Effects of the JNK inhibitor anthra[1,9-cd]pyrazol-6(2H)-one (SP-600125) on soluble guanylyl cyclase α₁ gene regulation and cGMP synthesis

Joshua S. Krumenacker,1 Alexander Kots,2 and Ferid Murad1,2

1Institute of Molecular Medicine, University of Texas–Houston Health Science Center; and 2Department of Integrative Biology and Pharmacology, University of Texas Medical School at Houston, Houston, Texas

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Krumenacker, Joshua S., Alexander Kots, and Ferid Murad. Effects of the JNK inhibitor anthra[1,9-cd]pyrazol-6(2H)-one (SP-600125) on soluble guanylyl cyclase α₁ gene regulation and cGMP synthesis. Am J Physiol Cell Physiol 289: C778–C784, 2005. First published May 11, 2005; doi:10.1152/ajpcell.00057.2005.—The decreased expression of the nitric oxide (NO) receptor, soluble guanylyl cyclase (sGC), occurs in response to multiple stimuli in vivo and in cell culture and correlates with various disease states such as hypertension, inflammation, and neurodegenerative disorders. The ability to understand and modulate sGC expression and cGMP levels in any of these conditions could be a valuable therapeutic tool. We demonstrate herein that the c-Jun NH2-terminal kinase JNK II inhibitor anthra[1,9-cd]pyrazol-6(2H)-one (SP-600125) completely blocked the decreased expression of sGCα₁-subunit mRNA by nerve growth factor (NGF) in PC12 cells. Inhibitors of the ERK and p38 MAPK pathways, PD-98059 and SB-203580, had no effect. SP-600125 also inhibited the NGF-mediated decrease in the expression of sGCα₁ protein as well as sGC activity in PC12 cells. Other experiments revealed that decreased sGCα₁ mRNA expression through a CAMP-mediated pathway, using forskolin, was not blocked by SP-600125. We also demonstrate that TNF-α/IL-1β stimulation of rat fetal lung (RFL-6) fibroblast cells resulted in sGCα₁ mRNA inhibition, which was blocked by SP-600125. Expression of a constitutively active JNKK2-JNK1 fusion protein in RFL-6 cells caused endogenous sGCα₁ mRNA levels to decrease, while a constitutively active ERK2 protein had no effect. Collectively, these data demonstrate that SP-600125 may influence the intracellular levels of the sGCα₁-subunit in certain cell types and may implicate a role for c-Jun kinase in the regulation of sGCα₁ expression.

The free radical gas nitric oxide (NO) is known to modulate multiple signaling events within cells and tissues that occur through either cGMP-dependent or cGMP-independent pathways. The cGMP-mediated effects of NO occur through the receptor, soluble guanylyl cyclase (sGC), which is stimulated upon NO binding up to 400-fold (8). The production of cGMP can then lead to activation of cGMP-dependent protein kinases, phosphodiesterases, or ion channels, depending on the stimulus and/or the type of cell that is responding (for review, see Ref. 12). Regulation of these signaling processes has been shown to occur at several levels, including at the level of expression of sGC.

As a NO receptor, sGC exists as an obligatory homodimeric protein composed of α- and β-subunits. Although sGCα₂- and β₂-subunits do exist, the α₁- and β₁-subunits appear to be the predominantly expressed isoforms. Each sGC subunit is the product of an independent gene, but the genes for the α₁- and β₁-subunits are located adjacent on the same chromosome in mammals (26).

Regulation of the sGCα₁ and sGCβ₁ mRNA and protein subunits has been reported in several in vivo and cell culture models through various pathways, first in response to cyclic nucleotides or NO donors (20, 27). We also have demonstrated that the mRNA for the sGCα₁- and β₁-subunits rapidly decreased in the uterus of estradiol-treated rats (13). Others have shown that nerve growth factor (NGF) treatment of PC12 cells and TNF-α/IL-1β treatment of rat pulmonary artery smooth muscle cells decreased the expression of the sGC transcripts (17, 28). However, many of the signaling mechanisms and cell-type specificities involved in the regulation of sGC expression have yet to be explained.

Vascular disease states are thought to involve decreased expression of sGC. For example, models of hypertension, atherosclerosis, and Alzheimer’s disease all correlate with decreased levels of sGC (2, 9, 19, 24). Downregulation of sGC also has been suggested in other processes, such as NGF-mediated neuronal differentiation and negative feedback regulation of NO-cGMP signaling (10, 17). The stimulation and cell type-specific effects on sGC regulation add to the already complex NO-cGMP signaling paradigm. Therefore, a compound that could affect sGC gene regulation would be useful as a tool to dissect the important cell signaling and gene regulatory pathways involved.

This article demonstrates that the c-Jun NH2-terminal kinase JNK II inhibitor anthra[1,9-cd]pyrazol-6(2H)-one (SP-600125) can block inhibition of sGCα₁ mRNA expression by NGF in PC12 cells. As a result, sGCα₁ protein levels and NO-stimulated sGC activity in NGF-treated cells were preserved. Additional experiments imply that JNK may affect sGCα₁ mRNA regulation.

EXPERIMENTAL PROCEDURES

Materials and plasmids. NGF was purchased from Invitrogen (Carlsbad, CA). TNF-α and IL-1β were purchased from Biosource (Camarillo, CA). SP-600125, PD-98059, SB-203580, and forskolin were all purchased from Calbiochem (La Jolla, CA). All other routine chemicals and molecular biological reagents were purchased from Sigma (St. Louis, MO). Plasmids that express a constitutively active JNKK2-JNK1 fusion protein and a constitutively active ERK2 protein were generously provided by Dr. Anning Lin (Ben May Institute, Chicago, IL) and Dr. Natalie Ahn (University of Colorado, Boulder, CO).

Cell cultures. Rat pheochromocytoma (PC12) and rat fetal lung (RFL-6) fibroblast cells were purchased from the American Type...
Culture Collection (Manassas, VA). PC12 cells were cultured in RPMI 1640 containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 10% heat-inactivated horse serum, and 5% fetal bovine serum. RFL-6 cells were cultured in Ham's F-12K medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 20% fetal bovine serum. Cells were maintained in a 37°C and 5% CO₂ atmosphere. For experiments, PC12 cells were plated at a density of 8 × 10⁵ cells/well in six-well dishes, incubated for 3 days in culture medium, and treated without further manipulation. RFL-6 cells were cultured in six-well plates until confluent and also were treated without further manipulation. In both cell lines, the inhibitors SP-600125, PD-98059, or SB-203580 were added to the culture medium 30 min before the addition of NGF (50 ng/ml), forskolin (10 μM), or the combination of TNF-α (100 ng/ml) and IL-1β (20 ng/ml).

**Real-time RT-PCR analysis of endogenous sGCα₁ mRNA.** sGCα₁ mRNA levels were determined using a rat-specific real-time RT-PCR assay as previously described (13). Briefly, 100 ng of total RNA for each sample, extracted using UltraSpec RNA isolation solution (Biotech Laboratories, Inc., Houston, TX), was reverse transcribed using gene-specific oligonucleotides. Real-time PCR reactions were performed using an Applied Biosystems Prism 7700 sequence detector (PerkinElmer, Boston, MA). The data were analyzed using Sequence Detector software. sGCα₁ mRNA levels were normalized to the housekeeping gene 36b4 (14), which also was detected using a real-time RT-PCR assay (a generous gift from Dr. Greg Shipley, University of Texas at Houston, Houston, TX), and expressed as relative mRNA expression per 100 ng of total RNA in each sample. 36b4 expression levels were unchanged between samples and did not change in PC12 or RFL-6 cells treated with NGF, TNF-α/IL-1β, SP, PD, or SB or in their combination treatments in this study (data not shown). To detect sGCα₁ mRNA levels in transiently transfected RFL-6 cells, the plates were washed twice in PBS, followed by RNA isolation. RNA was then used directly in the RT-PCR assay, and sGCα₁ mRNA levels were normalized to 36b4.

**Immunoblot analysis.** Total protein lysates were obtained by washing the cells twice in PBS and then immediately lysing them with 1× Laemmli loading buffer containing a protease inhibitor cocktail (RFL-6 cells) or hypotonic lysis buffer containing 20 mM Tris (pH 8.0), 500 μM sodium orthovanadate, 80 mM β-glycerophosphate, and protease inhibitor cocktail (PC12 cells). For RFL-6 cells, equal volumes of lysate were fractioned using 10% SDS-PAGE and then electrophoretically transferred to immunoblotting polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). PC12 cell lysates were quantified using the Bradford method, and 100 μg/lane were loaded using the same technique. Membranes were blocked at room temperature for 30 min with 5% milk and incubated overnight with antibodies to c-Jun (0.4 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Ser63 and phospho-Ser73 c-Jun (1:1,000 dilution; Cell Signaling Technology, Beverly, MA), ERK2 (0.4 μg/ml; Santa Cruz Biotechnology), sGCα₁ (1:10,000 dilution; Sigma), hemagglutinin (HA, 1:10,000 dilution; Roche, Indianapolis, IN), or GAPDH (1:10,000 dilution, clone 6C5; a generous gift from Dr. A. Katrukha, Hytest, Turku, Finland) in blocking buffer or 5% BSA in place of milk for the antiphosphorylated c-Jun antibodies. Membranes were then incubated with secondary antibodies to peroxidase-conjugated rabbit (for c-Jun, phospho-c-Jun, sGCα₁, and ERK2 antibodies, 1:5,000 dilution; Amersham, San Francisco, CA) or mouse (for HA and GAPDH antibodies, 1:5,000 dilution; Amersham) for 1 h at room temperature, followed by ECL detection (Amersham).

**Assay of sGC activity.** Frozen cell preparations were thawed on ice and sonicated in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 10% glycerol, and protease inhibitor cocktail (Roche). The homogenates were centrifuged at 100,000 g for 1 h at 4°C, and the supernatant (20–80 μg protein/sample assayed using the Bio-Rad dye-binding method) was used to measure sGC activity. Incubation medium (final volume 50 μl) contained 50 mM triethanolamine-HCl buffer, pH 7.4, 5 mM MgCl₂, 1 mM 3-isobutyl-1-methylxanthine, 1 mg/ml BSA, 1 mM GTP, 5 mM creatine phosphate, and 50 μM/ml creatine kinase. Reactions were started by addition of the cell extract, followed with or without the NO donor diethyline (DEA)-NO (100 μM). Samples were incubated for 10 min at 37°C and boiled for 2 min, and the amount of cGMP formed was determined by performing a radioimmunoassay, with the results expressed as picomolar cGMP formed per minute per milligram of protein (7).

**Transient transfection of RFL-6 cells.** RFL-6 cells were plated in six-well dishes such that their confluence reached ~80% after 18–24 h in maintenance medium. After this incubation, each well was transfected with 1 μg of DNA and 3 μl of Fugene-6 (Roche Diagnostics, Indianapolis, IN) per well according to the manufacturer's protocol. Four hours after addition of the transfection reagent and the DNA to each well, the medium was aspirated and new medium was added. Cells were collected for RNA isolation after 24 h. Using a GFP expression construct, we determined the transfection efficiency to be ~35–45%.

**Statistical analyses.** Data are presented as means ± SE or SD. ANOVA was used for all analyses, followed by the Scheffé’s post hoc test for comparison between groups.

**RESULTS**

SP-600125 blocks NGF-mediated decrease of sGCα₁ mRNA in PC12 cells. Previously, it was shown that NGF-mediated decrease of sGCα₁ mRNA in PC12 cells likely occurred through a Ras-dependent pathway (17). To determine whether the effect of NGF on sGCα₁ mRNA levels was dependent on activation of one of the Ras effector MAPK signaling pathways, we pretreated PC12 cells with 50 μM SP-600125, PD-98059, or SB-203580 30 min before the administration of NGF (50 ng/ml) for 4 h. This concentration (or lower), under similar conditions, has been shown to be effective for each inhibitor to block JNK, ERK, and p38 signaling (15, 18, 21, 25, 31). After incubation with each selective inhibitor, the cultures were collected and analyzed for sGCα₁ mRNA expression levels using RT-PCR. As shown in Fig. 1A, NGF led to an ~80% decrease in sGCα₁ steady-state mRNA levels as previously reported (17). Pretreatment with each MAPK pathway inhibitor revealed that while neither PD-98059 nor SB-203580 affected the decrease in sGCα₁ mRNA by NGF, SP-600125 completely blocked the effects of NGF. A concentration-dependent response was evident after pretreating PC12 cells with varying concentrations of SP-600125 before the addition of NGF to the culture medium and sGCα₁ mRNA determination (Fig. 1B). We found that a concentration as low as 5 μM was able to block the effects significantly and that 20 μM could completely block the effects of NGF on the steady-state levels of sGCα₁ mRNA. At 50 μM, we consistently measured levels of sGCα₁ mRNA above the levels in control (i.e., vehicle treated) cultures. We attribute this finding to the accumulation of ongoing transcription of the sGCα₁ gene, while the degradation of the preexisting transcripts, which normally proceeds in the absence of SP-600125, was halted.

We also confirmed that JNK signaling was stimulated by NGF in PC12 cells, shown previously, by measuring the phosphorylation of the downstream substrate, c-Jun (16). Figure 1C, top, demonstrates that considerable phosphorylated c-Jun was detected in basal conditions and that, after 30 min of NGF treatment to PC12 cells, there was increased detection of phosphorylated c-Jun proteins, which were identified using (in combination) phospho-specific (Ser63 and Ser73) c-Jun anti...
bodies. The NGF-induced phosphorylated bands were not present when cells were pretreated with SP-600125. The inhibitor blocked the stimulation of this signaling pathway by NGF in PC12 cells. Figure 1C, bottom, shows that total c-Jun levels also appeared to increase slightly after NGF treatment, similar to the findings in a previous report by Leppä et al. (16).

SP-600125 blocks the NGF-mediated decrease of sGCα1 protein and activity levels in PC12 cells. Because SP-600125 blocked the decrease of sGCα1 mRNA expression by NGF, we were curious to determine whether this was also reflected in the sGCα1 protein expression and, importantly, in NO-stimulated sGC activity. PC12 cells were pretreated with SP-600125 and stimulated with NGF for 24 h, a duration within which sGC protein and activity levels were decreased (17). Figure 2A shows that after a 24-h incubation with NGF, sGCα1 protein expression levels were noticeably decreased, an effect that was blocked by SP-600125. Densitometry results from three independent Western blot experiments revealed that NGF treatment resulted in the significant decrease of sGCα1 protein to ~60% of the controls, which were completely inhibited and even slightly increased in the presence of SP-600125 (Fig. 2B).

We measured both basal and NO-stimulated sGC activity in PC12 cell lysates treated with NGF, SP-600125, and the combination of these agents. Basal sGC activity was slightly decreased compared with control cultures in cells treated with

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Fig. 1. The JNK inhibitor antral[1,9-cd]pyrazol-6(2H)-one (SP-600125) blocks the decreased expression of soluble guanylyl cyclase (sGC) α1-subtype mRNA by nerve growth factor (NGF) in rat pheochromocytoma (PC12) cells. A: PC12 cells were pretreated with 50 μM MAPK pathway inhibitors SP-600125 (SP), SB-203580 (SB), or PD-98059 (PD) alone for 30 min before the addition of NGF or DMSO vehicle control (CTL) for 4 h. Cells were collected and analyzed for sGCα1 mRNA using real-time RT-PCR normalized to total RNA and the housekeeping gene 36b4 and presented as the mean percentage ± SD of three independently treated groups of cells. *P < 0.05, significantly different from control; ANOVA. B: PC12 cells were pretreated with various concentrations of SP-600125 30 min before the addition of NGF, NGF alone, 50 μM SP-600125 alone, or DMSO vehicle control for 4 h and analyzed for sGCα1 mRNA normalized to total RNA and the housekeeping gene 36b4 and are presented as the percentage of control ± SD of three independently treated groups of cells. #P < 0.05, significantly different from NGF-treated cells; ANOVA. C: PC12 cells were pretreated with 50 μM SP-600125 30 min before the addition of NGF, NGF alone, SP-600125 alone, or DMSO vehicle control for 30 min and analyzed for phosphorylated c-Jun (p-c-Jun) and total c-Jun levels in total cell extracts using Western blot analysis with anti-phospho-specific and total c-Jun antibodies.

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Fig. 2. Effect of SP-600125 on the NGF-mediated decrease of sGCα1 protein expression. A: PC12 cells were treated with SP-600125 (50 μM) alone, SP-600125 30 min before the addition of NGF, NGF alone, or DMSO vehicle control for 24 h. Cells were collected and analyzed for sGCα1 (A, top) and GAPDH (A, bottom) protein expression levels using SDS-PAGE and Western blot analysis. B: densitometric analyses were performed, and the results are expressed as means ± SE from three separate experiments. The control was designated as 100% for each experiment. *P < 0.05, significantly different from control; ANOVA.
NGF for 24 h. Treatment of cultures with SP-600125 both alone and before the addition of NGF resulted in significantly higher (twofold) basal sGC activity levels (Fig. 3A). Similar to the report of Liu et al. (17), we found that NO-stimulated sGC activity significantly decreased in lysates from NGF-treated PC12 cells to almost 50% of the control lysates. This decreased activity was completely blocked when cells were pretreated with SP-600125 (Fig. 3B).

To help determine whether the increased sGC activity observed with SP-600125 in the basal state (Fig. 3A) was due to increased specific activity or to the amount of sGC enzyme, we used the ratios of the average amount of protein levels (Fig. 2B) to the average sGC activity for each treatment. Results of three independent experiments are shown and expressed as means ± SE, with the control designated as 100% (A and B) or 1 (C). As a reference, sGC activity levels for the control in A were 22.4 and the control in B were 2,619 pmol·min⁻¹·mg of protein⁻¹. *P < 0.05, significantly different from control; ANOVA.

Fig. 3. Effect of SP-600125 on basal and nitric oxide (NO)-stimulated sGC activity in PC12 cell lysates in vitro. PC12 cells were treated with SP-600125 (50 μM) alone, SP-600125 30 min before the addition of NGF, NGF alone, or DMSO vehicle control for 24 h. Cell lysates were prepared and sGC activity was assayed in the absence (A) or presence (B) of NO donor diethylamine (DEA)-NO (100 μM). C: specific sGC activity using the ratios of the average sGC protein levels (Fig. 2B) to the average sGC activity for each treatment.

SP-600125 does not block forskolin-mediated decrease of sGCα1 mRNA levels in PC12 cells. Forskolin treatment of various cell types, including PC12 cells, causes decreased sGC mRNA expression through a cAMP-dependent mechanism (17, 20). As shown in Fig. 4, SP-600125 had no effect on the decrease in sGCα1 mRNA by forskolin. This finding demonstrates that the effects of SP-600125 in blocking sGCα1 regulation are specific to a NGF-stimulated pathway in PC12 cells. Because there was no increase in sGCα1 mRNA, this also supports the hypothesis that the increased mRNA levels with SP-600125 described in Fig. 1 are not likely due to nonspecific induction of sGCα1 gene transcription.

TNF-α and IL-1β decrease sGCα1 mRNA levels in RFL-6 cells, which are blocked by SP-600125. Takata et al. (28) demonstrated that the combination of the inflammatory cytokines TNF-α and IL-1β caused sGCα1 mRNA levels to decrease in pulmonary artery smooth muscle cells. We recognized that the combination of treatments of these cytokines also characteristically stimulates the JNK signaling pathway (30). To determine whether SP-600125 could block TNF-α/IL-1β-mediated decrease of sGCα1 mRNA levels, we used a rat fetal lung (RFL-6) fibroblast cell line. As shown in Fig. 5A, TNF-α/IL-1β treatment of RFL-6 cells for 6 h resulted in the
decrease of sGCα1 mRNA levels (45% of control) compared with control cultures. Similarly to the effects of NGF in PC12 cells, the decrease of sGCα1 mRNA by TNF-α/H9251/IL-1/H9252 stimulation could be prevented by pretreatment of RFL-6 cells with SP-600125.

TNF-α/H9251/IL-1/H9252 treatment of RFL-6 cells resulted in the activation of JNK signaling, which was identified by the migratory shift during SDS-PAGE of the phosphorylated forms of c-Jun after 3 h using anti-c-Jun antibodies (Fig. 5 B, top). These phosphorylated forms of c-Jun were not observed when the RFL-6 cultures were pretreated with SP-600125. The migratory shift of the c-Jun bands in Fig. 5 B, top, made identification of phosphorylated c-Jun possible without using phospho-specific antibodies. However, in PC12 cells (Fig. 1 C), we did not observe any migratory shift using anti-c-Jun antibodies and therefore used phospho-specific antibodies to visualize the phosphorylation effects. This finding could be due to the different durations of the different treatments (30 min for NGF, 3 h for TNF-α/IL-1β) or to the fact that these are two separate paradigms in which c-Jun phosphorylation was observed, resulting in different forms of modified c-Jun.

Expression of a constitutively active JNKK2-JNK1 fusion protein causes sGCα1 mRNA levels to decrease in RFL-6 cells. To investigate whether JNK signaling may lead to inhibition of sGCα1 mRNA expression in RFL-6 cells, we used a gene delivery approach with a plasmid containing a constitutively active JNKK2-JNK1 fusion protein, which specifically stimulates the JNK signaling pathway and not the ERK or p38 pathways (32). Therefore, we transiently transfected RFL-6 cells with the plasmid that expresses the JNKK2-JNK1 fusion protein or the parent plasmid, SRα, and collected samples for sGCα1 mRNA analysis after 24 h. Cultures transfected with the JNKK2-JNK1 fusion protein expressed 35% less endogenous sGCα1 mRNA transcripts compared with cells transfected with the parent control plasmid SRα (Fig. 6 A). In a parallel

![Fig. 5](http://ajpcell.physiology.org/)  
Fig. 5. TNF-α and IL-1β treatment to rat fetal lung (RFL-6) fibroblast cells causes sGCα1 mRNA levels to decrease, which is reversed by SP-600125. A: RFL-6 cells were pretreated with 50 μM SP-600125 30 min before the addition of TNF-α (100 ng/ml) and IL-1β (20 ng/ml), cytokines alone, SP-600125 alone, or DMSO vehicle control for 6 h and then were analyzed for sGCα1 mRNA using real-time RT-PCR, normalized to total RNA and the housekeeping gene 36b4, and presented as the mean percentage of control ± SD of three independently treated groups of cells. *P < 0.05, significantly different from control; ANOVA. B: RFL-6 cells were treated exactly as in A but were analyzed for phosphorylated c-Jun after 3 h using Western blot analysis with anti-c-Jun antibodies. GAPDH expression levels are shown as a loading control.

![Fig. 6](http://ajpcell.physiology.org/)  
Fig. 6. Expression of a constitutively active JNKK2-JNK1 fusion protein in RFL-6 cells causes sGCα1 mRNA levels to decrease. A: RFL-6 cells were transfected with either a plasmid that encodes active JNKK2-JNK1 fusion protein, a constitutively active ERK2 protein, or the control plasmids SRα or pCMV5, respectively. Cultures were collected after 24 h, analyzed for sGCα1 mRNA levels using real-time RT-PCR, normalized to the housekeeping gene 36b4, and presented as mean percentage of control ± SD of three independently transfected groups of cells. Nontransfected, cytokine-treated, and control cell groups from Fig. 5 A are also included for reference. *P < 0.05, significantly different from control and SRα; ANOVA. B: verification of expression of the JNKK2-JNK1 fusion protein and the constitutively active ERK2 (ERK2*) protein in transfected RFL-6 cells using anti-hemagglutinin and anti-ERK antibodies, respectively, for Western blot analysis. Endogenous ERK1 and ERK2 levels that were detected are also marked. C: identification of phosphorylated c-Jun levels in RFL-6 cells transfected with the JNKK2-JNK1 or SRα plasmids for 24 h using Western blot analysis with anti-c-Jun antibodies. GAPDH expression levels are shown as a loading control.
experiment, cells were transiently transfected with a plasmid that expressed a constitutively active ERK2 protein (5). Compared with the parent control plasmid, pCMV5, the endogenous sGCα1 mRNA expression levels were not significantly changed by constitutively active ERK2 expression after the 24-h transfection period (Fig. 6A). Considering that the transfection efficiency in RFL-6 cells was ~35–45%, we did not expect more of a decrease in sGCα1 mRNA expression in the cell preparations transfected with the JNKK2-JNK1 plasmid. During the transfection incubation, the cell number did not change among the cells transfected with JNKK2-JNK1, ERK2, or control plasmids, and the total RNA collected from cells also was not significantly different. Therefore, we do not think that cell viability was a factor in the changed expression level of sGCα1 mRNA. Both the constitutively active JNKK2-JNK1 and ERK2 plasmids were expressed at abundant levels in a parallel experiment in which they were detected using anti-HA (for the JNKK2-JNK1 fusion protein) and anti-ERK2 (for the active ERK2 protein) antibodies (Fig. 6B).

Phosphorylation of c-Jun was present in cells transfected with the JNKK2-JNK1 expression plasmid compared with the SRα control plasmid (Fig. 6C). These data suggest that through exogenous expression, the expression of endogenous sGCα1 mRNA can be decreased by stimulating JNK signaling.

DISCUSSION

The NO-cGMP signaling pathway is known to be regulated not only at the level of enzyme activity of sGC by NO but also at the level of mRNA for the sGCα1- and β1-subunits. Although numerous treatments and disease models in tissue cultures or animals have been used to describe sGC regulation, very little is known about how to control this phenomenon. Preservation of sGC levels and activity could possibly be useful in disease states such as hypertension, Alzheimer’s dementia, atherosclerosis, and inflammation, all of which appear to correlate with decreased sGC expression (3, 9, 19, 24, 28).

Previously, it was shown that NGF treatment of PC12 cells resulted in the inhibition of sGC expression, which did not occur in a stable PC12 cell line that expressed a dominant inhibitory Ras mutant protein (17). Ras-dependent cell signaling has been identified as having the ability to proceed through any of the downstream MAPK effectors, including ERK, JNK, or p38 (29). Herein we have shown that not only was sGC downregulation blocked by SP-600125 but also expression of a constitutively active JNK enzyme caused sGC downregulation. We also have collected data indicating that UV radiation, a common stress signal known to activate JNK signaling (23), caused sGCα1 mRNA levels to decrease after 4 h (data not shown). It is interesting that many of the conditions that lead to sGC mRNA regulation also have been shown to stimulate the JNK signaling pathway, including NGF, estradiol, and the cytokines TNF-α and IL-1β (16, 22, 30). Our results suggest that JNK signaling could be important in the regulation of sGC, but more work needs to be done to expand on the data reported herein. The specificity of SP-600125 to the JNK signaling pathway has been challenged (1), and we have not ruled out the contribution of a nonspecific effect of this compound. Because so little is known about the pathways involved in sGC mRNA regulation, additional studies are required to determine whether a nonspecific effect independent of JNK could contribute to sGC regulation.

It is reasonable to predict that sGC gene regulation will prove to be complex, dependent not only on the stimulation but also on the cell or tissue type as well as the intracellular machinery. For instance, estradiol treatment of rats caused sGC regulation in the uterus, but not in several other tissues known to contain estrogen receptors (13). Each cell may also contain more than one signaling pathway that can facilitate sGC regulation, exemplified in this study by the ability of SP-600125 to block sGC regulation by NGF but not by forskolin. It is possible that distinct pathways that regulate sGC expression could converge on a common downstream mechanism. Of note, both cGMP- and cAMP-mediated sGC mRNA regulation was shown to involve the decreased expression of the RNA binding protein HuR (10, 11). However, cGMP does not necessarily cause sGC regulation in all cell types, such as PC12 cells (17). We analyzed HuR protein expression levels in RFL-6 cells treated with either forskolin or TNF-α/IL-1β after 4 and 8 h of treatment. Our findings indicate that HuR protein levels did not decrease in RFL-6 cells in these conditions. This finding could imply that HuR may not have been involved in sGC regulation in this study. Importantly, these studies were performed in a different cell type that may use distinct signaling pathways.

The complexity and importance of sGC gene regulation will probably become apparent as more studies are conducted. For example, while our present study focused on the regulation of the sGCα1-subunit, there are three additional sGC isoforms, including α2, β1, and β2. Whether these isoforms are regulated in a similar manner remains to be resolved. It would be interesting to determine whether preservation of sGC expression could be achieved using SP-600125 in the setting of disease states in which sGC downregulation is found, such as β-amyloid deposition in Alzheimer’s disease (3). JNK pathway inhibitors are already being tested for their therapeutic potential in neurodegenerative and inflammatory conditions (4). Work by Fiscus et al. (6) suggests that preservation of cGMP levels in neuronal cell types may play a role in the survival of these cells and could be a mechanism that contributes to the beneficial effects of JNK pathway inhibitors.

We hope that the data described herein will shed some light on the signaling mechanisms of sGCα1 gene regulation. In addition, the SP-600125 compound will provide a useful tool to study the intracellular components involved in sGC regulation in culture models, which may help to improve the understanding of the role of sGC in the various disease states mentioned above.

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