ATA, myelin basic protein (MBP), and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Methods

Cell Culture. RAW 264.7 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in an atmosphere of 5% CO_2. Cells were seeded into 24-well plates for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and nitrite assays, into 35-mm dishes for immunoblots, and into 10-cm dishes for electrophoretic mobility shift assay (EMSA).

Nitrite Assay. Measurement of nitrite production as an assay of NO release was performed. Accumulation of nitrite in the medium was determined by colorimetric assay with Griess reagent. The cells were treated with LPS, ATA, and/or the indicated agents for different intervals. Aliquots of conditioned media were mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine in 5% phosphoric acid]. Nitrite concentrations were determined by comparison with the OD_{550} using standard solutions of sodium nitrite prepared in cell culture me-

dium. Each experiment was performed in duplicate and repeated at least three times.

Measurement of Cell Viability. Cell viability was assessed by MTT assay. Briefly, cells plated in 24-well plates were incubated for 1 day before the addition of LPS or sodium nitroprusside (SNP) in the absence or presence of ATA. After different periods of incubation, MTT (1 mg/ml) was added for 60 min, then the culture medium was removed, and cells were dissolved in dimethyl sulfoxide and shaken for 10 min. The OD at 550 and 630 nm was measured using a microplate reader. The net absorbance ($OD_{550} - OD_{630}$) indicates the enzymatic activity of mitochondria and implicates the cell viability.

Apoptotic Analysis. Annexin V is a protein that binds to phosphatidylserine (PS) residues, which are exposed on the cell surface of apoptotic, but not normal, cells. In living cells, the distribution of the PS group in the plasma membrane is asymmetrical, such that the groups are directed toward the inside of the cell. During apoptosis, this asymmetry is lost, and the PS groups are exposed to the exterior of the cell membrane. The binding of PS with annexin V is therefore an established biochemical marker of apoptosis. After agent treatment, cells were washed twice with PBS, pH 7.4, and resuspended in staining buffer containing 50 μg/ml propidium iodide and 0.025 μg/ml annexin V-fluorescein isothiocyanate (FITC). The double labeling was performed at room temperature for 15 min in the dark before flow cytometric analysis.

Immunoblotting Analysis. After agent treatment, the medium was aspirated. Cells were rinsed twice with ice-cold PBS, and 100 μl of whole-cell lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was then added to each well. After cell harvest, cell lysates were centrifuged. Equal amounts of the soluble protein were denatured in SDS, electrophoresed on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) containing 5% nonfat milk for 1 h at room temperature. After immunoblotting with the first specific antibodies, membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. After three washes with TBST, the protein bands were detected with enhanced chemiluminescence detection reagent. To make sure equal amounts of sample protein were applied for electrophoresis and immunoblotting, β-tubulin was used as an internal control.

RT-PCR. The expression of iNOS mRNA was determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Macrophages treated with LPS (1 μg/ml) in the presence or absence of ATA were homogenized with 1 ml of RNAzol B reagent (Invitrogen), and total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction. RT was performed using StrataScript RT-PCR kit (Stratagene, La Jolla, CA), and 10 μg of total RNA was reverse transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding iNOS and β-actin genes were amplified using PCR. The oligonucleotide primers used correspond to the mouse macrophages iNOS (5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′ and 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′) and mouse β-actin (5′-GAC TAC CTC ATG AAG ATC CT-3′ and 5′-CCA CAT CTG CTG GAA GGT GG-3′). PCR was performed in a final volume of 50 μl containing *Taq*DNA polymerase buffer, all four dNTPs, oligonucleotide primers, *Taq*DNA polymerase, and RT products. After an initial denaturing for 2 min at 94°C, 35 cycles of amplification (94°C for 45 s, 65°C for 45 s, and 72°C for 2 min) were performed followed by a 10-min extension at 72°C. PCR products were analyzed on 2% agarose gel. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Immunoprecipitation and Kinase Assay. To determine the effect of ATA on IKK in vivo, after stimulation, anti-IKKα and anti-IKKβ (1 μg each) with protein A/G agarose beads were added to the prepared cell extracts as mentioned above. Immunoprecipitation proceeded at 4°C overnight. The precipitated beads were washed three times with 1 ml of ice-cold cell lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 2 mM dithiothreitol). The immune-complex kinase assay of one half of the immunoprecipitates was performed at 30°C for 30 min in 20 μl of kinase reaction buffer containing 1 μg of GST-IκBα, 25 μM ATP, and 3 μCi of [γ-^{32}P]ATP. The reaction was terminated with 5× Laemmli sample buffer, and the products were resolved by 12% SDS-PAGE gel electrophoresis. The phosphorylated IκBα was visualized by autoradiography. The other half of the immunoprecipitates was subjected to SDS-PAGE and immunoblotting to verify that equal amounts of kinases were undergoing kinase reaction.

In some experiments, to investigate the direct effect of ATA on kinase activity, cells were stimulated with 1 μg/ml LPS for 15 min. After cell extraction, anti-IKKα/IKKβ, anti-ERK, and anti-p38 MAPK with protein A/G agarose beads were used, respectively, to precipitate each type of kinase. In the in vitro kinase reactions, ATA (different concentrations) and substrates (GST-IκBα for IKK, MBP for ERK and p38 MAPK) were included.

Preparation of Nuclear Extracts and EMSAs. Nuclear extracts from stimulated or nonstimulated macrophages were prepared by cell lysis followed by nuclear lysis; cells were suspended in 30 μl of buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride; vortexed vigorously for 15 s; allowed to stand at 4°C for 10 min; and centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride for 20 min on ice, and then the lysates were centrifuged at 15,000 rpm for 2 min. The supernatants containing the solubilized nuclear proteins were stored at −70°C until used for EMSAs. Binding tests for NF-κB, AP-1, and STAT-1 were performed. Briefly, binding reaction mixtures (15 μl) contained 0.25 μg of poly(dI-dC) (Amersham Biosciences) and 20,000 dpm of ^{32}P-labeled DNA probe in binding buffer consisting of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol, and 75 mM KCl; the binding reaction was started by the addition of cell extracts and continued for 30 min. Samples were analyzed on native 5% polyacrylamide gels. For supershift experiments, 5 μg of p65, p50, c-fos, c-jun, or STAT-1 antibody were mixed with the nuclear extract proteins.

Inducible NOS Protein Stability Analysis. Macrophages were stimulated with LPS (1 μg/ml) for 24 h. Anisomycin (3 μg/ml) was applied to the medium thereafter, to interrupt further protein synthesis. One hour later, ATA (300 μM) was added immediately after anisomycin for extra 4-, 8-, and 12-h incubations, respectively. Total protein was prepared at the indicated time points and processed for Western blotting as described above.

Laser Flow Cytometric Analysis of LPS Binding. RAW 264.7 cells were pretreated with ATA for 30 min before the incubation with FITC-conjugated LPS at 4°C for 1 h. The cells were then washed with and suspended in PBS. The fluorescence intensity was analyzed by a laser flow cytometer.

Statistical Evaluation. Values are expressed as the mean ± S.E.M. of at least three experiments. Analysis of variance was used to assess the statistical significance of the differences, with a p value of <0.05 being considered statistically significant.

Results

ATA More Effectively Inhibits Cell Death Caused by LPS Than That Caused by SNP. LPS can cause macrophage cell death in a concentration- and time-dependent manner. MTT assays revealed that at concentrations higher than 10 μg/ml, an apparent cell death (of approximately 75%)

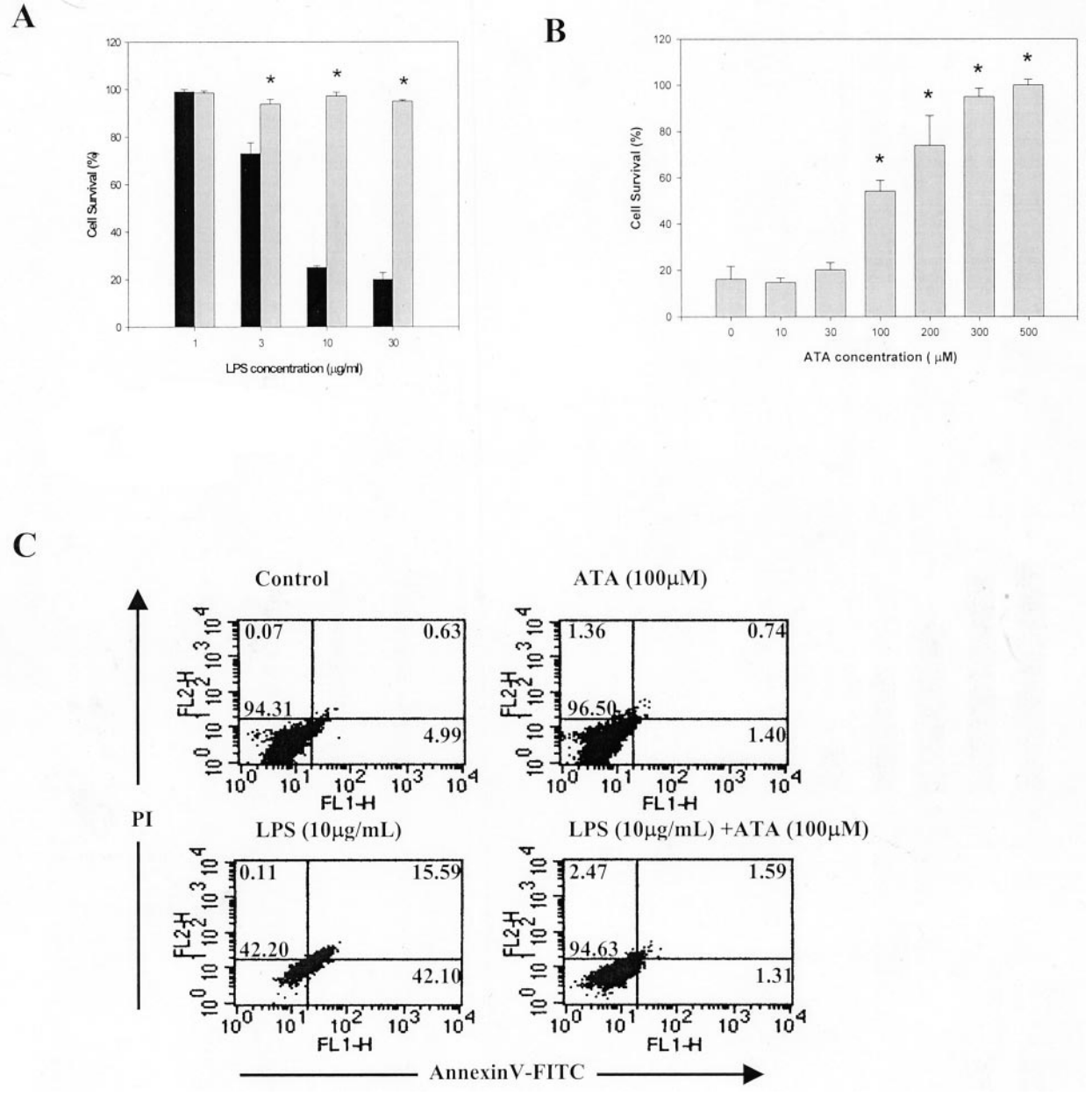

Fig. 1. ATA prevents cell death in response to LPS. A, confluent RAW 264.7 cells were treated with LPS at the concentrations indicated in the absence or presence of 300 μM ATA for 24 h. Filled bars, control; gray bars, ATA (300 μM). B, cells were treated with 10 μg/ml LPS together with different concentrations of ATA for 24 h. After incubation, cell viability was determined by MTT assay. The data represent the mean ± S.E.M. of at least three independent experiments. ⋆, $p < 0.05$ indicates significant protection of the LPS response. C, after LPS and/or ATA treatment for 24 h, cells were labeled by annexin V-FITC and propidium iodide and analyzed by flow cytometry. The lower-left quadrant (annexin V^-, PI^-) shows the viable cells. The lower-right quadrant (annexin V^+, PI^-) represents the early apoptotic cells. The upper-right quadrant (annexin V^+, PI^+) contains advanced apoptotic and necrotic cells.

was detected within 24 h of incubation (Fig. 1A). The toxicity of 3 μg/ml LPS was minimal, with 30% cell death being detected after 24 h of incubation (Fig. 1A). ATA itself did not affect cell viability within 24 h of incubation at concentrations up to 500 μM (data not shown). Coincubation of 300 μM ATA with LPS, however, significantly reduced cell death caused by LPS (Fig. 1A). The cell-protective effect of ATA exhibited a concentration-dependent manner. The threshold concentration for significant protection against 10 μg/ml LPS was 100 μM (Fig. 1B).

Previous studies have extensively documented the apoptotic features caused by LPS in vivo and in vitro (Slomiany et al., 1998; Karahashi and Amano, 2000). To confirm that cells underwent apoptosis after LPS incubation, propidium iodide and annexin V staining was measured. Figure 1C shows that the percentage of living cells from LPS treatment was lower compared with those observed in cells cultured with vehicle (42 versus 94%). ATA treatment in the presence of LPS significantly increased the percentage of living cells compared with the LPS group (94 versus 42%).

To explore whether cell protection by ATA is related to the abrogation of NO-dependent apoptotic pathways, we examined its effect on SNP, which is a direct NO generator (Feelisch, 1998). As shown in Fig. 2, after 12 or 24 h of incubation, cell viability was significantly diminished by 1 mM SNP. When concentrations were increased 2-fold (2 mM), almost all cells died within 24 h. In contrast to the full effective protection of cells from LPS treatment, ATA gave weak protection to SNP-treated cells; the percentage of cells rescued by 500 μM ATA was about 10 to 20%.

Cell Protection by ATA Is Accompanied by the Inhibition of NO Production. Because a high concentration of LPS-induced macrophage death was dependent on NO production caused by induced iNOS expression, we next examined the effect of ATA on nitrite level in the culture medium. Addressing this issue will provide information helpful to understanding why ATA protects macrophages against LPS more effectively than against SNP. Figure 3A shows that LPS concentration- and time-dependently induced NO production within 24 h of incubation, and this action of LPS was inhibited by 300 μM ATA. Figure 3B also shows that ATA inhibition of NO production manifested a concentration dependence with IC_{50} values of 26, 97, and 200 μM, respectively, for 0.1, 1, and 10 μg/ml LPS. Parallel to the NO reduction by ATA, immunoblotting analysis showed that the LPS-induced iNOS protein level was diminished by ATA in a concentration-dependent fashion (Fig. 3C).

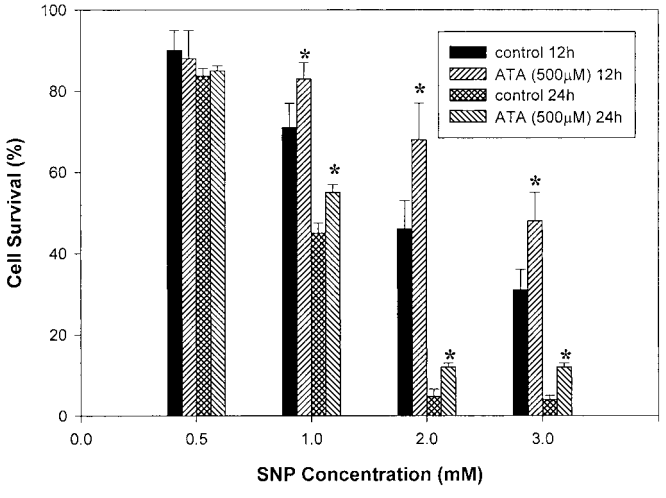

Fig. 2. ATA prevents cell death in response to SNP. Confluent RAW 264.7 cells were treated with SNP at the concentrations indicated in the absence or presence of 500 μM ATA for 12 or 24 h. After incubation, cell viability was determined by MTT assay. The data represent the mean ± S.E.M. of at least three independent experiments. ⋆, $p < 0.05$ indicates significant protection of the SNP response.

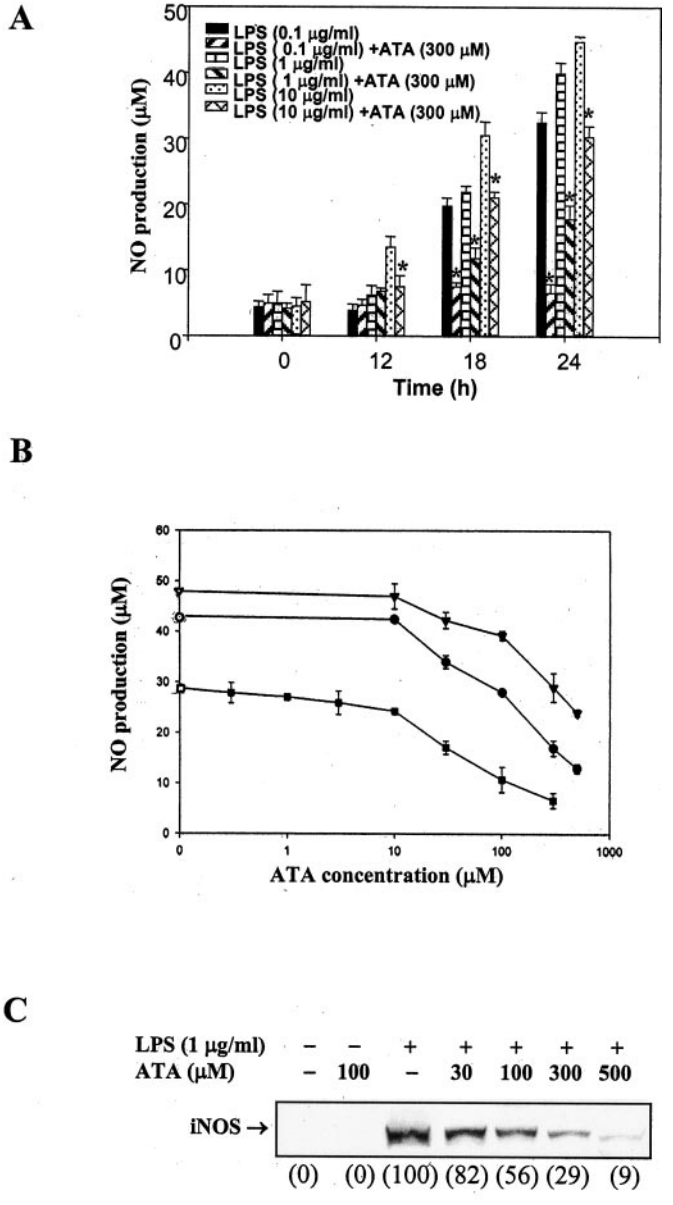

Fig. 3. ATA inhibits NO production and iNOS protein induction. A, time-dependent effects of LPS and ATA on NO production. RAW 264.7 cells were treated with 0.1 to 10 μg/ml LPS alone or in combination with 300 μM ATA for different periods. B, concentration-dependent effects of LPS and ATA on NO production. RAW 264.7 cells were treated with various concentrations of LPS (0.1, 1, and 10 μg/ml) in the absence or presence of ATA at the concentrations indicated for 24 h. The data represent the mean ± S.E.M. of at least three independent experiments. ⋆, $p < 0.05$ indicates significant reduction of NO production in response to LPS. ■, LPS (0.1 μg/ml); ●, LPS (1 μg/ml); ▼, LPS (10 μg/ml). C, cells were incubated with LPS (1 μg/ml) with or without ATA (30–500 μM) for 24 h. LPS-induced iNOS protein expression in ATA-treated RAW 264.7 cells was determined by immunoblotting analysis and quantified by densitometer. The results are representative of three independent experiments.

ATA Does Not Alter the Binding of FITC-Labeled LPS. To determine the mechanism of ATA inhibition of NO production and iNOS expression in LPS-stimulated RAW 264.7 cells, we studied the binding of FITC-labeled LPS (10 and 20 μg/ml). Laser flow cytometric analysis demonstrated that there was no significant difference in the binding of FITC-labeled LPS in cells treated with 300 μM ATA or not (Fig. 4). This result suggests that ATA might affect the intracellular signal transduction of LPS rather than interfere with LPS binding to the receptors in plasma membrane.

NO Inhibition by ATA Occurs at the Transcriptional Level. Because NO production from macrophages occurs via gene expression of iNOS, the regulatory action of ATA on iNOS transcription was assessed. First, we tested the protective efficiency of ATA when iNOS-inducing signal transduction pathways were evoked by LPS earlier. When ATA (300 μM) was added at increasing intervals after the stimulation of macrophages with LPS (10 μg/ml), the inhibition of NO production decreased as the interval lengthened, up to 12 h (Fig. 5A). This result suggests that ATA alters iNOS expression by macrophages rather than having a direct effect on nitric-oxide synthase (NOS) activity. Consistent with the kinetic paradigm, the efficacy of ATA-induced cell protection was abrogated as ATA treatment was delayed by 6 h after LPS stimulation (Fig. 5B). Pretreatment with ATA for 6, 10, or 24 h elicited equivalent protection as coincubation of ATA with LPS simultaneously (data not shown). These results suggest that transcriptional inhibition of the iNOS gene is the major mechanism for ATA protection from cell death caused by LPS treatment.

Second, transcriptional inhibition was directly documented from iNOS mRNA measurement. RT-PCR analysis showed the reduction of LPS-induced iNOS mRNA expression by ATA (Fig. 6A). To explore the possible action of ATA on the stability of induced iNOS protein, cells containing a high amount of iNOS after LPS stimulation were washed with fresh medium to remove LPS and treated with anisomycin (3 μg/ml) to stop continuous iNOS transcription. Under these circumstances, the gradual decline of iNOS protein in both control and ATA-treated groups was seen. As normalized to the time-dependent basal level of β-tubulin, we found that the turnover half-life of iNOS (around 10 h) was not significantly changed by ATA (300 μM) (Fig. 6B).

ATA Inhibits LPS-Activated NF-κB and AP-1. It has been demonstrated that full activation of iNOS promoter by cytokines required NF-κB and AP-1 transcription factor binding sites (Goldring et al., 1996; Kristof et al., 2001). To understand the effects on NF-κB and AP-1 activation, analysis of nuclear extract was carried out by EMSA. Figure 7, as reflected from the shift assay by incubating reaction mixtures with antibodies for transcriptional subunit, indicates that NF-κB in RAW 264.7 cells is composed of p65 and p50, whereas AP-1 is a heterodimer of c-fos and c-jun. After LPS treatment, the nuclear translocation of NF-κB (Fig. 7A) and AP-1 (Fig. 7B), respectively, were inhibited by the presence of ATA within 1 and 2 h of incubation. Apart from NF-κB and AP-1, STAT-1 is another key transcriptional factor for cytokine IFN-γ to elicit its proinflammatory action on macrophages. As shown in Fig. 7C, the nuclear translocation and DNA-binding activity of STAT-1 was unaltered by LPS or ATA, in contrast to the positive activation with IFN-γ.

ATA Inhibits LPS-Caused IκB Degradation and IKK, ERK, and p38 MAPK Activation. In supporting the inhibitory action on NF-κB, the upstream signaling effectors were altered by ATA. Figure 8A shows that the IKK activity,

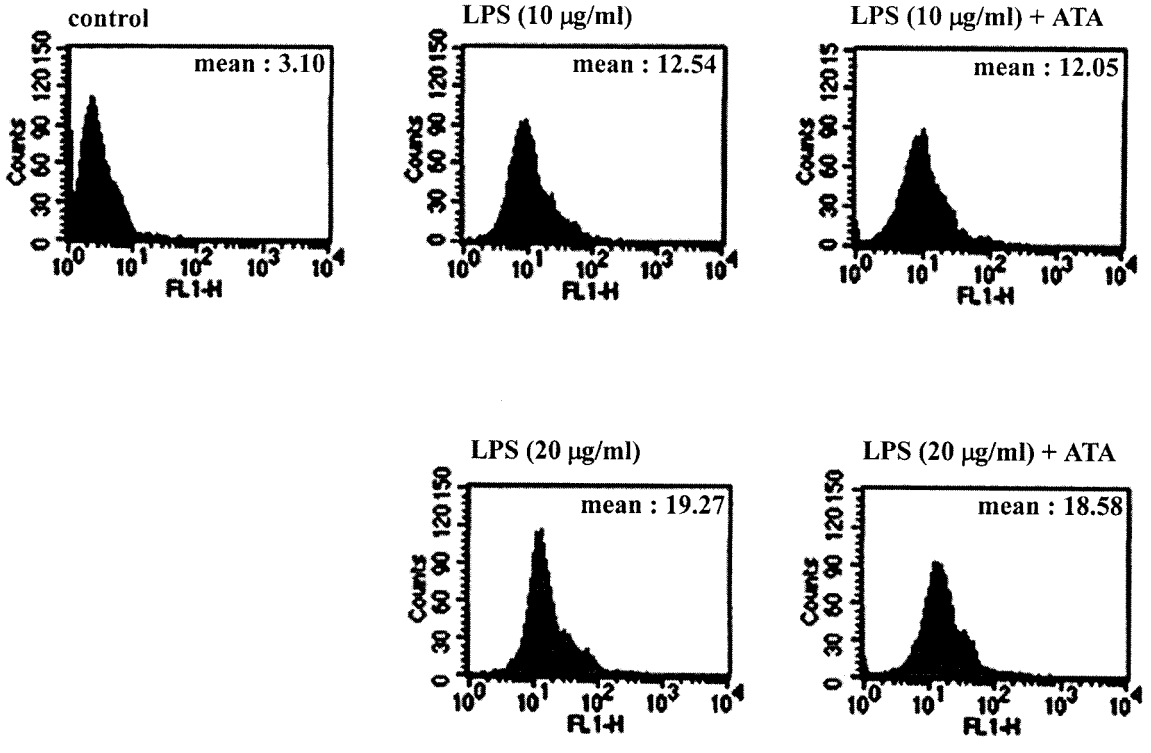

Fig. 4. ATA does not alter the binding of FITC-conjugated LPS. RAW 264.7 cells were treated with 10 or 20 μg/ml FITC-conjugated LPS in the absence or presence of 300 μM ATA for 1 h at 4°C. Then, the binding ability of LPS was analyzed by flow cytometry. The mean value of the fluorescence intensity is shown.

which is a convergent molecule for NF-κB activation in response to many stimuli, including LPS, was reduced by ATA. Upon LPS induction of IKK activation, IκBα and β were rapidly degraded, followed by a gradual recovery at 45 min and 1 h, respectively (Fig. 8B). Coaddition of ATA with LPS led to abrogation of IκB degradation.

Because ERK and p38 MAPK pathways have been implicated in the activation of AP-1 (Kristof et al., 2001), NF-κB (Carter et al., 1999; Zhao and Lee, 1999), and iNOS expression (Chen et al., 1998, 1999, 2001; Kristof et al., 2001), the involvement of both proteins in ATA action needs to be examined. Figure 9 shows that ERK and p38 MAPK activation by 1 μg/ml LPS, as indexed by their phosphorylation state, was diminished by coincubation with ATA (100 μM). ATA treatment alone did not affect the basal state of ERK and p38 MAPK phosphorylation.

Direct Inhibition of IKK, ERK, and p38 MAPK by ATA. To further dissect the mechanism contributing to in vivo kinase inhibition, the direct effect of ATA on these kinases was studied. Results from Fig. 10 show that ATA was able to inhibit all three kinases directly, and the effective concentrations required were 10 μM for ERK and p38 MAPK inhibition and 1 μM for IKK inhibition.

Discussion

NO is generated by a family of NOS isozymes that convert L-arginine to L-citruline and NO. Certain cytokines, mi-

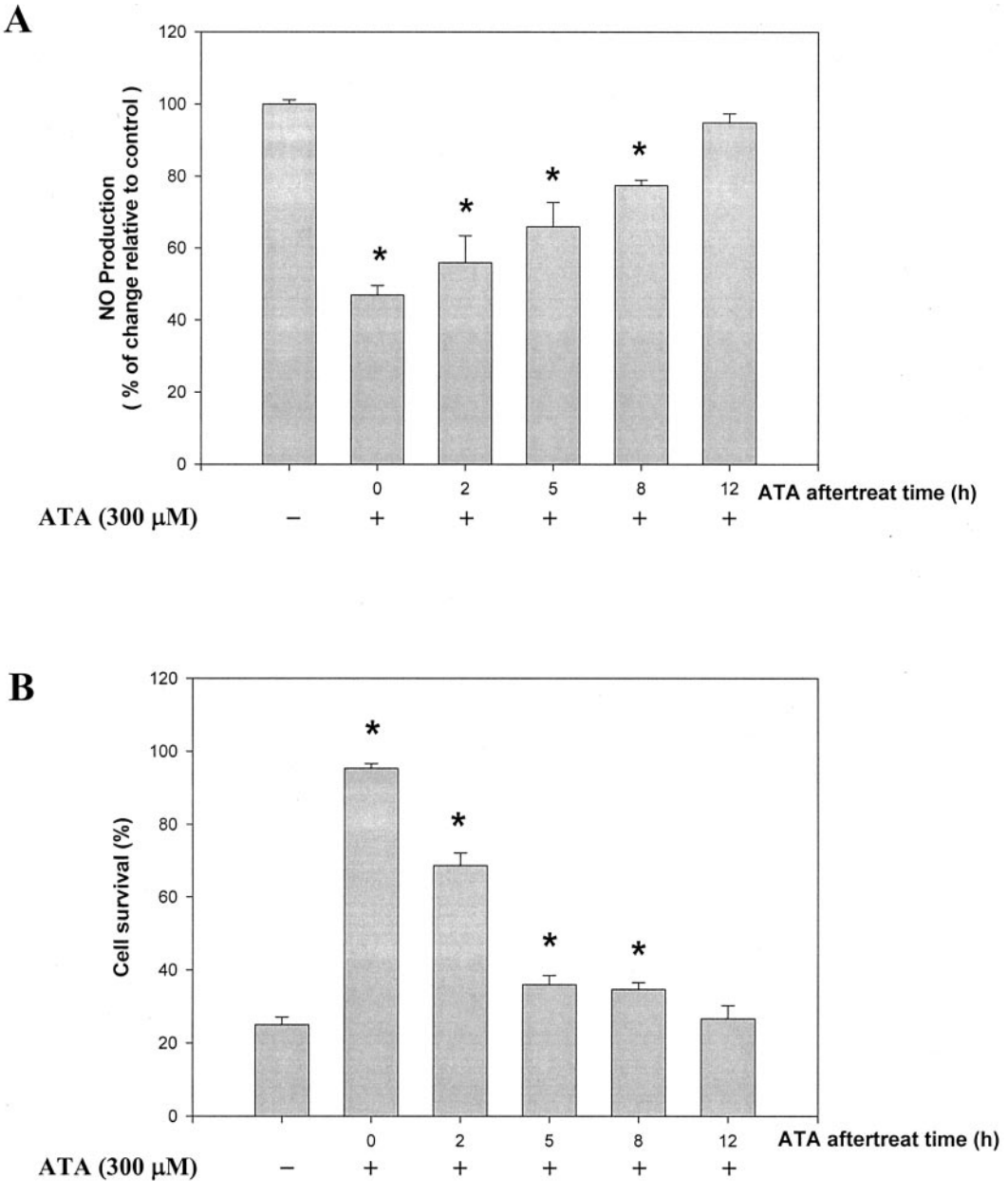

Fig. 5. Effects of ATA post-treatment on NO production and cell protection. RAW 264.7 cells were treated with 10 μg/ml LPS followed by the delayed addition of ATA (300 μM), with intervals indicated. Twenty-four hours after LPS addition, NO production was determined by Griess method (A), and cell viability was determined by MTT assay (B). The data represent the mean ± S.E.M. of at least three independent experiments. ⋆, $p < 0.05$ indicates significant reduction of NO production or cell viability compared with the group without ATA addition.

crobes, or microbial products trigger the expression of an inducible form of NOS (iNOS), which results in a high output of NO production from macrophages. By reacting with DNA, proteins, and lipids, NO impairs normal cellular functions and thus exerts its cytotoxic effects (Nathan, 1997; Albina and Reichner, 1998). Because the cytotoxic effect of NO is

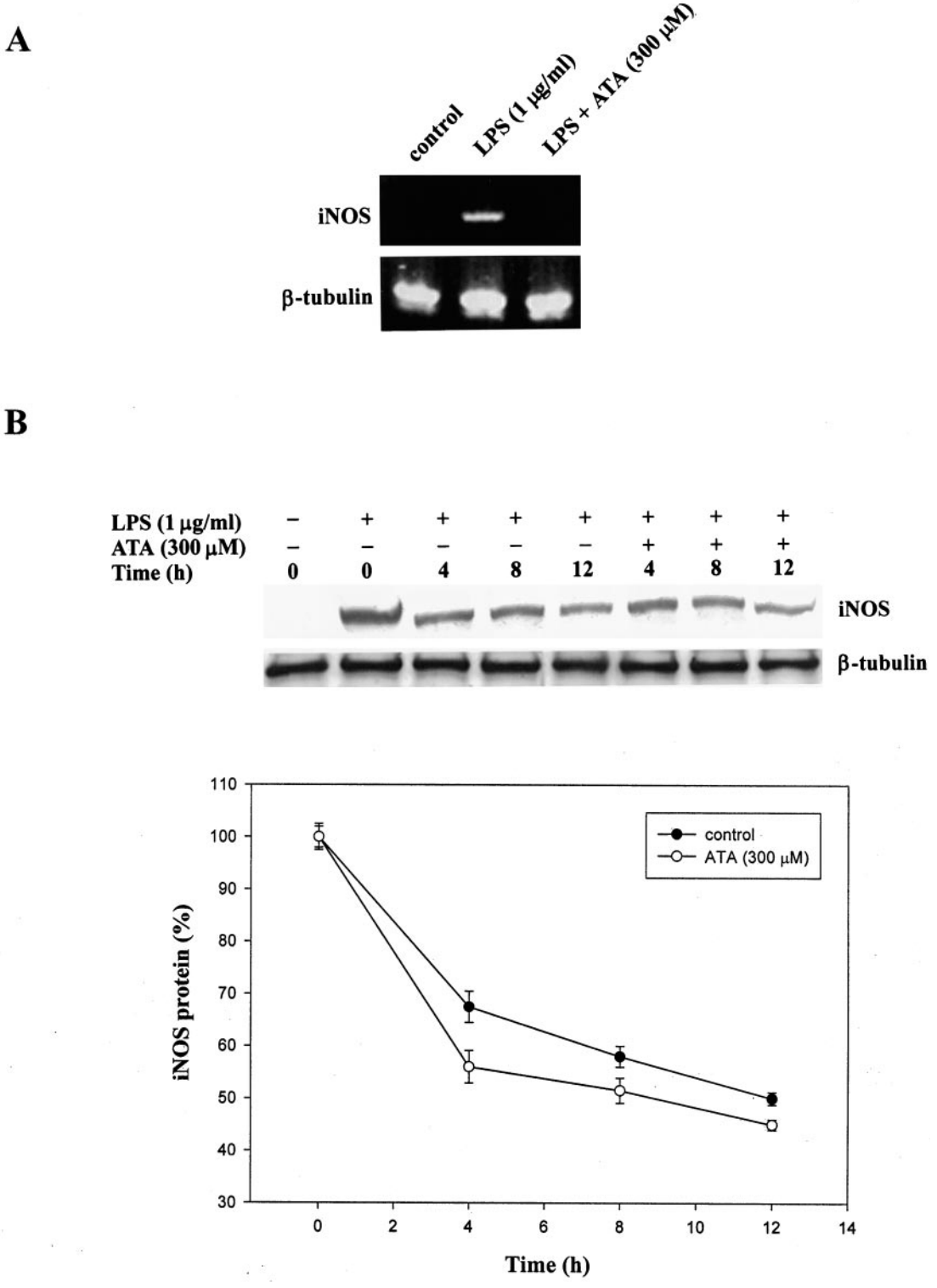

Fig. 6. Inducible NOS mRNA expression but not protein stability is inhibited by ATA. A, LPS-induced iNOS mRNA expression in ATA-treated RAW 264.7 cells was analyzed by RT-PCR. After 8 h of incubation with LPS (1 μg/ml) and ATA (300 μM), RNA was extracted and analyzed for iNOS expression by RT-PCR. The samples were also analyzed for β-actin as an internal control. B, ATA does not affect iNOS protein stability. In iNOS-expressing cells, after LPS (1 μg/ml) treatment for 24 h, LPS was washed out, and anisomycin (3 μg/ml) was included together with ATA (300 μM) or not for 4, 8, or 12 h. At these time points, cell lysates were subjected to SDS-PAGE. iNOS and β-tubulin proteins were determined by immunoblotting followed by quantification with densitometer. iNOS level was quantitatively normalized by β-tubulin immunoreactivity of the same time point. The results are mean ± S.E.M. of three independent experiments.

nonspecific, the consequences of overproduction of NO can be detrimental to the host instead of its pivotal role in the normal function of the host defense system. Therefore, a precise regulation of NO production under pathophysiological conditions would be critical for the survival of host cells. In this aspect, multiple ways to decrease NO production, including inhibition of iNOS enzyme activity (Albina and Reichner, 1998), depletion of arginine substrate by arginase (Boucher et al., 1999; Gotoh and Mori, 1999), and transcriptional down-regulation of iNOS gene expression by endogenous or exogenous manipulations, have been documented, with great interest, as strategies for developing anti-inflammatory agents. Therefore, in this study, we explored the action of ATA on NO-dependent apoptosis of murine macro-

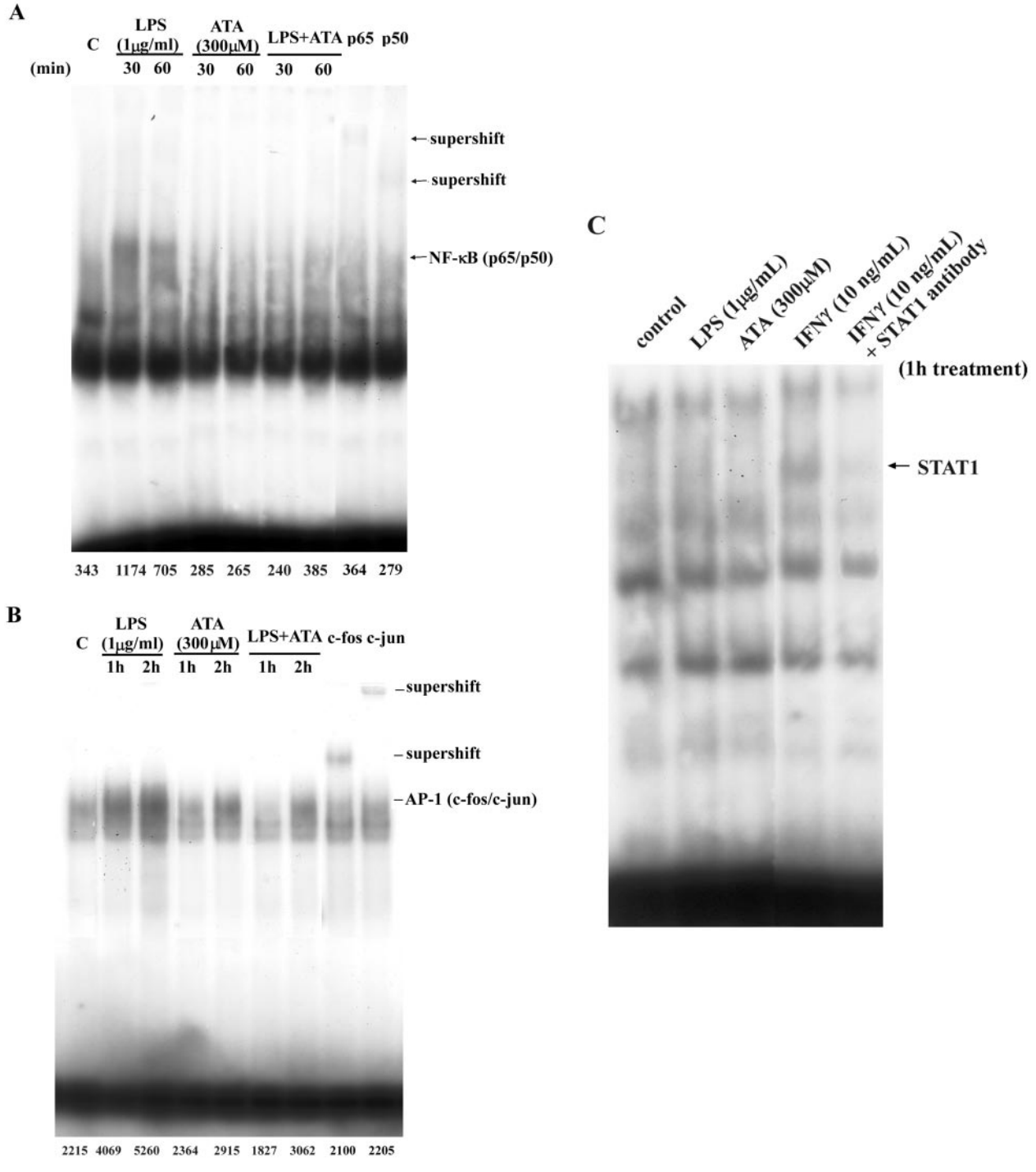

Fig. 7. ATA inhibits LPS-mediated NF-κB and AP-1 activation. Nuclear extracts from cell lysates were assayed for binding activity with specific oligonucleotides containing respective binding sequences for NF-κB (A), AP-1 (B), and STAT-1 (C). RAW 264.7 cells were treated with 1 μg/ml LPS, 10 ng/ml IFN-γ, and/or 300 μM ATA for different time intervals. Equivalent amounts of nuclear extracts were analyzed by EMSA. Specific antibodies for p65, p50, c-fos, c-jun, and STAT-1 were included in the binding solution, and the retarded gel mobilities of NF-κB and AP-1 are indicated. The data below each lane indicate the density of each transcription factor bound with specific oligonucleotide. The results are representative of three independent experiments.

phages, which has provided the best-studied example for the regulation of NO production.

NO production in large quantities after transcriptional induction of iNOS by LPS and cytokines, or direct supply by spontaneously decomposing NO donors, is established to be involved in macrophage death (Albina and Reichner, 1998; Hortelano et al., 1999). In LPS-activated macrophages, NO mediates many pathways responsible for apoptosis, including Bax and p53 up-regulation (Hortelano et al., 1999; Xaus et al., 2000). Likewise, morphological characteristics of apoptosis, such as nuclear condensation, blebbing of the nuclear membrane, and internucleosomal DNA fragmentation, also occur in macrophages treated with NO donor (Cui et al., 1994; Mebmer and Brune, 1996; Hortelano et al., 1999). Not only evidence deduced from cell culture system but also increased macrophage apoptosis during sepsis seems to be mediated by a high amount of NO production (Williams et al., 1997).

In this study, annexin V/propidium iodide staining has confirmed the apoptosis of LPS (Fig. 1C). In addition, confirming previous reports (Albina and Reichner, 1998; Feelisch, 1998; Hortelano et al., 1999), our results in this study show that RAW 264.7 macrophages undergo apoptosis caused by the large amounts of NO produced by LPS and SNP (Figs. 2 and 3). LPS-induced vulnerability is dependent on the amount and onset of NO production. Although 24-h treatment of LPS at 0.1 μg/ml results in 60 to 75% production of nitrite compared with 10 μg/ml LPS, cell toxicity within this time period was not significant (Fig. 3, A and B). A longer incubation period for 0.1 μg/ml LPS is necessary to cause cell death, and less than 10% cell viability was detected by a 72-h incubation (data not shown). Additionally, treatment of the iNOS inhibitor L-NAME (300 μM) blunted the cell death induced by LPS (10 μg/ml) (data not shown), strongly implicating the detrimental factor of NO in this apoptotic model.

Establishing apoptosis as the type of macrophage cell death by LPS and SNP permits the beneficial use of ATA as

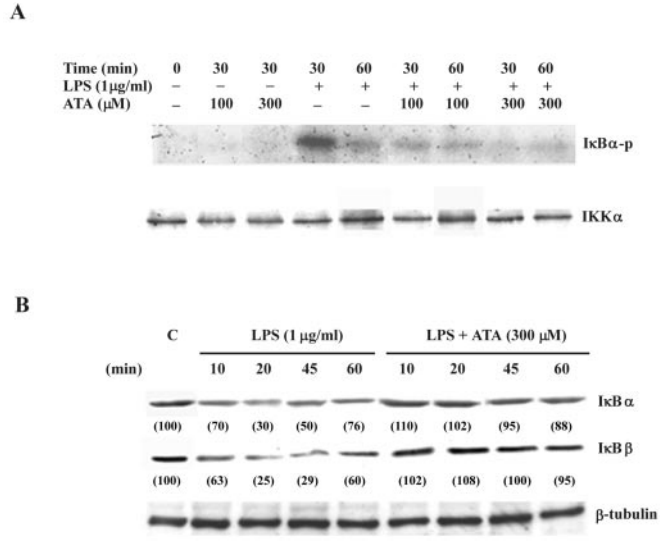

Fig. 8. Inhibition of LPS-induced IKK activation and the degradation of IκBs by ATA. Cells were treated with LPS (1 μg/ml) and/or ATA (100 or 300 μM) for the indicated times. A, cell lysates were immunoprecipitated with polyclonal IKKα antibody, and the immunoprecipitates were divided into two equal parts. One part was resolved for immunoblotting with IKKα, and the other part was subjected to kinase assays using GST-IκBα as a substrate. B, equal amounts of cytosolic samples were subjected to SDS-PAGE, and the immunoreactivities of IκBα, IκBβ, and β-tubulin were measured. The results are representative of three different experiments.

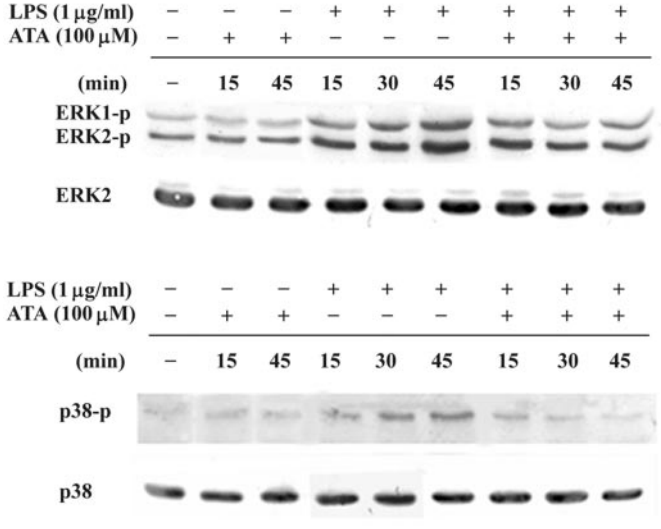

Fig. 9. Inhibition of LPS-induced ERK and p38 MAPK phosphorylation by ATA. After quiescent cells were treated with 1 μg/ml LPS and/or 100 μM ATA for different periods, total cell lysates were subjected to SDS-PAGE followed by immunoblotting with phosphorylation-specific ERK or p38 MAPK antibody, or antibodies that recognized ERK2 or p38 MAPK. The results are representative of three different experiments.

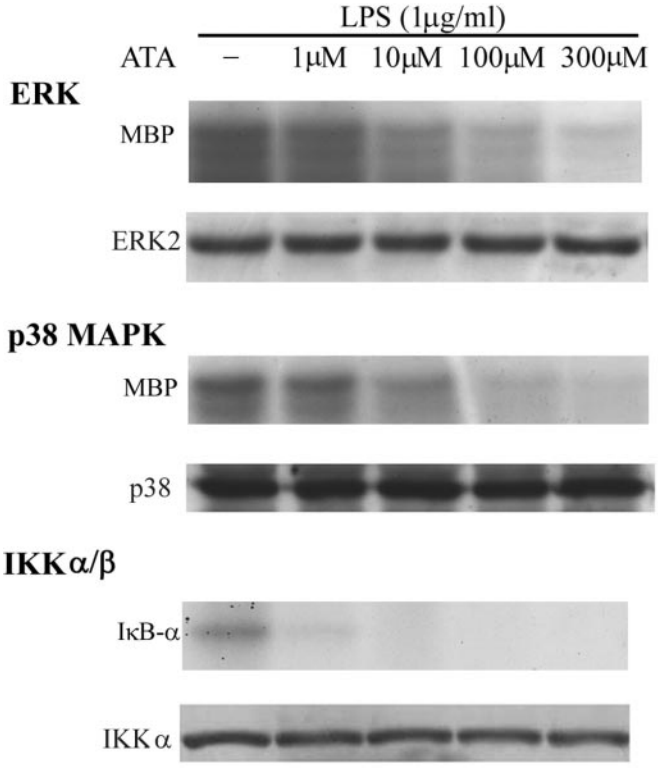

Fig. 10. ATA inhibits ERK, p38 MAPK, and IKK in vitro. Using specific antibody and protein A/B beads, each type of activated kinase was immunoprecipitated from the cell lysates prepared from RAW 264.7 cells, which were pretreated with 1 μg/ml LPS for 15 min. Equal amounts of immune complexes were aliquoted for testing kinase levels and effects of ATA on kinase activities. In the kinase mixture, either MBP (for ERK and p38 MAPK) or GST-IκBα (for IKK) was used as a kinase substrate. The results are representative of three different experiments.

an antidote. MTT assays demonstrate that ATA can significantly reduce cell vulnerability in response to LPS and SNP (Fig. 1, A and B; Fig. 2). Annexin V/propidium iodide staining further supports the results of MTT assay (Fig. 1C). Interestingly, we found that the inhibition of apoptosis exerted by ATA in LPS-activated macrophages is more prominent than in SNP-treated cells (Figs. 1 and 2). Compared with the partial protection of SNP-treated cells within 24 h of incubation, ATA almost completely rescues cells under insult from LPS. These results suggest that ATA not only moderately inhibits NO-dependent processes for execution of cell apoptosis, possibly through endonuclease inhibition as described previously (Vincent and Maiese, 1999), but, most importantly, also regulates NO production caused by iNOS induction by LPS.

Inducible NOS is an important signaling protein subjected to transcriptional regulation by cytokines and LPS. The kinetic study on protecting LPS-induced cell death shows that ATA is not very effective when added up to 5 to 8 h after LPS stimulation. Addition of ATA 12 h after stimulation with LPS completely fails to prevent apoptosis (Fig. 5A). This time-dependent cell protection is correlated to the extent of NO inhibition by ATA (Fig. 5B) and strongly suggests that transcriptional inhibition of iNOS gene expression is the primary mechanism for ATA action. The reduced level of iNOS mRNA after the addition of ATA in cells exposed to LPS (Fig. 6A) is direct evidence to support this point.

In investigating the signal transduction pathways responsible for iNOS induction by LPS, at least three main signaling pathways have been established and are as follows: 1) NIK → IKK → IκB phosphorylation and ubiquitin-dependent proteasome degradation → NF-κB activation; 2) TAK1 → MKK3/6 → p38 MAPK; and 3) MEK → ERK1/2 (Beutler, 2000; O'Neill and Dinarello, 2000). Evidence indicates that, secondary to the stimulation by LPS, transcription factors NF-κB and AP-1 are critical and act in a coordinated manner for the induced expression of iNOS (Goldring et al., 1996; Kristof et al., 2001). Many studies, including ours, have extensively dissected the upstream signal kinases contributing to both factor *trans*-activation and iNOS gene expression. All these results strongly indicate the crucial roles of ERK and p38 MAPK in AP-1 and NF-κB activation, IKK in NF-κB activation, and all three kinases in iNOS gene expression (Chen et al., 1998, 1999, 2001; Vanden Berghe et al., 1998; Carter et al., 1999; Zhao and Lee, 1999; Kristof et al., 2001). Our results here indicate that ATA can inhibit not only NF-κB and AP-1 activation induced by LPS (Fig. 7) but also the upstream kinases, IKK, ERK, and p38 MAPK, both in vivo and in vitro (Figs. 8–10). Reasonably, the concentration sufficient to elicit kinase inhibition (1–10 μM) is at least around 10-fold lower than that for NO reduction (30–100 μM). Thus, based on ATA inhibition on these essential signal pathways, it is perceivable for ATA to inhibit iNOS gene expression. To assess whether these inhibitory actions of ATA are caused by its nonspecific interference of LPS binding to macrophages, binding studies with FITC-conjugated LPS have ruled out this possibility (Fig. 4).

In this study, we have demonstrated for the first time that endonuclease inhibitor ATA at similar concentration ranges can not only protect macrophages from NO-dependent apoptosis in response to SNP but also inhibit iNOS induction and NO production in response to LPS. The latter action is caused by the interruption of the activation of upstream signal kinases IKK, ERK, and p38 MAPK, and these lead to the inhibition of NF-κB and AP-1 activation. The iNOS inhibitory action of ATA provides a permissive action on anti-apoptosis and possibly also on anti-inflammation.

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