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c-Jun NH_2-Terminal Kinase Inhibitor Anthra(1,9-*cd*)pyrazol-6(2H)-one Reduces Inducible Nitric-Oxide Synthase Expression by Destabilizing mRNA in Activated Macrophages

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ABSTRACT

In this study, we investigated the role of c-Jun NH_2-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family, in lipopolysaccharide (LPS)-stimulated inducible nitric-oxide synthase (iNOS) expression and nitric oxide (NO) production in J774 murine macrophages. Anthra(1,9-*cd*)pyrazol-6(2H)-one (SP600125), a pharmacological inhibitor of JNK, inhibited phosphorylation of c-Jun with an IC_{50} of 5 to 10 μM. At the same concentrations, SP600125 inhibited LPS-induced iNOS protein expression and NO production. SP600125 had no effect on the activation of nuclear factor κB, which is an important transcription factor for iNOS expression. SP600125 had no significant effect on iNOS mRNA levels if measured 4 h after LPS. In contrast, SP600125 reduced iNOS mRNA levels >90% when measured 8 h after LPS. These data suggest that SP600125 reduced iNOS mRNA stability, and this was confirmed in the mRNA degradation assay using actinomycin D, in which SP600125 reduced the iNOS mRNA half-life from 5 to 2 h. These results show that the JNK pathway is involved in the up-regulation of LPS-induced iNOS expression and NO production by a mechanism related to the stabilization of iNOS mRNA.

Nitric oxide (NO) is an inflammatory mediator that acts as a cytotoxic molecule and a modulator of innate and lymphocyte-mediated immune response (Moilanen et al., 1999). Inducible nitric-oxide synthase (iNOS) is expressed in various cells in response to cytokines and bacterial products, and it produces high amounts of NO for prolonged times (MacMicking et al., 1997).

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that are an important part of intracellular signaling pathways, connecting extracellular signals to intracellular regulatory proteins (Su and Karin, 1996). MAPK family members extracellular signal-regulated kinases 1/2 (Erk1/2) and p38 MAPK are known to participate in the regulation of LPS-induced iNOS expression and NO production (Larsen et al., 1998; Lahti et al., 2000; Chan and Riches, 2001b; Lahti et al., 2002). The third signal transduction pathway of the MAPK family is the c-Jun NH_2-terminal kinase (JNK) pathway, which is also activated primarily by cellular stress and cytokines, and its downstream targets include transcription factors important in cytokine expression (Kyriakis and Avruch, 1996). The role of the JNK pathway in iNOS expression has been investigated previously with the use of a biochemical approach to inhibit the JNK pathway. In human astrocytes, transfection with the dominant-negative JNK construct inhibited interleukin 1 (IL-1)-induced iNOS expression (Hua et al., 2002), whereas in RAW264.7 murine macrophages, transfection with dominant-negative JNK had no effect on bovine type I collagen-stimulated iNOS expression (Cho et al., 2002). SP600125 is a recently discovered pharmacological inhibitor of JNK and can be used as a tool in evaluating the role of JNK in physiological processes (Bennett et al., 2001).

The aim of this study was to investigate the role of JNK pathway in the regulation of iNOS expression and NO production. We used SP600125 as a pharmacological tool to inhibit the JNK pathway and studied its effects on LPS-

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ABBREVIATIONS: NO, nitric oxide; JNK, c-Jun NH_2-terminal kinase; iNOS, inducible nitric-oxide synthase; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; SP600125, anthra(1,9-*cd*)pyrazol-6(2H)-one; NF-κB, nuclear factor κB; Erk1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; RT, reverse transcriptase; TAMRA, 5-carboxytetramethylrhodamine; 6-FAM, 6-carboxyfluorescein; MKK, mitogen-activated protein kinase kinase; JRE, c-Jun NH_2-terminal kinase responsive element; IFN-γ, interferon-γ; UTR, untranslated region.

induced iNOS expression and NO production in J774 macrophages. The results show that JNK is an important factor in the regulation of iNOS mRNA stability.

Materials and Methods

Materials. Reagents were obtained as follows: SP600125, N^1-methyl-substituted pyrazolanthrone (N^1-methyl-1,9-pyrazoloanthrone), PD98059, and SB203580 were from Calbiochem (San Diego, CA); rabbit polyclonal mouse iNOS, c-Jun and JNK1 antibodies, and goat anti-rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and rabbit polyclonal phospho–stress-activated protein kinase/JNK (Thr183/Tyr185), phospho-Erk1/2, Erk1/2, phospho-p38 MAPK, p38 MAPK, and phospho-c-Jun (Ser63) II antibodies were from Cell Signaling Technology Inc. (Beverly, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. J774 macrophages were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with glutamax-I (Cambrex Bioproducts Europe, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen, Carlsbad, CA). Cells were seeded on 24-well plates for nitrite measurements and on six-well plates for Western blot and RT-PCR; they were then grown for 72 h to confluence before experiments.

Nitrite Assays. At indicated time points, the culture medium was collected for nitrite measurement, which was used as a measure of NO production. Culture medium (100 μl) was incubated with 100 μl of Griess reagent (0.1% napthalethylenediamine dihydrochloride, 1% sulfanilamine, and 2.5% H_3PO_4), and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

Preparation of Cell Lysates. At indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate, and 10 mM n-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (14,500g for 15 min), and supernatants were mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 1% glycerol, 2% SDS, 0.025% bromphenol blue, and 5% β-mercaptoethanol) and boiled for 5 min. Protein concentrations in the samples were measured by the Coomassie blue method (Bradford, 1976).

Western Blotting. Protein (30 μg) was loaded onto 10% SDS-polyacrylamide electrophoresis gel and was electrophoresed for 4 h at 100 V in a buffer containing 95 mM Tris-HCl, 960 mM glycine, and 0.5% SDS. After electrophoresis, the proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) with semidry blotter at 2.5 mA/cm^2 for 60 min. After transfer, the membrane was blocked in TBST (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% bovine serum albumin for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4°C overnight. Thereafter the membrane was washed 4x with TBST for 5 min, incubated with secondary antibody in the blocking solution for 0.5 h at room temperature, and washed four times with TBST for 5 min. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA). The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1.

RNA Extraction and Real-Time RT-PCR. At indicated time points, cell monolayers were rapidly washed with ice-cold PBS, and cells were homogenized using QIAshredder (QIAGEN, Valencia, CA). RNA extraction was carried out with the use of RNeasy kit for isolation of total RNA (QIAGEN). Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA). Reverse-transcriptase (RT) reaction parameters were as follows: incubation at 25°C for 10 min, RT at 48°C for 30 min, and RT inactivation at 95°C for 5 min. cDNA obtained from the RT reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5′-CCTGGTACGGGCATTGCT-3′, 5′-GCTCATGCGGCCTCCTT-3′ (forward and reverse mouse iNOS primers, respectively, both 300 nM), 5′-CAGCAGCGGCTCCATGACTCCC-3′ (mouse iNOS probe of 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-GGTTTTGGTGAGGTTGAATCCATA-3′, 5′-TCTTCACTGGCTCCTCTTCCTTAA-3′ (forward and reverse mouse AUF1 primers, respectively, both 300 nM), 5′-CAGAACCCACGCCTCTTATTGGTCTTCTTG-3′(mouse AUF1 probe of 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-TGTCCCCGGCAATGCT-3′, 5′-TCACGAATCACTTTCACATTGGT-3′ (forward and reverse mouse HuR primers, respectively, both 300 nM), and 5′-CCTCATCGGCGTCTTGCCCAA-3′ (mouse HuR probe of 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probes were obtained from TaqMan Rodent GAPDH Control Reagents kit (Applied Biosystems) and were used in the following concentrations: forward and reverse GAPDH primers, both 300 nM, and GAPDH probe, 50 nM, containing VIC as 5′-reporter dye and TAMRA as 3′-quencher. PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate.

A standard curve method was used to determine the relative mRNA levels as described in Applied Biosystems User Bulletin number 2. In short, a standard curve for each gene was created using mRNA isolated from LPS-stimulated J774 macrophages. Isolated RNA was reverse-transcribed as described. Dilution series were made from obtained cDNA ranging from 10 ng to 1 pg and were subjected to real-time PCR as described. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated from the standard curve.

Preparation of Nuclear Extracts. J774 macrophages were seeded on 10-cm dishes and grown for 72 h to confluence before the experiments. Cells were incubated in the presence of the tested compounds for 3 h. Thereafter, the cells were rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1 mM EGTA, 1 mM Na_2VO_4, and 1 mM NaF). After incubation for 10 min on ice, the cells were vortexed for 30 s, and the nuclei were separated by centrifugation at 4°C, 21,000g for 10 s. Nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1 mM EGTA, 1 mM Na_3VO_4, and 1 mM NaF) and incubated for 20 min on ice. Nuclei were vortexed for 30 s, and nuclear extracts were obtained by centrifugation at 4°C and 21,000g for 2 min. Protein content of the nuclear extracts were measured by use of the Coomassie blue method (Bradford, 1976).

Electrophoretic Mobility Shift Assay. Transcription factor consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) for NF-κB (Promega, Madison, WI) were 5′-^{32}P–end-labeled with DNA 5′-End Labeling Kit (Roche Diagnostics, Indianapolis, IN). For binding reactions, 5 μg of nuclear extract was incubated in 20 μl of total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM dithio-

threitol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl, and 10% glycerol for 20 min at room temperature. ^{32}P-labeled oligonucleotide probe (0.2 ng) was added, and the reaction mixture was incubated for 10 min. Protein/DNA complexes were separated from DNA probe by electrophoresis on a native 4% polyacrylamide gel. The gel was dried and autoradiographed using an intensifying screen at −70°C. The quantitation of densities of specific bands was carried out with the use of FluorChem software version 3.1.

Statistics. Results are expressed as the mean ± S.E.M. When indicated, statistical significance was calculated by analyses of variance supported by the Bonferroni multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

LPS Stimulation Activates JNK in J774 Macrophages. We investigated the activation kinetics of JNK and phosphorylation of c-Jun after LPS stimulation in J774 macrophages by Western blotting with the use of antibodies directed against Thr183/Tyr185 phosphorylated (activated) JNK and Ser63 phosphorylated c-Jun. JNK activity was rapidly increased after LPS, reaching the maximum in 30 min and reducing thereafter (Fig. 1), although low levels of activated JNK were present in cells several hours after LPS stimulation. C-Jun is a direct target of JNK, which phosphorylates c-Jun at residue Ser63 (Derijard et al., 1994). Parallel to the activation of JNK, levels of phosphorylated c-Jun were increased in cells after LPS stimulation, and increased levels of phosphorylated c-Jun in cells could be detected for hours.

SP600125 Inhibits LPS-Induced JNK-Dependent Phosphorylation of c-Jun. To test the ability of SP600125 to inhibit JNK activity in intact J774 macrophages, we measured the phosphorylation of c-Jun residue Ser63 by Western blot. SP600125 inhibited the LPS-induced Ser63 phosphorylation of c-Jun in a concentration-dependent manner, and the reduction in phosphorylation was 40% at 5 μM concentration and >90% at 20 μM concentration (Fig. 2). We also tested whether SP600125 had any unspecific effects on the activation of other MAPKs. We used Western blot to measure LPS-induced activation of Erk1/2 and p38 MAPK. SP600125 had no significant effect on the activation of Erk1/2, whereas PD98059 (10 μM), which is an inhibitor of Erk1/2 activation (Alessi et al., 1995), markedly inhibited the activation of Erk1/2 (Fig. 3). SB203580 is an inhibitor of p38 MAPK(Cuenda et al., 1995), which can also inhibit p38 MAPK activation by inhibiting p38 MAPK autophosphorylation (Ge et al., 2002). SP600125 had no effect on LPS-induced activation of p38 MAPK, whereas SB203580 (1 μM) markedly inhibited 38 MAPK activation.

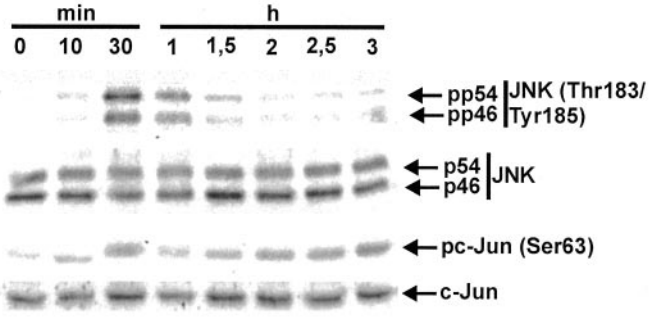

Fig. 1. The effect of LPS on JNK activation. J774 macrophages were stimulated with LPS (1 μg/ml), and incubations were terminated at the indicated time points. Parallel immunoblots were run from the same cell lysates using antibodies against Thr183/Tyr185-phosphorylated JNK (pp54 and pp46), total JNK (p54 and p46), Ser63-phosphorylated c-Jun (pc-Jun), and total c-Jun. Data are representative of two experiments with similar results.

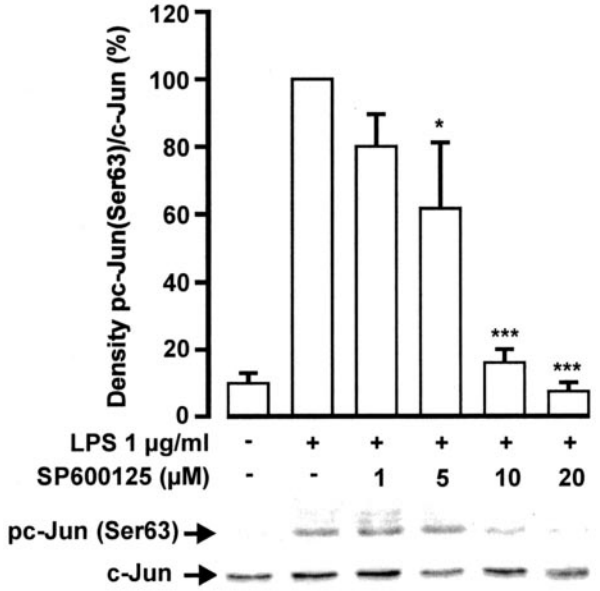

Fig. 2. The effect of SP600125 on JNK activity. J774 macrophages were treated with various concentrations of SP600125 for 30 min before stimulation with LPS (1 μg/ml). Incubations were terminated after 2.5 h, and parallel immunoblots were run from the same cell lysates using antibodies against Ser63-phosphorylated c-Jun (pc-Jun) and total c-Jun. The chemiluminescent signal was quantified as described under *Materials and Methods*. Phosphorylated c-Jun values were normalized to c-Jun values. Results are expressed as mean ± S.E.M. (unstimulated, $n = 4$; others, $n = 6$). *, $P < 0.05$; ***, $P < 0.001$ compared with cells treated with LPS only.

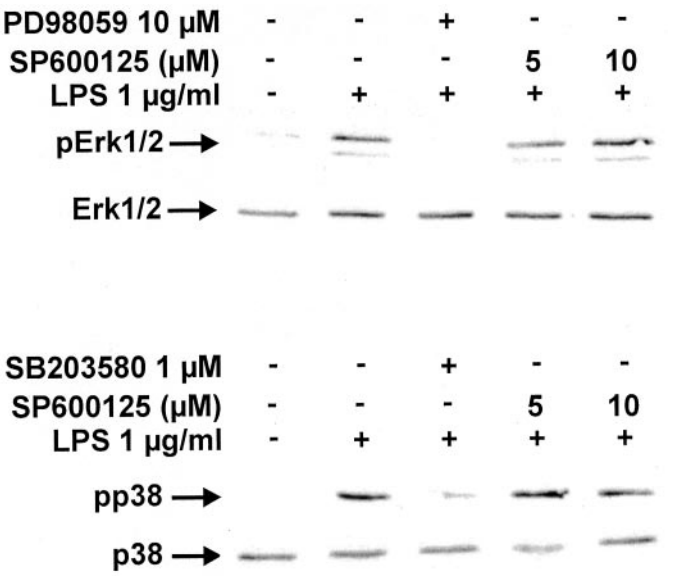

Fig. 3. The effect of SP600125 on Erk1/2 and p38 MAPK activation. J774 macrophages were treated with PD98059 (10 μM), SB203580 (1 μM), or SP600125 (5–10 μM) for 30 min before stimulation with LPS (1 μg/ml). Incubations were terminated after 15 min, and parallel immunoblots were run from the same cell lysates using antibodies against Thr/Tyr-phosphorylated Erk1/2 (pErk1/2), total Erk1/2, Thr/Tyr phosphorylated p38 MAPK (pp38), and total p38. Results are representative of three separate experiments with similar results.

SP600125 Inhibits LPS-Induced NO Production and iNOS Expression. To investigate the role of JNK in LPS-induced NO production, J774 macrophages were stimulated with LPS and treated with various concentrations of SP600125. Figure 4A shows that SP600125 had a concentration-dependent inhibitory effect on LPS-induced NO production at a concentration range of 1 to 20 μM. The inhibitory effect was maximal when SP600125 was added to cells between time points of 30 min before or 1 h after LPS stimulation (Fig. 4B). The inhibitory effect was reduced if the compound was added to cells 2 h or longer after LPS stimulation and was only 17% when added 6 h after LPS. To test whether the inhibitory effect of SP600125 was related to JNK inhibition, we used N^1-methyl substituted pyrazolanthrone as a control compound. It is structurally related to SP600125, but it is a >100-fold less potent inhibitor of JNK compared with SP600125 (Bennett et al., 2001). N^1-methyl-1,9-pyrazoloanthrone (20 μM) inhibited NO production slightly, but the effect was considerably less potent than the effect of SP600125 at the same concentration (80% inhibition) (Fig. 4C).

In the further studies, we measured the effect of SP600125 on iNOS protein expression. Western blots using antibody

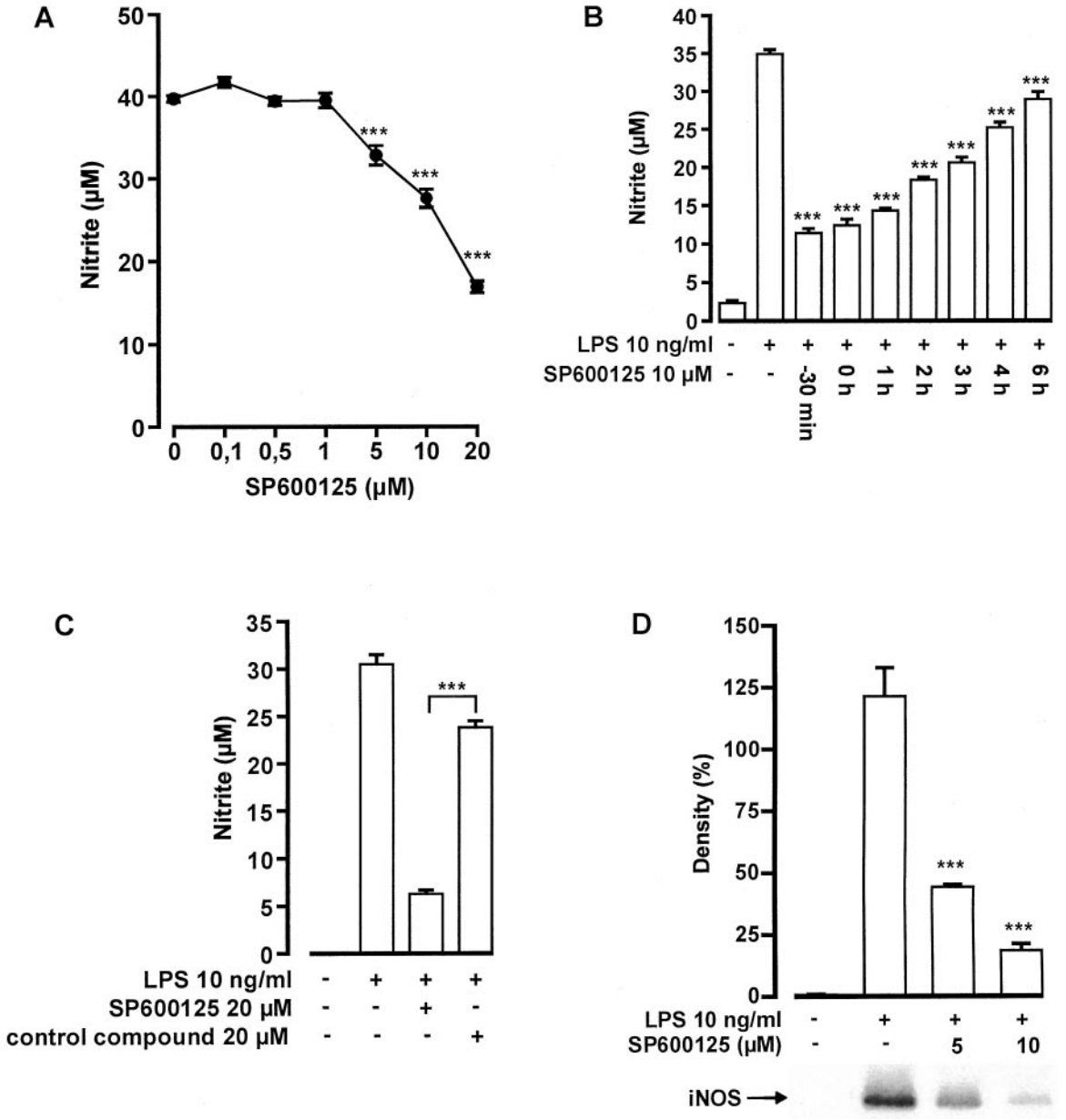

Fig. 4. The effect of SP600125 on LPS-induced NO production and iNOS protein expression. A, cells were stimulated with LPS (10 ng/ml) and treated with various concentrations of SP600125. After 24 h of incubation, the nitrite concentrations were measured as a marker of NO production. Values shown are mean ± S.E.M. (n = 6). B, SP600125 (10 μM) was added to cells 30 min before or at the indicated time points after LPS. Twenty-four hours after the addition of LPS, the nitrite concentrations were measured as a marker of NO production. Values shown are mean ± S.E.M. (n = 6). C, cells were stimulated with LPS (10 ng/ml) and treated with SP600125 (20 μM) or N^1-methyl-1,9-pyrazoloanthrone (control compound) (20 μM). After 24 h of incubation, the nitrite concentrations were measured as a marker of NO production. Values shown are mean ± S.E.M. (n = 6). D, cells were incubated with or without SP600125 for 30 min before stimulation with LPS. After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under *Materials and Methods*. Values shown are mean ± S.E.M. (n = 3). ***, $P < 0.001$ compared with cells treated with the respective control.

against iNOS showed a concentration-dependent reduction in LPS-induced iNOS expression that was similar to the reduction in NO production (Fig. 4D).

SP600125 Inhibits iNOS mRNA Expression in an NF-κB–Independent Manner. We used real-time RT-PCR to investigate the effect of SP600125 on LPS-induced iNOS mRNA expression at two different time points. SP600125 had no marked effect on LPS-induced iNOS mRNA expression when measured 4 h after LPS stimulation (Fig. 5). In contrast, at the 8-h time point, the iNOS mRNA levels were drastically reduced in SP600125-treated cells. In addition, we measured the effects of SP600125 on the activation of NF-κB that is an essential transcription factor for LPS-induced iNOS expression (Kim et al., 1997). In the gel-shift assay, SP600125 had no inhibitory effect on NF-κB nuclear translocation and DNA binding activity (Fig. 6). The lack of effect of SP600125 on mRNA expression at the 4-h time point and the marked reduction seen in levels of iNOS mRNA at the 8-h time point suggest that SP600125 may not have a direct effect on iNOS transcription, but it rather regulates iNOS mRNA stability.

SP600125 Decreases iNOS mRNA Half-Life in a Cycloheximide-Sensitive Manner. To test whether SP600125 decreases iNOS mRNA half-life, J774 cells were stimulated with LPS, and after 6 h, actinomycin D, an inhibitor of transcription, was added. Cells were then incubated further for 0, 2, 4, and 6 h before extraction of total RNA. Real-time RT-PCR analysis showed that when transcription was blocked with actinomycin D, the relative levels of iNOS mRNA were decreasing faster in SP600125-treated cells compared with untreated cells, indicating reduced mRNA stability (Fig. 7). The half-life of iNOS mRNA was approximately 5 h in cells treated with LPS only, but it was reduced to 2 h when treated with a combination of LPS and SP600125.

The protein-synthesis inhibitor cycloheximide has been shown to stabilize iNOS mRNA (Evans et al., 1994). To investigate whether the effect of SP600125 could be reversed by cycloheximide, LPS-stimulated J774 cells were treated with both compounds, and RNA was isolated after 8 h of incubation. Cycloheximide increased LPS-induced iNOS mRNA expression by 50%. However, in the presence of cycloheximide, SP600125 had no effect on iNOS mRNA expression (Fig. 8A).

Cycloheximide has also been described to activate JNK (Newton et al., 1997). Therefore, we wanted to investigate whether cycloheximide could enhance LPS-induced JNK activity and whether SP600125 would still inhibit JNK under such conditions. Western blots of Thr183/Tyr185-phosphorylated JNK and Ser63-phosphorylated c-Jun showed that cycloheximide enhanced LPS-induced JNK activation compared with cells treated with LPS alone. SP600125 markedly inhibited LPS-induced JNK activity also in cycloheximide-treated cells, and the levels of phosphorylated c-Jun were reduced to approximately the same levels as those in cells treated with LPS alone (Fig. 8B).

SP600125 Has No Effect on mRNA Expression of AUF1 and HuR in LPS-Treated Cells. The 3′ untranslated region (3′-UTR) of iNOS mRNA contains several AU-rich sequence elements (Evans et al., 1994), which have been shown to bind mRNA stability-regulating proteins HuR (Rodriguez-Pascual et al., 2000) and AUF1 (Kleinert et al., 2002). Cycloheximide was able to reverse the effect of

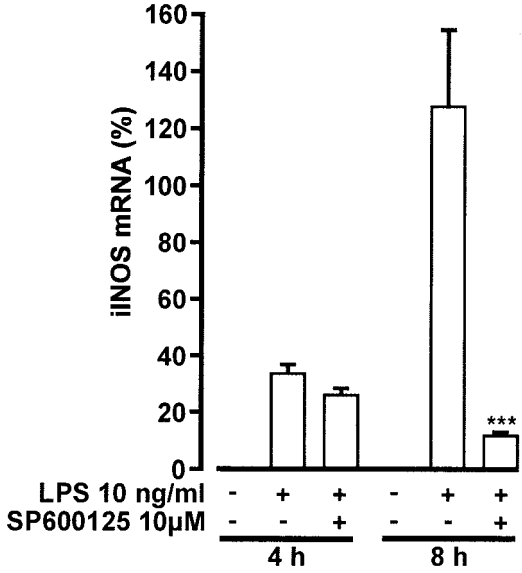

Fig. 5. The effect of SP600125 on LPS-induced iNOS mRNA expression. Cells were incubated with SP600125 for 30 min before stimulation with LPS. Incubations were terminated at the indicated time points, and the extracted total RNA was subjected to real-time RT-PCR. iNOS mRNA levels were normalized against GAPDH. Values shown are mean ± S.E.M. ($n = 3$). ***, $P < 0.001$ compared with cells treated with LPS only.

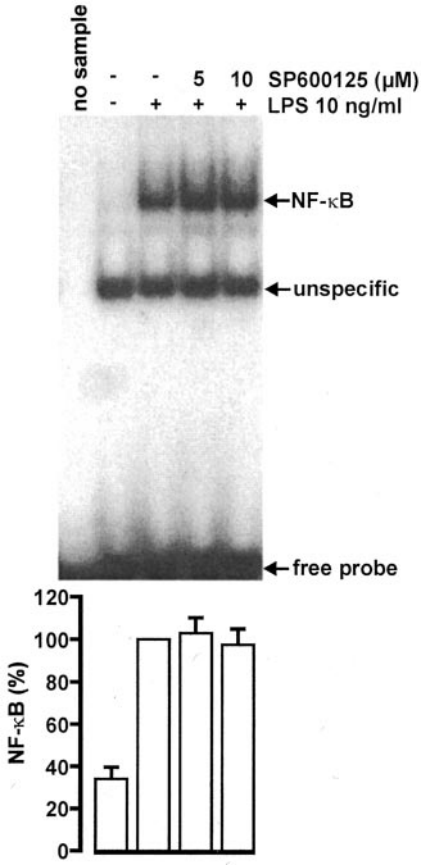

Fig. 6. The effect of SP600125 on NF-κB activity. Cells were incubated with SP600125 for 30 min before stimulation with LPS. Cells were incubated for a further 3 h before the preparation of nuclear extracts. NF-κB DNA binding activity was analyzed by electrophoretic mobility shift assay. Densities of specific bands were quantified as described under *Materials and Methods*. Values shown are mean ± S.E.M. ($n = 4$).

SP600125 on iNOS mRNA, suggesting that protein synthesis is required for stabilizing iNOS mRNA. Therefore, we investigated whether SP600125 has an effect on mRNA expression of HuR and AUF1. LPS stimulation or combination of LPS and SP600125 had no effect on mRNA levels of HuR and AUF1 compared with unstimulated cells (Fig. 9).

Discussion

In the present study, we have shown that JNK inhibitor SP600125 down-regulates LPS-induced iNOS expression and NO production in J774 macrophages by reducing iNOS mRNA stability.

SP600125 inhibited LPS-induced c-Jun phosphorylation in J774 macrophages with an IC_{50} of 5 to 10 μM, which is similar to the IC_{50} value previously reported in Jurkat T cells (Bennett et al., 2001). In the same report, partial inhibition of other MAPK pathways was observed only when SP600125 was used at concentrations greater than 25 μM. The effects of SP600125 on iNOS expression that were observed in the present study were evident at 5 to 10 μM concentrations, and we did not observe any inhibitory effect on other MAPKs at these concentrations. Furthermore, N^1-methyl-1,9-pyrazoloanthrone, which is chemically related to SP600125 but is a >100-fold less potent inhibitor of JNK, had only a minimal inhibitory effect on LPS-induced NO production at 20 μM concentration. These results suggest that the effect of SP600125 on LPS-induced iNOS expression is specific to the inhibition of JNK.

SP600125 inhibited iNOS protein expression and NO production in a concentration-dependent manner. Interestingly, SP600125 had practically no effect on LPS-induced iNOS mRNA expression when measured 4 h after the addition of LPS, suggesting that SP600125 had no direct effect on iNOS gene transcription. In addition, SP600125 had no effect on nuclear translocation and DNA binding activity of NF-κB, which is an essential transcription factor for iNOS (Kim et al., 1997). In contrast, a significant reduction in iNOS mRNA levels was observed in SP600125-treated cells 8 h after LPS stimulation. This suggested that SP600125 treatment facilitated iNOS mRNA degradation, and this hypothesis was confirmed by the mRNA degradation assay. The half-life of iNOS mRNA was reduced from 5 to 2 h.

Our results are in line with previous results by Hua et al. (2002), who observed that IL-1– and interferon-γ (IFN-γ)–stimulated NO production and iNOS protein expression were reduced in human fetal astrocytes transfected with dominant-negative JNK. In contrast, dominant-negative JNK had no effect on bovine type I collagen-induced iNOS expression in RAW264.7 murine macrophages (Cho et al., 2002). Participation of the JNK pathway in iNOS expression has also been evaluated by using dominant-negative mutants of MAP ki-

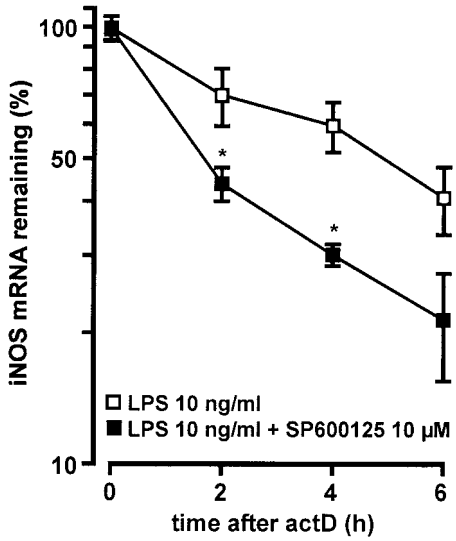

Fig. 7. Effect of SP600125 on iNOS mRNA stability. A, cells were incubated with SP600125 for 30 min before stimulation with LPS. Actinomycin D (actD) (0.5 μg/ml) was added to cells 6 h after LPS. Incubations were terminated and total RNA isolated at indicated time points after actinomycin D. iNOS and GAPDH mRNA was measured by real time RT-PCR. iNOS mRNA levels were normalized against GAPDH. Values are mean ± S.E.M. (n = 6). *, $P < 0.05$ compared with the respective control in the absence of SP600125.

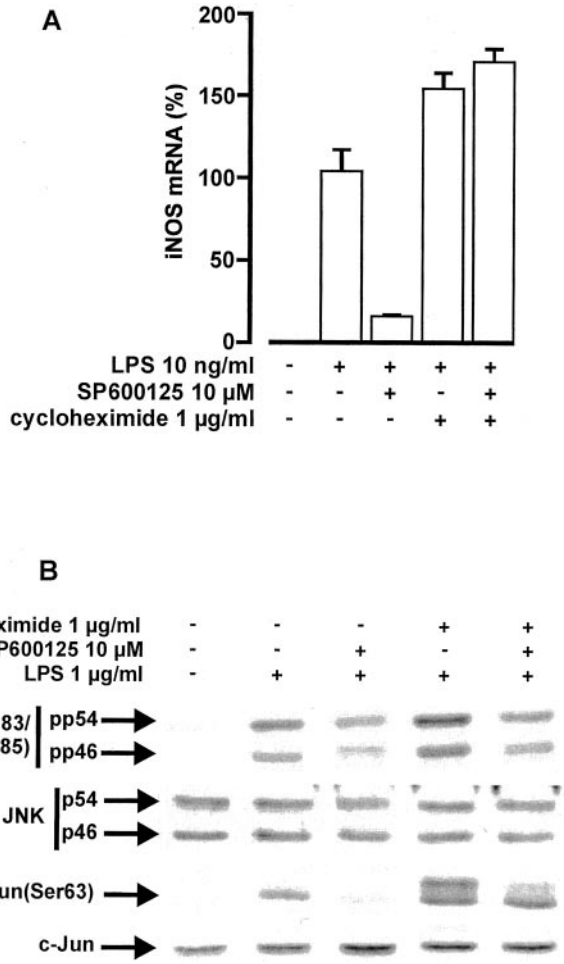

Fig. 8. The effect of cycloheximide on iNOS mRNA expression and JNK activity. A, cells were incubated with SP600125 for 30 min before the addition of LPS with and without cycloheximide. Incubations were terminated after 8 h, and total RNA was isolated. iNOS and GAPDH mRNA levels were measured by real-time RT-PCR. iNOS mRNA levels were normalized against GAPDH. Values shown are mean ± S.E.M. (n = 3). B, cells were treated with SP600125 (10 μM) for 30 min before stimulation with LPS (1 μg/ml) and cycloheximide (1 μg/ml). Incubations were terminated after 30 min (for JNK blots) and after 2.5 h (for c-Jun blots), and parallel immunoblots were run from same-cell lysates using antibodies against Thr183/Tyr185-phosphorylated JNK (pp54 and pp46), total JNK (p54 and p46), Ser63-phosphorylated c-Jun (pc-Jun), and total c-Jun. The results are representative of three separate experiments with similar results.

nase kinase 4 and 7 (MKK4 and MKK7), which are upstream activators of JNK. IFN-γ and LPS-induced iNOS promoter activity in RAW264.7 cells was reduced by dominant-negative MKK4 (Chan and Riches, 2001b), whereas lipoarabinomannan and IFN-γ-induced iNOS promoter activity was reduced by dominant-negative MKK7 but not MKK4 in the same cells (Chan et al., 2001a). These differences might result from cell-type and stimulus-dependent differences in the regulation of iNOS expression.

There is now an increased interest in the regulation of iNOS mRNA stability, which seems to be a significant mechanism in the regulation of iNOS expression. The events leading to the stabilization of iNOS mRNA and the factors regulating iNOS mRNA stability are poorly known. Dexamethasone (Korhonen et al., 2002), protein kinase Cδ (Carpenter et al., 2001), and β-adrenergic stimulation (Gustafsson and Brunton, 2000) have been shown to regulate iNOS mRNA stability. The stability of iNOS mRNA has been reported to be translation- and transcription-dependent (Evans et al., 1994; Park and Murphy, 1996). In the present study, SP600125 reduced iNOS mRNA stability in LPS-treated cells, suggesting a role for JNK in the stabilization of iNOS mRNA. Because the effect of SP600125 on iNOS mRNA expression was evident at the 8- but not at the 4-h time point and SP600125 had no effect on iNOS mRNA expression in cycloheximide-treated cells, we hypothesized that the stabilization of iNOS mRNA requires protein synthesis. However, cycloheximide has also been reported to stabilize mRNAs in a protein synthesis-independent manner (Ragheb et al., 1999). In addition, cycloheximide enhanced LPS-induced JNK activity, which was inhibited by SP600125. These data open up the possibility that the enhancing effect of cycloheximide on LPS-induced iNOS mRNA expression may be caused by the enhanced activation of JNK. However, in cells treated with a combination of cycloheximide and LPS, SP600125 significantly reduced JNK activity but had no effect on iNOS mRNA expression. Therefore, we concluded that the inhibitory effect of SP600125 on iNOS mRNA stability is probably dependent on de novo protein synthesis.

iNOS mRNA stabilizing factors HuR and AUF1 have been shown to bind to AU-rich sequence elements in the 3′ untranslated region of human iNOS mRNA and to stabilize iNOS mRNA (Rodriguez-Pascual et al., 2000; Kleinert et al., 2002). Because SP600125 seemed to destabilize iNOS mRNA by a protein synthesis-dependent manner, we measured the effects of SP600125 on AUF1 and HuR mRNA levels. SP600125 had no effect on the mRNA levels of these two factors in LPS-treated cells. Thus, JNK seems not to regulate the expression of these factors, and the reduced stability of iNOS mRNA by SP600125 is not mediated by the altered expression of HuR and AUF1. However, it is possible that JNK regulates the activity of these factors directly or indirectly by other means, e.g., by phosphorylation. Previously, JNK has been shown to participate in the stabilization of mRNAs of vascular endothelial growth factor, IL-2, and IL-3 (Chen et al., 1998; Ming et al., 1998; Pages et al., 2000). The JNK-mediated stabilization of IL-2 mRNA was reported to be dependent on JNK-responsive element (JRE) in the 5′-UTR of IL-2 mRNA and in the binding of Y box-binding protein and nucleolin to 5′-JRE (Chen et al., 2000). The nucleotide sequence in 5′-JRE that is necessary to mediate the effect of JNK has not been defined, and to our knowledge, 5′-UTR of iNOS mRNA has not been reported to contain JRE. The mechanism by which JNK mediates the stabilization of iNOS mRNA remains to be clarified.

In summary, this is the first study to show that SP600125, an inhibitor of JNK, reduces LPS-induced iNOS expression and NO production by facilitating iNOS mRNA degradation. The results suggest that JNK is an important part of the signaling pathway involved in the LPS-induced iNOS mRNA stabilization.

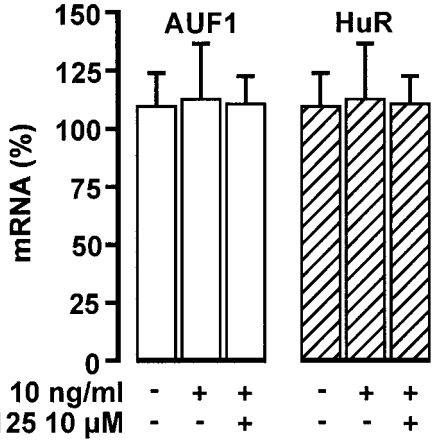

Fig. 9. The effect of SP600125 on HuR and AUF1 mRNA expression. Cells were incubated with SP600125 for 30 min before stimulation with LPS. Incubations were terminated after 4 h, and total RNA was isolated. HuR, AUF1, and GAPDH mRNA levels were measured with the use of real-time RT-PCR. HuR and AUF1 mRNA levels were normalized against GAPDH levels. Values shown are mean ± S.E.M. (n = 3).

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