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Post-Transcriptional Regulation of Human Inducible Nitric-Oxide Synthase Expression by the Jun N-terminal Kinase

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ABSTRACT

Human inducible nitric-oxide synthase (iNOS) expression is regulated both at transcriptional and post-transcriptional levels. In the present study, the effect of Jun N-terminal kinase (JNK) on human iNOS expression was investigated. In A549/8 human alveolar epithelial cells, both the inhibition of JNK by a pharmacological inhibitor anthra[1,9-*cd*]pyrazol-6(2*H*)-one1,9-pyrazoloanthrone (SP600125) and small interfering RNA (siRNA)-mediated down-regulation of JNK led to a reduction of iNOS mRNA and protein expression. iNOS promoter activity was not affected by these treatments. Hence, JNK seems to regulate iNOS expression through post-transcriptional mechanisms by stabilizing iNOS mRNA. Our laboratory has shown recently that a cytokine-induced RNA binding protein tristetraprolin (TTP) is a major positive regulator of human iNOS expression by stabilizing iNOS mRNA. Therefore, the effect of JNK inhibition by SP600125 or down-regulation by siRNA on TTP expression was investigated. Both SP600125 and siRNA targeted at JNK resulted in a reduction of TTP protein expression without affecting the amount of TTP mRNA. These data suggest a post-transcriptional control of TTP expression by JNK. Moreover, the modulation of JNK signaling by SP600125 or siRNA did not change p38 phosphorylation. In summary, the results suggest that JNK regulates human iNOS expression by stabilizing iNOS mRNA possibly by a TTP-dependent mechanism.

Post-transcriptional mechanisms have been published to be of central importance in the regulation of gene expression. Genes regulated by post-transcriptional mechanisms include a wide variety of functional repertoire such as cytokines, growth factors, and genes regulating cell cycle and development. In general, mRNAs of these genes contain AU-rich elements (ARE) in their 3′-untranslated region (3′-UTR), making them inherently unstable and allowing rapid and precise regulation of gene expression (Wilusz and Wilusz, 2004; Barreau et al., 2006). In mammalian cells, the 3′- to 5′-exonucleolytic decay catalyzed by the exosome is believed to be the major mRNA degradation pathway for ARE-containing mRNAs (Mukherjee et al., 2002). This pathway involves proteins binding to the 3′-UTR and modulating the interaction between these mRNAs and the exosome (Chen et al., 2001).

Proteins shown to regulate the stability of such inherently unstable mRNAs include members of the embryonic lethal abnormal vision protein family (most important HuR), the KH-type splicing regulatory protein (KSRP), tristetraprolin (TTP), the polypyrimidine tract binding protein (PTB, also known as hnRNP I), and the ARE/poly-(U)-binding protein/degradation factor 1 (also known as hnRNP D) (Wilusz and Wilusz, 2004; Barreau et al., 2006). One important signaling pathway involved in the post-transcriptional regulation of inherently unstable mRNAs is the p38 mitogen-activated protein kinase (MAPK) pathway (Clark et al., 2003). The

ABBREVIATIONS: ARE, AU-rich element; C_T, cycle threshold; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside; CM, cytokine mixture containing interferon-γ, interleukin-1β, and tumor necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IL, interleukin; iNOS, inducible nitric-oxide synthase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PTB, polypyrimidine tract binding protein; siRNA, small interfering RNA; shRNA, short hairpin RNA; SP600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one-1,9-pyrazoloanthrone; TNF-α, tumor necrosis factor α; TTP, tristetraprolin; UTR, untranslated region; kb, kilobase(s); qRT-PCR, quantitative reverse transcriptase/real-time polymerase chain reaction; DMSO, dimethyl sulfoxide; KSRP, KH-type splicing regulatory protein.

importance of mRNA stability in the regulation of inflammatory process has also been shown in vivo (Carballo et al., 1998; Katsanou et al., 2005).

Jun N-terminal kinase (JNK) is a subfamily of MAPKs. There are three different JNKs, namely JNK1, JNK2, and JNK3, all encoded by different genes. JNK1 and JNK2 are expressed ubiquitously, whereas the expression of JNK3 is restricted to heart, brain, and testis. All JNKs are involved in the regulation of immune responses and apoptosis (Pearson et al., 2001). Although p38 MAPK is considered to be the major factor that mediates mRNA stabilization, there are reports showing that JNK is also involved in post-transcriptional gene regulation. Using dominant-negative isoforms or over-expressing JNK it has been shown that JNK regulates the stability of IL-3 mRNA and vascular endothelial growth factor mRNA by a 3'-UTR-dependent mechanism (Ming et al., 1998; Pages et al., 2000). In Jurkat T-cells, JNK has been shown to stabilize IL-2 mRNA via binding of nucleolin and Y box-binding protein to the 5'-UTR of IL-2 mRNA (Chen et al., 2000).

Inducible nitric-oxide synthase (iNOS) is an important enzyme regulating physiological and pathophysiological processes in mammalian cells, and its expression is associated with several inflammatory diseases (Bogdan, 2001; Korhonen et al., 2005). Post-transcriptional mechanisms have been shown to play an important role in the regulation of iNOS expression in response to inflammatory stimuli (cytokines, etc.). The 3'-UTR of the human iNOS mRNA contains five AREs, which are centrally involved in the regulation of iNOS mRNA stability (Kleinert et al., 2004). HuR, KSRP, and PTB have been shown to modulate human iNOS mRNA stability by binding to this 3'-UTR (Rodriguez-Pascual et al., 2000; Linker et al., 2005; Pautz et al., 2006). In addition, TTP has been shown to play an important role in the regulation of iNOS mRNA stability and thereby in the overall iNOS expression (Fechir et al., 2005a). In murine macrophages, pharmacological inhibition of JNK by SP600125 destabilized iNOS mRNA and consequently down-regulated iNOS expression (Lahti et al., 2003). Because human iNOS expression is pivotally regulated post-transcriptionally and there are data showing that JNK is involved in the regulation of mRNA stability, we hypothesized that JNK may regulate human iNOS expression at the post-transcriptional level. In the present study, we provide data showing that JNK is involved in the induction of human iNOS expression by stabilizing iNOS mRNA, and the results suggest that the effect may be mediated through the regulation of TTP expression.

Materials and Methods

Reagents. Trypsin, glutamine, pyruvate solution, bovine serum albumin, 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB), and horseradish peroxidase-coupled anti-rabbit and anti-mouse IgG were purchased from Sigma (Deisenhofen, Germany). Monoclonal anti-iNOS antibody was obtained from R&D systems (Wiesbaden, Germany). The monoclonal anti-GAPDH antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Complete EDTA-free protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Mannheim, Germany). The QuantiTect Probe reverse transcription-polymerase chain reaction kit and the HiPerFect transfection reagent were from QIAGEN (Hilden, Germany). All oligonucleotides and dual-labeled probes were from MWG Biotech (Ebersberg, Germany). MessageMuter shRNAi Production Kit and

AmpliScribe T7-*Flash* transcription kit were purchased from Epicenter Biotechnologies (Madison, WI). Quick Spin Columns for RNA purification were purchased from Roche Applied Systems (Mannheim, Germany). dNTPs were purchased from GE Healthcare (Freiburg, Germany). Human interferon-γ, IL-1β, and TNF-α were obtained from Strathmann (Hannover, Germany). Fetal calf serum, DMEM, and RPMI 1640 medium were purchased from PAN Systems (Nürnberg, Germany). SP600125 was purchased from Calbiochem (Bad Soden, Germany). The Bradford reagent mix for determination of protein concentration was obtained from Bio-Rad Laboratories (Munich, Germany). JNK1, p38, and phospho-p38 antibodies were purchased from Cell Signaling Technology (Danvers, MA). The polyclonal antitristetraprolin antibody was a kind gift of Dr. William Rigby (Department of Medicine, Dartmouth Medical School, Lebanon, NH).

Cell Culture, Cytokine Treatment, and Nitrite Measurement. A549/8 human alveolar epithelial cells were grown in DMEM with 2 mM L-glutamine, penicillin/streptomycin, and 5% heat-inactivated fetal bovine serum to ~80% confluence. Eighteen hours before cytokine stimulation, cells were washed with phosphate-buffered saline and incubated with DMEM containing 2 mM L-glutamine in the absence of serum and phenol red. iNOS expression was induced with a cytokine mixture (CM) containing 100 U/ml interferon-γ, 50 U/ml IL-1β, and 10 ng/ml TNF-α for the corresponding time periods depending on the experiment. In all experiments in which SP600125 was used, cells were treated with SP600125 (1–10 μM) or vehicle (DMSO 1:1000) 30 min before and during cytokine incubation. Afterward, the supernatant of the cells (500 μl) was used to measure nitrite (NO_2^-) by the Griess reaction or the Sievers Nitric Oxide Analyzer (ADInstruments, Spechbach, Germany).

Analysis of Human iNOS Promoter Activity in Stably Transfected Cells. To investigate the effect of JNK inhibitor SP600125 and siRNA-mediated down-regulation of JNK on iNOS promoter activity and iNOS mRNA expression, pools of stably transfected A549/8 cells containing a 16-kb fragment of the human iNOS promoter (GenBank accession number AC005697) cloned in front of a luciferase reporter gene were used (Hausding et al., 2000). These A549/8-pNOS2(16)Luc cells were grown in DMEM with 2 mM L-glutamine, penicillin/streptomycin, and 5% heat-inactivated fetal bovine serum to ~80% confluence. Serum starvation and preincubations with SP600125 and cytokine treatment were carried out as described above.

RNA Isolation and Quantitative Reverse Transcription/Polymerase Chain Reaction. A549/8 cells and A549/8-pNOS2(16)Luc cells were washed twice with phosphate-buffered saline, and total cellular RNA was isolated by guanidinium thiocyanate/phenol/chloroform extraction as described previously (Rodriguez-Pascual et al., 2000). One-Step reverse transcription-polymerase chain reaction was performed in 25-μl reactions in a 96-well spectrofluorometric thermal cycler (iCycler; Bio-Rad Laboratories). The following oligonucleotides were purchased from MWG-Biotech: iNOS: sense, TGCAGACACGTGCGTTACTCC; antisense, GGTAGCCAGCATAGCGGATG; probe, TGGCAAGCACGACTTC-CGGGTG; GAPDH: sense, CCCATGTTCGTCATGGGTGT; antisense, TGGTCATGAGTCCTTCCACGATA; probe, CTGCACCAC-CAACTGCTTAGCACCC; TTP: sense, TTCGCCCACTGCAACCTC; antisense, CGCCCACTCTCTGAGAAGGTC; probe, CCCCTCGCGC-TACAAGACTGAGCTATG; luciferase: sense, AAAAAGTTGCGCG-GAGGAG; antisense, TTTTTCTTGCGTCGAGTTTTCC; probe, TGT-GTTTGTGGACGAAGTACCGAAAGGTCTTAC; JNK1: sense, CCACCAAAGATCCCTGACAAG; antisense, TGGATGCTGAGAGC-CATTG; probe, ACGGGGGGCAGCCCTCTCCTTTAG; and JNK2: sense, TGGATTGGGAAGAAAGAAGC; antisense, CGTCGAG-GCATCAAGACTG; probe, TTCCACTGAGCAGACGCTGGCC.

Each experimental reaction was performed in triplicate. All primer/probes sets had efficiencies of 100% (±10%). To calculate the relative expression of iNOS, luciferase, TTP, JNK1, or JNK2 mRNA in A549/8 cells, the $2^{(-\Delta\Delta C_T)}$ method (Livak and Schmittgen, 2001)

was used. According to this method, the C_T values for iNOS, luciferase, TTP, JNK1, or JNK2 mRNA expression in each sample were normalized to the C_T values of GAPDH mRNA in the same sample.

DRB Assay. To investigate the stability of iNOS mRNA, A549/8 cells were cultured as described previously, and iNOS expression was induced with cytokines for 4 h. An inhibitor of transcription (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside, DRB; final concentration, 25 μg/ml) was then added. The cells were further incubated for the time periods indicated, and total RNA was extracted. Relative amounts of iNOS and GAPDH mRNA were analyzed with quantitative reverse transcriptase/real-time PCR (qRT-PCR). iNOS mRNA was normalized to GAPDH mRNA, and time point 0 h (DRB addition) was set as 100%.

Western Blot. To study protein expression, A549/8 cells were lysed. Total cellular proteins (10–20 μg) were separated in 7.5% SDS polyacrylamide gel and transferred to nitrocellulose membrane by semidry electroblotting. Further steps were performed as described previously (Rodriguez-Pascual et al., 2000). iNOS and GAPDH were detected with monoclonal anti-iNOS and anti-GAPDH antibodies, respectively. JNK1, p38, phospho-p38, and TTP were detected with rabbit polyclonal JNK1 antibody, rabbit polyclonal anti-p38 antibody, rabbit polyclonal anti-phospho-p38 antibody, and rabbit polyclonal anti-TTP antibody (Brooks et al., 2002), respectively. Immune complexes were detected by using anti-mouse and anti-rabbit horseradish peroxidase-conjugated immunoglobulin. The immunoreactive proteins were visualized by the enhanced chemiluminescence detection system (Amersham Biosciences).

Down-Regulation of JNK1 and JNK2 Expression by siRNA. Small hairpin RNAs (shRNAs) were produced in vitro using chemically synthesized DNA oligonucleotide templates and the Message-Muter shRNA Production Kit as described by the manufacturer. Transcription templates were designed such that they contained T7 promoter sequences at the 3′-end. The target sequence for the down-regulation of both JNK isoforms was GAAAGAATGTCCTACCTTCT, which is found in both JNK1 mRNA (nucleotides 393–412) and JNK2 mRNA (nucleotides 425–444) (Gururajan et al., 2005). As a control, shRNAs with target sequence against GFP (GGGCAAGCTGACCCT-GAAGTT) were synthesized. shRNAs were purified with phenol/chloroform and precipitated with ethanol and then further purified with Quick Spin Columns. A549/8-pNOS2(16)Luc cells were grown to ~80% confluence, and then cells were transiently transfected with shRNAs using HiPerFect Transfection Reagent according to the manufacturer's instructions. Cells were incubated for 6 h, medium was replaced with fresh culture medium with serum to remove the transfection reagent, and cells were further incubated for 24 h. The culture medium was then replaced with medium without serum and phenol red, and cells were further incubated for 18 h. Then cells were stimulated with cytokines as described above.

Statistics. Data represent means ± S.E.M. Statistical differences were determined by factorial analysis of variance followed by Fisher's protected least-significant-difference test for comparison of multiple means. Statistical probability is expressed as $*$, $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$.

Results

Pharmacological Inhibition of JNK by SP600125 Reduced Human iNOS Expression by Affecting iNOS mRNA Stability in Cytokine-Treated A549/8 Human Alveolar Epithelial Cells. To test the effect of JNK inhibitor SP600125 on iNOS expression, A549/8 human alveolar epithelial cells able to express iNOS in response to cytokines (Kleinert et al., 1996) were used. To investigate the effect of SP600125 on iNOS mRNA expression, cells were preincubated with SP600125 for 30 min and then stimulated with CM for 6 h. Total RNA was extracted and iNOS mRNA levels were determined by qRT-PCR. SP600125 inhibited the ex-

pression of iNOS mRNA in a dose-dependent manner in A549/8 cells (Fig. 1A). To investigate the effect of SP600125 on iNOS protein expression, cells were preincubated with SP600125 for 30 min and stimulated with the CM for 12 h. Total cellular protein was then extracted, and iNOS expression was detected by Western blot. SP600125 (10 μM) inhibited iNOS protein expression (Fig. 1B). The effect of SP600125 on NO production in A549/8 cells was also tested. Cells were preincubated with SP600125 (or DMSO) for 30 min, stimulated with CM, and incubated for 24 h. NO production was determined as nitrite levels in the culture medium. SP600125 inhibited NO production in A549/8 cells (Fig. 1C).

Human iNOS mRNA expression in response to cytokines is a result of activation of iNOS promoter and the stabilization of the synthesized mRNA. In fact, mRNA stability plays a major role in the overall regulation of iNOS mRNA expression (Kleinert et al., 2004). To investigate whether the inhibitory effect of SP600125 on iNOS expression is at the level of promoter activity or mRNA stability, we used A549/8 cells stably transfected with pNOS2(16)Luc, a construct containing the 16-kb human iNOS promoter cloned in front of luciferase (Hausding et al., 2000). This allows the differentiation between regulation of human iNOS promoter activity and mRNA stability in the same cell. According to the results from untransfected A549/8 cells, SP600125 also inhibited cytokine-induced iNOS mRNA expression in a dose-dependent manner in A549/8-pNOS2(16)Luc cells (Fig. 2A). However, cytokine-induced increase in luciferase mRNA, which reflects iNOS promoter activity, was barely affected by JNK inhibitor SP600125 (Fig. 2A). This indicates that the major regulation of iNOS expression by JNK is mediated through iNOS mRNA stabilization. To further investigate the effect of SP600125 on iNOS mRNA stability, transcription was blocked by DRB (25 μg/ml). In these experiments, cells were preincubated with SP600125 for 30 min followed by cytokine stimulation for 4 h. After that, DRB was added to the cells. Cells were harvested for RNA extraction 0, 2, and 4 h after the addition of DRB. In cells treated with SP600125, iNOS mRNA expression was clearly reduced 4 h after DRB addition, indicating an effect of JNK on iNOS mRNA stability (Fig. 2B). Taken together, pharmacological inhibition of JNK in A549/8 cells resulted in the reduction of iNOS expression because of the destabilization of iNOS mRNA.

Down-Regulation of JNK1 and JNK2 by siRNA Reduced iNOS Expression in A549/8 Human Alveolar Epithelial Cells. To further confirm the finding that JNK regulates human iNOS expression through stabilizing iNOS mRNA, we used siRNA to down-regulate JNK1 and JNK2 expression. A549/8 cells were transiently transfected with siRNA targeted at JNK1 and JNK2 and incubated for 48 h. To monitor the down-regulation of JNK expression, mRNA and protein expression were analyzed. siRNA incubation reduced the mRNA and protein expression of JNK1 (Fig. 3, A and B) and mRNA expression of JNK2 (Fig. 3C). siRNA-mediated JNK down-regulation clearly reduced iNOS mRNA expression compared with control cells treated with anti-GFP siRNA (Fig. 4A). In addition, down-regulation of JNK1 and JNK2 resulted in inhibition of cytokine-induced iNOS protein expression in these cells (Fig. 4B). Consistent with the results obtained for pharmacological inhibition of JNK by SP600125, luciferase mRNA levels were not significantly
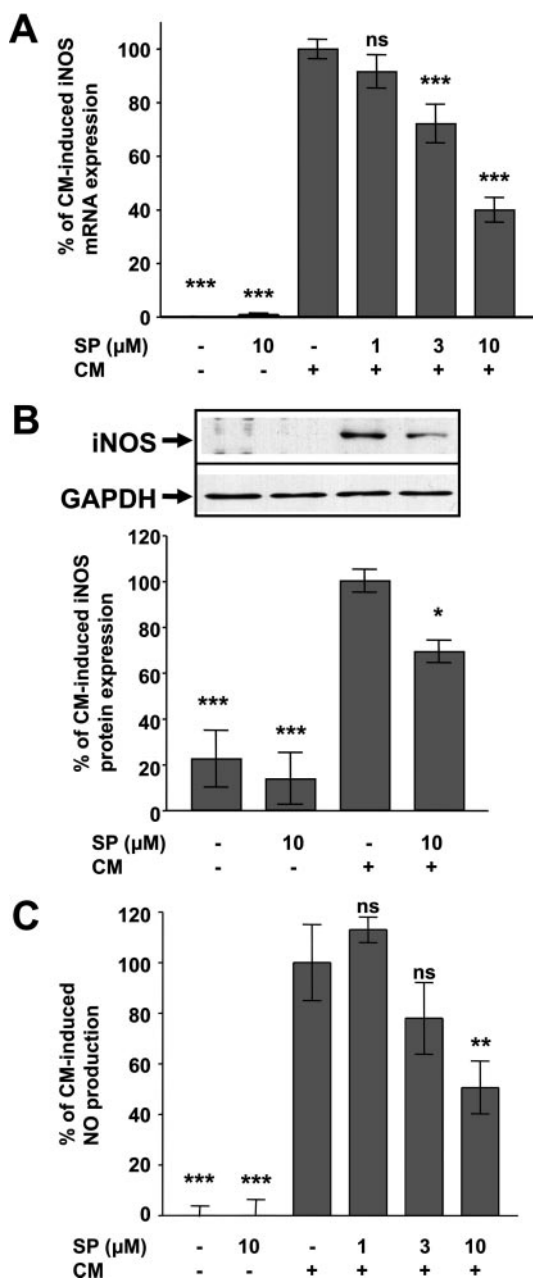
Fig. 1. Inhibition of iNOS expression and NO production by the JNK inhibitor SP600125 in A549/8 cells. Cell culture, serum starvation, and preincubation with SP600125 (1–10 μM) or vehicle (DSMO; 1:1000) were carried out as described under *Materials and Methods*. A, cells were incubated with or without CM in the presence of either SP600125 or vehicle for 6 h and then harvested for total RNA extraction. iNOS mRNA expression was detected by qRT-PCR, and results were normalized to GAPDH mRNA and expressed as a percentage of CM, mean $\pm$ S.E.M. ***, $p < 0.001$; ns, not significant versus cells treated with CM; $n = 6$. B, cells were incubated with or without CM in the presence of either SP600125 or vehicle for 12 h and then harvested for total cellular protein extraction. iNOS and GAPDH expression was detected by Western blot using monoclonal anti-iNOS and anti-GAPDH antibodies, respectively. The gel is a representative of four separate experiments with similar results. Protein expression was quantified by densitometric analysis using Quantity One 4.4.1 (Bio-Rad). Results are normalized to GAPDH and expressed as a percentage of CM, mean $\pm$ S.E.M. ***, $p < 0.001$; *, $p < 0.05$ versus cells treated with CM; $n = 4$. C, a summary of nitrite measurements using supernatants from A549/8 cells incubated with or without CM in the presence of either SP600125 (1–10 μM) or vehicle for 24 h. The nitrite values of supernatants from untreated cells were subtracted from those treated with SP600125/CM. Results are expressed as a percentage of CM, mean $\pm$ S.E.M. **, $p < 0.01$; ns, not significant versus cells treated with CM; $n = 8$.

changed by JNK knockdown (Fig. 4A). These results support the idea that JNK is a positive regulator of iNOS expression in A549/8 cells, and the effect is mainly mediated by mRNA stabilization.

SP600125 and Anti-JNK siRNA Reduced Protein Expression of TTP. TTP has been shown to positively regulate iNOS expression in human cells (Fechir et al., 2005a). Therefore, a possible effect of JNK1 and JNK2 inhibition on TTP expression was investigated. Both pharmacological inhibition of JNK and down-regulation of JNK by siRNA resulted in a reduction of TTP protein expression (Fig. 5, B and E) but had no effect on TTP mRNA expression (Fig. 5, A and D). Because TTP-expression is regulated by p38 MAPK (Fechir et al., 2005a), we further analyzed the effect of JNK inhibition/down-regulation on p38 MAPK activity. Neither
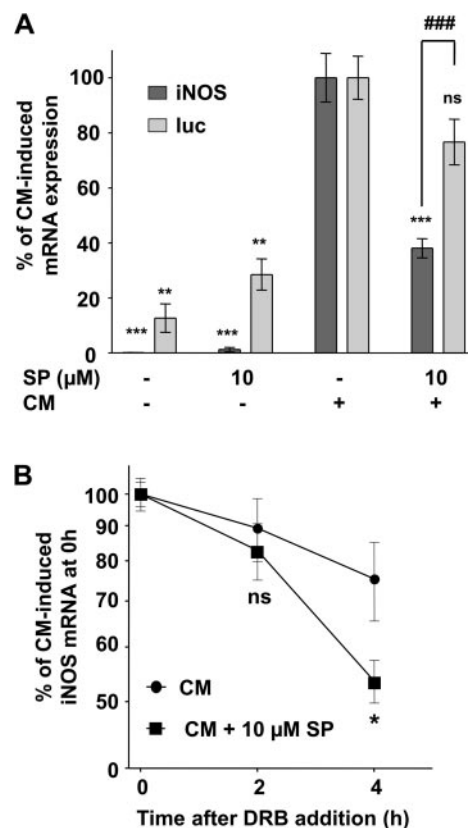

Fig. 2. Effects of the JNK inhibitor SP600125 on cytokine-induced iNOS promoter activity, iNOS mRNA expression, and mRNA stability in A549/8-pNOS2(16)Luc cells. A, cell culture, serum starvation, and preincubation with SP600125 (10 μM) or vehicle (DSMO; 1:1000) were carried out as described under *Materials and Methods*. Cells were incubated with or without CM in the presence of either SP600125 or vehicle for 6 h followed by RNA extraction. iNOS mRNA (■) and luciferase mRNA (□) expressions was measured by qRT-PCR and results are normalized to GAPDH mRNA and expressed as a percentage of CM, mean $\pm$ S.E.M. ***, $p < 0.001$; **, $p < 0.01$; ns, not significant versus cells treated with CM; ###, $p < 0.001$ for cells treated with SP600125 compared with cells treated without SP600125; $n = 6$. B, cell culture, starvation, and preincubation with SP600125 (10 μM, ■) or vehicle (DMSO; ●) were carried out as described under *Materials and Methods*. Then, cells were stimulated with CM in the presence of either SP600125 (10 μM, ■) or vehicle (DMSO; ●) for 4 h. Then DRB (an inhibitor of transcription; 25 μg/ml) was added to the cells, and cells were further incubated for the time periods indicated. After that, cells were harvested, and total RNA was extracted. iNOS and GAPDH expression was measured by qRT-PCR. Results are normalized to GAPDH mRNA and expressed as a percentage of CM, mean $\pm$ S.E.M. *, $p < 0.05$; ns, not significant versus cells treated with CM at the same time point; $n = 8$.

SP600125 nor siRNA against JNK affected p38 MAPK expression or phosphorylation in response to CM (Fig. 5, C and F). Therefore JNK seems to regulate TTP expression independent of p38 MAPK by a mechanism modulating the translation of the TTP mRNA or the stability of the TTP protein.

Discussion

In the present study, we provided data showing that JNK positively regulates human iNOS expression by stabilizing iNOS mRNA in A549/8 cells. In these cells, JNK also regulates the expression of TTP, a factor known to regulate human iNOS mRNA stability.

To examine a possible effect of JNK on human iNOS expression we used the JNK inhibitor SP600125 at concentrations between 1 and 10 μM. SP600125 in these concentrations dose-dependently inhibited both iNOS mRNA and protein expression and NO production (Fig. 1). Performing in vitro inhibition assays with isolated kinases, SP600125 has
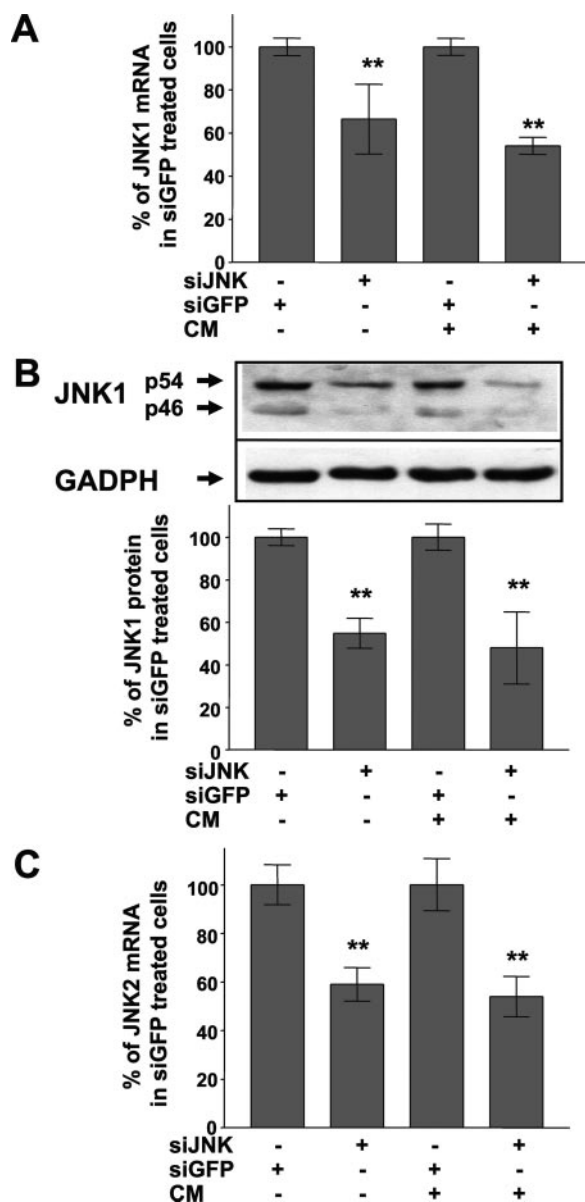
Fig. 3. Down-regulation of JNK expression by siRNA. Cell culture, transfections of shRNAs, and serum starvation were carried out as described under *Materials and Methods*. Then, cells were incubated with or without CM for 6 and 12 h for RNA and protein extractions, respectively. A, expression of JNK1 mRNA in cells transfected with shRNAs targeted at JNK. Data (mean ± S.E.M.) represent JNK1 mRNA expression normalized to GAPDH mRNA, and cells treated with siGFP or CM + siGFP were set as 100% (∗∗, $p < 0.01$ versus cells treated with siGFP, $n = 6$). B, expression of JNK1 protein in cells transfected with shRNAs targeted at JNK. JNK1 and GAPDH were detected with polyclonal anti-JNK1 and monoclonal GAPDH antibodies, respectively. The gel is a representative result from four separate experiments with similar results. Protein expression was quantified by densitometric analysis using Quantity One 4.4.1 (Bio-Rad). Results were normalized to GAPDH, and cells treated with siGFP or CM + siGFP were set as 100%, mean ± S.E.M. ∗∗, $p < 0.01$ versus cells treated with siGFP, $n = 4$. C, expression of JNK2 mRNA in cells transfected with shRNAs against JNK. Data (mean ± S.E.M.) represent JNK2 mRNA expression normalized to GAPDH mRNA, and cells treated with siGFP or CM + siGFP were set as 100% (∗∗, $p < 0.01$ versus cells treated with siGFP, $n = 6$.
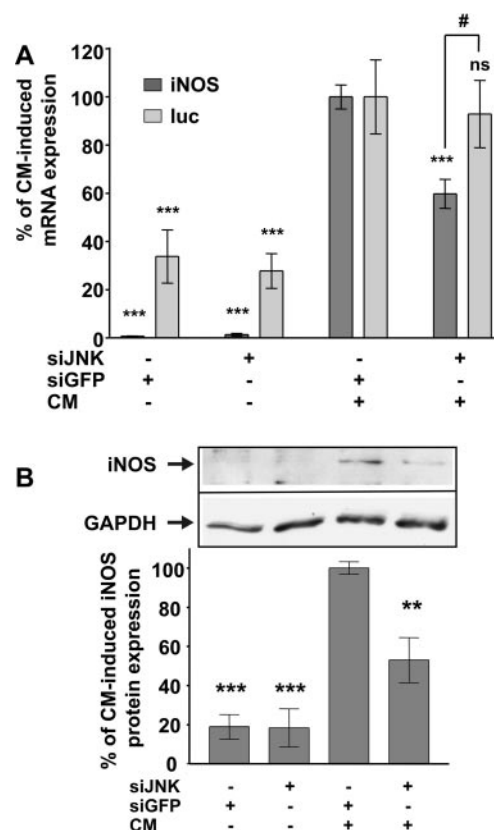

Fig. 4. Effects of siRNA-mediated down-regulation of JNK expression on cytokine-induced iNOS promoter activity and iNOS mRNA and protein expression in A549/8-pNOS2(16)Luc cells. Cell culture, transfections of shRNAs, and serum starvation were carried out as described under *Materials and Methods*. Then, cells were incubated with or without CM for 6 and 12 h for RNA and protein extractions, respectively. A, effect of down-regulation of JNK by siRNA on iNOS promoter activity and iNOS mRNA expression. iNOS mRNA (■) and luciferase mRNA (□) expressions were measured by qRT-PCR. Data (mean ± S.E.M.) represent iNOS and luciferase mRNA expressions normalized to GAPDH mRNA, and cells treated with CM + siGFP were set as 100%. ∗∗∗, $p < 0.001$; ns, not significant versus cells treated with siGFP; #, $p < 0.05$ luciferase mRNA expression versus iNOS mRNA expression in cells treated with CM and siJNK; $n = 6$. B, effect of down-regulation of JNK by siRNA on iNOS protein expression in A549/8 cells. iNOS and GAPDH expression were detected by Western blot using monoclonal anti-iNOS and anti-GAPDH antibodies, respectively. The gel is a representative result from four separate experiments with similar results. Protein expression was quantified by densitometric analysis using Quantity One 4.4.1 (Bio-Rad). Results are normalized to GAPDH and expressed as a percentage of CM and siGFP-treated cells, mean ± S.E.M. ∗∗∗, $p < 0.001$; ∗∗, $p < 0.01$ versus cells treated with CM and siGFP; $n = 4$.

been shown previously to inhibit JNK1 and JNK2. However, with higher concentrations, SP600125 inhibits several other signaling proteins also (Bennett et al., 2001). In the present study, the finding that inhibition of JNK by SP600125 resulted in the inhibition of iNOS expression was tested also with siRNA approach. In experiments with siRNA targeted at JNK1 and JNK2, human iNOS expression was inhibited confirming that JNK is involved in the regulation of iNOS

expression (Fig. 4). In addition, using JNK inhibitor SP600125, we did not detect any change in the cytokine-induced expression or phosphorylation of p38 MAPK (Fig. 5C). Taken together, the inhibition of iNOS expression and destabilization of iNOS mRNA by SP600125 (Figs. 1 and 2) are due to inhibition of JNK activity and not to other effects of the pharmacological compound used.

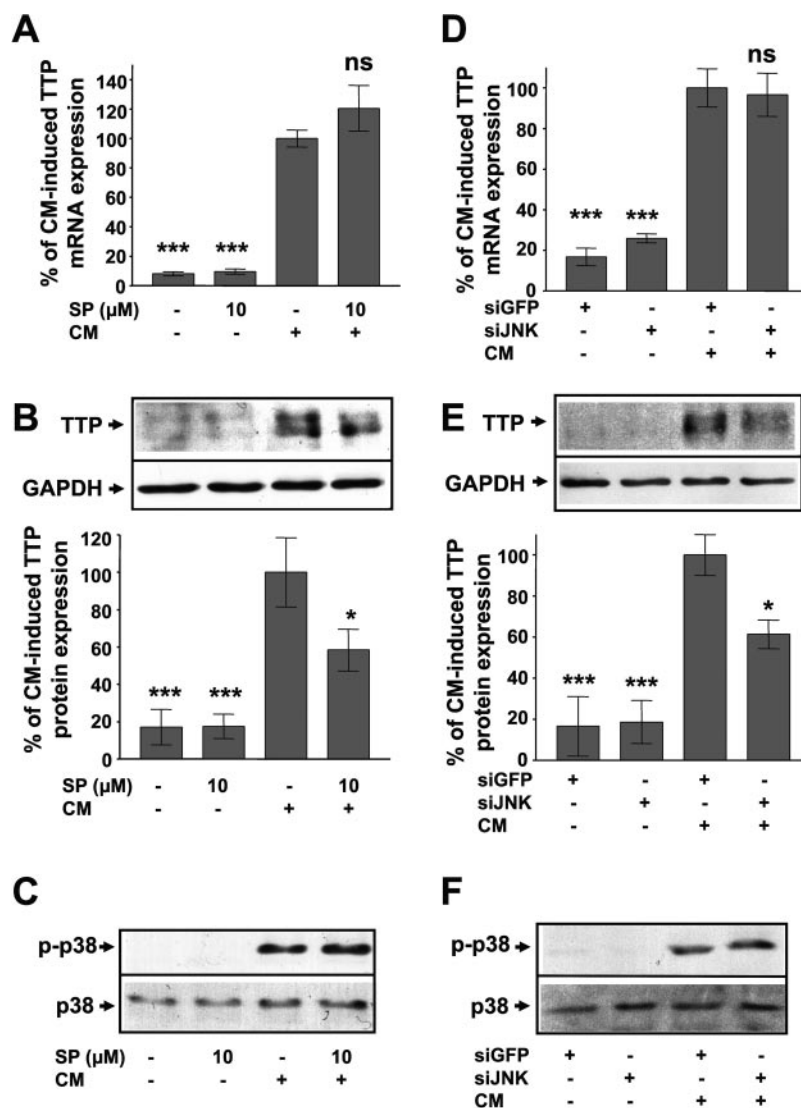JNK has been shown to regulate mRNA stability of IL-3

Fig. 5. Effect of modulation of JNK signaling using the JNK inhibitor SP600125 or siRNA-mediated down-regulation of JNK expression on TTP expression and p38 phosphorylation in A549/8 cells. Cell culture, serum starvation, and preincubation with SP600125 (10 μM) or vehicle (DSMO 1:1000) and transfection of shRNAs were carried out as described under *Materials and Methods*. A and D, effect of JNK inhibitor SP600125 and siJNK treatment on TTP mRNA expression in A549/8 cells. In experiments with SP600125, cells were stimulated with or without CM in the presence of either SP600125 or vehicle for 6 h followed by RNA extraction. In transfection experiment with shRNAs targeted at JNK, cells were incubated with or without CM for 6 h followed by RNA extraction. TTP mRNA expression was measured by qRT-PCR. Data (mean $\pm$ S.E.M.) represent TTP mRNA expression normalized to GAPDH mRNA, and cells treated with CM were set as 100%. ∗∗∗, $p < 0.001$; ns, not significant versus cells treated with CM; $n = 6$. B and E, effect of JNK inhibitor SP600125 and down-regulation of JNK expression by siRNA on TTP protein expression in A549/8 cells. In experiments with SP600125, cells were stimulated with or without CM in the presence of either SP600125 or vehicle for 12 h followed by protein extraction. In transfection experiment with shRNAs targeted at JNK, cells were incubated with or without CM for 12 h followed by protein extraction. TTP and GAPDH expressions were detected by Western blot using polyclonal anti-TTP and monoclonal anti-GAPDH antibodies, respectively. The gel is a representative result from four separate experiments with similar results. Protein expression was quantified by densitometric analysis using Quantity One 4.4.1 (Bio-Rad). Results are normalized to GAPDH and are expressed as a percentage of CM, mean $\pm$ S.E.M. ∗∗∗, $p < 0.001$; ∗, $p < 0.05$ versus cells treated with CM; $n = 4$. C and F, effect of JNK inhibitor SP600125 and down-regulation of JNK expression by siRNA on p38 phosphorylation in A549/8 cells. In experiments with SP600125, cells were stimulated with or without CM in the presence of either SP600125 or vehicle for 30 min followed by protein extraction. In transfection experiments with shRNAs targeted at JNK, cells were incubated with or without CM for 30 min followed by protein extraction. Cells were incubated with or without CM for 30 min followed by protein extraction. phospho-p38 and total p38 were detected by Western blot using polyclonal phosphospecific anti-p38 and anti-p38 antibodies. The gel is a representative result from four separate experiments with similar results.

mRNA in a mast cell line (Ming et al., 1998) and IL-2 mRNA in Jurkat T-cells (Chen et al., 2000). It is interesting that the stabilization of IL-2 mRNA in Jurkat T-cells was mediated by binding of nucleolin and Y box-binding protein to the 5′-UTR of IL-2 mRNA. In addition, the JNK inhibitor SP600125 has also been suggested to destabilize murine iNOS and cyclooxygenase-2 mRNAs (Lahti et al., 2003; Nieminen et al., 2006). In the present study, we used A549/8 cells stably transfected with a luciferase construct under control of the 16-kb human iNOS promoter. Using this cell line, it is possible to differentiate the promoter activity and mRNA stability in the mRNA expression in the same cell in response to cytokine stimulation. Cytokine stimulation increased iNOS promoter activity only weakly (maximal 10-fold; Figs. 2A and 4A), whereas iNOS mRNA expression was markedly enhanced (at least 100-fold), indicating that iNOS mRNA expression in response to cytokines is critically dependent on the stabilization of iNOS mRNA, as shown in previous reports (Linn et al., 1997; Rodriguez-Pascual et al., 2000). By incubation of these A549/8-pNOS2(16)Luc cells, we were able to show that SP600125-mediated inhibition of JNK activity and siRNA-mediated knockdown of JNK1 and JNK2 expression resulted in marked inhibition of cytokine-induced human iNOS mRNA expression. However these treatments do not significantly modify human iNOS promoter activity (Figs. 2A and 4A). In addition, DRB experiments directly analyzing mRNA stability showed that incubation of A549/8 cells with SP600125 destabilized cytokine-induced iNOS mRNA (Fig. 2B). Taken together, these results suggest that JNK positively regulates cytokine-induced iNOS expression and NO production in A549/8 human alveolar epithelial cells by enhancing iNOS mRNA stability.

The 3′-UTR of the human iNOS mRNA contains five AREs, which are known to be central *cis*-acting elements in the regulation of the stability of unstable mRNAs. We have shown that HuR, a member of embryonic lethal abnormal vision protein family, KSRP, the T-cell intracellular antigen-1-related protein, and PTB regulate human iNOS mRNA stability by binding to the iNOS 3′-UTR (Rodriguez-Pascual et al., 2000; Fechir et al., 2005b; Linker et al., 2005; Pautz et al., 2006). Another important protein in the regulation of mRNA turnover is TTP. Its importance for the mRNA stability and consequently protein expression of TNF-α and granulocyte/macrophage-colony stimulating factor has been demonstrated in vivo (Carballo et al., 1998, 2000). We have shown recently that TTP is involved in the cytokine-induced stabilization of human iNOS mRNA. TTP does not directly bind to the iNOS 3′-UTR; rather, it acts by governing the interaction of the destabilizing protein KSRP and the iNOS mRNA (Fechir et al., 2005a; Linker et al., 2005). Therefore, we analyzed the effects of SP600125-mediated inhibition of JNK activity and siRNA-mediated knockdown of JNK1 and JNK2 expression on TTP expression. It is interesting that inhibition of JNK by SP600125 or down-regulation of JNK1 and JNK2 expression by siRNA did not affect cytokine-induced TTP mRNA expression (Fig. 5, A and D) but significantly reduced cytokine-induced TTP protein expression (Fig. 5, B and E). These data suggest that JNK may be involved in the control of the translation of TTP or the stability of the TTP protein. JNK-mediated regulation of translation or protein stability has been published previously for other proteins. According to recent findings in cardiomyo-

cytes from $JNK1^{-/-}$ and $JNK2^{-/-}$ mice, microsomal prostaglandin E2 synthase expression is dependent on the mRNA stabilization by JNK, and JNK also controls its translation (Degousee et al., 2006). The stability of c-Myc protein is also regulated (at least partially) by JNK (Alarcon-Vargas and Ronai, 2004). TTP expression has been shown to be regulated by the p38 MAPK-mitogen-activated protein kinase-activated protein kinase 2 pathway through a post-transcriptional mechanism in the murine cells (Tchen et al., 2004; Hitti et al., 2006). In addition, in a recent publication, TTP subcellular localization and protein stability was reported to be regulated by p38 MAPK and extracellular signal-regulated kinase (Brook et al., 2006). Both JNK inhibition by SP600125 and siRNA-mediated down-regulation of JNK inhibited the TTP protein expression without affecting the phosphorylation status or expression of p38 MAPK. This suggests that JNK regulates TTP expression independently of p38 MAPK and probably by regulation of TTP mRNA translatability and/or TTP protein stability.

In conclusion, cytokine stimulation leads to the stabilization of human iNOS mRNA. This stabilization is, at least partially, dependent on JNK suggesting that JNK is a positive regulator of iNOS expression in human cells. Furthermore, our results suggest that JNK regulates TTP protein expression and that TTP may be involved in the JNK-mediated stabilization of iNOS mRNA.

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