Characterization of a Novel Type of Endogenous Activator of Soluble Guanylyl Cyclase*

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Nitric oxide (NO) remains the only firmly established endogenous modulator of soluble guanylyl cyclase (sGC) activity, but physiological, structural, and biochemical evidence now suggests that in vivo regulation of sGC involves direct interaction with other factors. We searched for such endogenous modulators in human umbilical vein endothelial cells and COS-7 cells. The cytosolic fraction of both cell types stimulated the activity of semipurified sGC severalfold in the absence or presence of a saturating concentration of NO. The cytosolic factor was sensitive to proteinase K and destroyed by boiling, suggesting that it contains a protein component. Size exclusion chromatography revealed peaks of activity between 40 and 70 kDa. The sGC-activating effect was further purified by ion exchange chromatography. In the presence of the benzylindazole YC-1 or NO, the partially purified factor synergistically activated sGC, suggesting that this factor had a mode of activation different from that of YC-1 or NO. Four candidate activators were identified from the final purification step by matrix-assisted laser desorption ionization mass spectrometry analysis. Using an sGC affinity matrix, one of them, the molecular chaperone Hsp70, was shown to directly interact with sGC. This interaction was further confirmed by co-immunoprecipitation in lung tissues and by co-localization in smooth muscle cells. sGC and Hsp70 co-localized at the plasma membrane, supporting the idea that sGC can be translocated to the membrane. Hsp70 co-purifies with the sGC-activating effect, and immunodepletion of Hsp70 from COS-7 cytosol coincided with a marked attenuation of the sGC-activating effect, yet the effect was not rescued by the addition of pure Hsp70. Thus, Hsp70 is a novel sGC-interacting protein that is responsible for the sGC-activating effect, probably in association with other factors or after covalent modification.

The cellular processes that are regulated by NO are central to many aspects of biology and disease, particularly in the cardiovascular and central nervous system. Despite the widely recognized importance of NO, little is known about the mechanism of regulation of the NO receptor, the soluble guanylyl cyclase (sGC)1 (1–3). sGC is a heterodimeric enzyme formed by α and β subunits, the latter containing the heme where NO binds. Upon binding of NO, activity of sGC increases several hundred-fold over basal levels to produce the second messenger cGMP from the substrate GTP (4, 5).

Conflicting data have emerged from the studies of mechanisms of regulation of sGC in vivo and in vitro. In vivo, the rate of dissociation of NO from the heme occurs in minutes, perhaps seconds (6), whereas in cerebellar cells, dissociation is 25-fold faster (7, 8). Similarly, desensitization of sGC has been characterized in vivo but has not been observed with the purified form of the enzyme (9). These discrepancies between in vivo and in vitro data suggest the involvement of endogenous modulators of sGC. Several years ago, an allosteric inhibitor of sGC was isolated from bovine lung, but its identity has yet to be determined (10).

Significant progress in understanding the regulation of sGC was achieved with the discovery of YC-1, a benzylindazole first identified by its capacity to increase the production of cGMP in intact platelets (11). YC-1 synergistically activates sGC in the presence of NO, apparently by slowing down the dissociation rate of NO from the heme and by increasing the efficacy of NO stimulation (6, 12). YC-1 can also stimulate the sGC activity independently of NO (13). Furthermore, YC-1 has the ability to potentiate stimulation of sGC by carbon monoxide (CO) (13, 14). CO by itself activates sGC poorly, but in the presence of YC-1, it stimulates sGC as much as NO. These observations raise the possibility that endogenous analogs of YC-1 could enhance responsiveness to NO, activate sGC independently of NO, or potentiate CO activation, providing a cGMP-signaling pathway distinct from that mediated by NO. Recently, studies reported the existence in endothelial cells of a heat-labile activator of sGC (15). However, its cGMP-promoting effect was dependent on YC-1.

We describe herein a search for endogenous modulators that led to the identification of an interaction between sGC and the molecular chaperone Hsp70.

EXPERIMENTAL PROCEDURES

Cell Culture

Human umbilical vein cells (HUVEC) were grown in endothelial cell basal medium prepared with the EGM Bulletkit (Clonetics). COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum (ICN) and 1% penicillin/streptomycin/amphotericin (Mediatech). Human umbilical vein (HUVEC/CC-2519) cells were from BioWhittaker, and COS-7 cells (CRL-1651) and rat smooth muscle cells (CRL-2018) were purchased from ATCC. SF21 cells (IPLB-SF21 Clontech) were cultured in S900-II SFM medium

S-nitroso-N-acetylpenicillamine; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DTT, dithiothreitol; HUVEC, human umbilical vein cells; β-ME, β-mercaptoethanol; IBMX, 3-isobutyl-1-methylxanthine; FPLC, fast protein liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MS, mass spectrometry; PDI, protein-disulfide isomerase; RIA, radioimmunoassay.

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1 The abbreviations used are: sGC, soluble guanylyl cyclase; SNAP,
**Preparation of Cytosolic Fractions**

Cells were grown in 100-mm dishes until 90–100% confluence. HUVEC cells were used at passages 2–10. Cells were carefully washed four times with 8 ml of 1 ml of dithioreitol (DTT) before being scraped from plates and sonicated in the same buffer. The cytosolic fraction was separated from membranes by centrifugation at 16,000 × g for 10 min at 4 °C. Protein concentration was estimated by Bradford assay (Sigma) using bovine serum albumin as a standard. Cytosols of HUVEC and COS-7 cells were diluted to 0.3 and 1 mg/ml, respectively.

**sGC Expression and Purification**

Viruses containing the rat α1 subunit and β1 subunit, the latter carrying six histidines at its carboxyl terminus, were generated. The α1/β1-His6-sGC was expressed in an SF21/baculovirus system and purified using Talon cobalt resin (Clontech) followed by MonoQ 5/5 FPLC (Amersham Biosciences), as previously described (12). Fractions with the highest sGC activity were pooled in 10% glycerol and 5 mM DTT and snap frozen.

**sGC Activity Assay**

sGC activity was determined by formation of [α-32P]cGMP from [α-32P]GTP, as previously described (12). Reactions were performed for 10 min at 30 °C in a final volume of 100 μl of HEPES, pH 8.0, reaction buffer containing 500 μM GTP, 1 mM DTT, and 5 mM MgCl2. For initial characterization, the NO donor Glyco-SNAP-2 at 100 μM (Calbiochem) was used to stimulate the sGC activity. To determine the mechanisms of activation of the partially purified factor, the NO donor SNAP (Calbiochem) was used at a lower concentration (1 μM). Typically, 5 μl of purified sGC (10 ng/μl) was used in each assay reaction. No variation in the pH of the reaction (pH 8.0) was observed after the addition of the extract and completion of the enzymatic assay.

For initial characterization, the NO donor SNAP at 100 μM was used to determine the sGC-purified preparation did not contain DTT or glycerol. To study the direct interaction between the partially purified factor, NO concentrations-response curves of the sGC activity were measured in the presence of nine different concentrations of the NO donor SNAP (from 0.03 to 300 μM). The NO concentration-response curve was similarly present in the presence of 10 μg of COS-7 cytosol. All concentration-response experiments were performed in duplicate, and each experiment was repeated three times. The EC50 values were calculated from these concentration-response curves and corresponded to the concentration of SNAP that half-maximally activates the enzyme.

To investigate the effect of calcium, 5 mM EGTA was added to reaction buffer containing 9 mM MgCl2. Free calcium concentrations were calculated using Maxchelator software.

**Protease Treatment**

Cytosolic extracts (1 mg/ml) were incubated with 200 μg/ml proteinase K (Invitrogen) at 50 °C for 30 min. The reactions were stopped by the addition of 5 mM phenylmethylsulfonyl fluoride (Sigma). We determined that phenylmethylsulfonyl fluoride does not interfere with sGC activity (data not shown).

**Characterization of the Cytosolic sGC-activating Effect**

To determine whether the sGC-activating effect was independent of NO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; Calbiochem) was used as NO scavenger at 450 μM. We verified that PTIO lowered GC activity stimulated with 1 μM SNAP to its basal level.

**Gel Filtration**

For gel filtration chromatography, Sephacryl S-100 HR 16/60 column (Amersham Biosciences) was used. Proteins were eluted with 50 mM HEPES, pH 8.0, buffer containing 150 mM NaCl using FPLC at 0.75 ml/min. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), and myoglobin (17.5 kDa) from Sigma were used as standards.

**sGC-Hsp70 Interaction Studies**

Affinity Binding—1 μg of purified sGC-His6-tag and 400 μg of desalted COS-7 cytosol were mixed for 1 h at 4 °C. 100 μl of Talon resin equilibrated in 50 mM HEPES, pH 8.0, containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 35 μg/ml phenylmethylsulfonyl fluoride) and 0.015% β-ME were added to samples and incubated for 20 min at 4 °C. Resin was collected in HandeeSpin Cup Columns (Pierce) by centrifugation at 1000 × g for 1 min and washed twice with 1 ml of wash buffer containing 0.5 mM NaCl, 50 mM Tris-HCl, pH 8.0, protease inhibitors, and 0.015% β-ME and once with the same buffer containing 50 mM NaCl. The resin bound complex was eluted with 100 μl of 150 mM imidazole buffer, pH 8.0, containing 50 mM NaCl. Eluted proteins were resolved on 10% SDS-PAGE and analyzed by immunoblotting with anti-Hsp70 (mouse monoclonal; Abcam), anti-β1 subunit of sGC (rabbit polyclonal; kindly provided by Dr. David L. Garbers), anti-protein-disulphide isomerase (mouse monoclonal; Abcam), and anti-βγ actin (rabbit polyclonal; Abcam). To measure the activity of the eluted samples, the same protocol was used, but the resin-bound complex was eluted with 50 mM EDTA in a 25 mM Tris, pH 8.0, buffer containing 50 mM NaCl and 0.015% β-ME. For these affinity experiments, the sGC-purified preparation did not contain DTT or glycerol.

To study the direct interaction between pure Hsp70 (Stressgen) and sGC, the above protocol was modified as follows: 1 μg of purified sGC-His6-tag was passed through 200 μl of Talon cobalt resin equilibrated with 2 ml of PBS. The column was washed twice with 50 mM Tris-HCl, pH 8.0, buffer containing 50 mM NaCl prior to applying 2 μg of pure Hsp70. The column was washed twice with 50 mM Tris-HCl, pH 8.0, buffer containing 500 mM and 150 mM NaCl. Proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 50 mM NaCl and 50 mM EDTA.

**Co-immunoprecipitation**—Rat livers were minced and then disrupted by Dounce homogenizer in 10 ml of cold lysis buffer: PBS buffer containing protease inhibitors, 1 mM DTT, 1 mM EDTA, and 150 mM NaCl. Lysates were centrifuged at 10,000 × g for 10 min and then at 100,000 × g for 1 h at 4 °C. The protein concentration of lung lysates was between 10 and 12 mg/ml. If not used fresh, rat lung lysates were snap frozen and stored at −80 °C. Lung lysates (2 mg) were precleared with Protein A-Sepharose 4B beads, following the supplier’s protocol (Amersham Biosciences). Preeclared lung lysates were incubated with nonimmune serum or mouse monoclonal anti-Hsp70 (1 μg/ml lung extract) for 1 h at 4 °C. Protein A beads were added to samples for 1 h at 4 °C. Beads were pelleted by centrifugation at 12,000 × g for 20 s and
washed in three alternating cycles of lysis buffer containing 1 M NaCl and no NaCl. Proteins were eluted in 1% SDS, 100 mM DTT and incubated at 85 °C for 5 min. Samples were resolved on 10% SDS-PAGE and analyzed by immunoblotting with anti-β1 subunit of sGC.

Immunodepletion—Typically, 100 μg of desalted COS-7 cytosol was precleared with Protein A-Sepharose and IgG (1:100 dilution) prior to incubation with 4 μl of anti-Hsp70 (serial dilutions 1:5 to 1:100) or with nonimmune serum (0.2 μg/ml of cytosol) overnight at 4 °C. Complexes were immunoprecipitated with 100 μl of protein A-Sepharose 4B, as above. Supernatants were tested for their effects on sGC activity. To assess the efficiency of immunodepletion, supernatants were resolved on SDS 10% SDS-PAGE and analyzed by immunoblotting with anti-Hsp70.

Effect of Purified Hsp70 on sGC Activity—50 ng of sGC and various concentrations (10, 50, or 100 ng) of purified Hsp70 (Stressgen) were incubated at room temperature for 5 min. sGC activity was then measured in basal and NO-stimulated conditions and in the absence or presence of 100 μM ATP, since some functions of Hsp70 involve ATPase activity (18).

Immunocytochemistry and Confocal Microscopy—Smooth muscle cells (CRL-2018; ATCC) were plated on round 18CIR coverslips in 12-well tissue culture plates. The coverslips were fixed in 4% paraformaldehyde, washed three times in 1–2 ml of PBS for 5 min, and then permeabilized with 100% acetone for 10 min at −20 °C. The cells were washed three times in PBS and incubated in a humidified container for 30 min at room temperature in blocking solution (PBS containing 2% normal goat serum and 1% bovine serum albumin (BSA)) to prevent nonspecific binding of the primary antibodies. The coverslips were washed twice more in buffer (PBS containing 0.2% normal goat serum and 0.1% BSA). The primary antibodies against the β1 subunit of sGC (1:50 dilution) and Hsp70 (1:100 dilution) were diluted in the buffer and applied to the coverslips for a 2-h incubation at room temperature in a humidified container. Negative controls either lacked the primary antibodies (for anti-Hsp70), or a blocking peptide was applied (the C-terminal 15 amino acids for anti-Hsp70).

RESULTS

Search for a Cellular Modulator of sGC Activity—We started our search for an endogenous modulator of sGC activity in cytosolic fractions of HUVEC. These cells express components linked to the NO-eGMP pathway. Cytosolic fractions from

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**FIG. 1.** Effect of HUVEC and COS-7 cytosolic fractions on basal and NO-stimulated activity of the soluble guanylyl cyclase. A, 5 μl of semipurified sGC (50 ng) was incubated in the presence of 6 μg of HUVEC cytosol and was assayed for cGMP formation in basal (left panel) and NO-stimulated (right panel) conditions, as described under “Experimental Procedures.” B, same experiment as in A with 20 μg of COS-7 cytosolic fraction. The preparation of purified sGC used in this experiment was different from that shown in A. This accounts for the lower GC activity observed. For NO-stimulated conditions, the NO donor GlycoSNAP-2 was used at 100 μM. Results are the mean ± S.E. of three experiments, with each measurement performed in duplicate.

COS-7 cells were initially used as a negative control because they do not express detectable levels of nitric-oxide synthase or sGC. Cytosolic fractions of HUVEC and COS-7 cells were tested for their effect on the activity of semipurified sGC in basal and stimulated conditions. For stimulated conditions, the NO donor GlycoSNAP-2 was used at 100 μM to maximally activate the sGC (a plateau is reached at 10–30 μM with this NO donor) (12).

A strong stimulatory effect of HUVEC cytosolic fractions was observed on the basal and NO-stimulated activity of sGC as shown in Fig. 1A. 6 μg of HUVEC cytosol induced a 9-fold increase in basal activity of sGC and was assayed for cGMP formation in basal (left panel) and NO-stimulated (right panel) conditions, as described under “Experimental Procedures.” B, same experiment as in A with 20 μg of COS-7 cytosolic fraction. The preparation of purified sGC used in this experiment was different from that shown in A. This accounts for the lower GC activity observed. For NO-stimulated conditions, the NO donor GlycoSNAP-2 was used at 100 μM. Results are the mean ± S.E. of three experiments, with each measurement performed in duplicate.

**A:** HUVEC

**B:** COS-7 cells

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2 M. Condrescu, personal communication.
HUVEC cytosols were able to further increase maximally stimulated sGC activity constituted preliminary evidence that this activating factor may have a different mode of action than that of NO. The endogenous GC activity of the HUVEC cytosol was not significantly higher than the background of the ([α-32P]cyclase assay and remained unchanged in the presence of Glyco-SNAP-2 (Fig. 1A), indicating that endogenous sGC of HUVEC was not responsible for the observed severalfold increase in purified sGC activity.

Surprisingly, an equivalent enhancement of sGC activity was triggered by cytosolic extracts isolated from COS-7 cells, initially used as a negative control (Fig. 1B). Basal sGC activity was increased 8-fold in the presence of 20 μg COS-7 cytosol. When the enzyme was maximally stimulated with Glyco-SNAP-2, cGMP production by sGC was increased more than 2-fold by the addition of cytosol (Fig. 1B). COS-7 cells had no detectable endogenous GC activity under our assay conditions.

Stimulating effect of the cytosol on sGC activity was retained after centrifugation at 100,000 × g for 1 h and was absent from the membrane fraction, suggesting that the sGC activator is not tightly associated with membranes.

Several components in the complete medium used for cell cultures may have the potential to increase enzymatic activity. Thus, we measured the sGC activity in the presence of serial dilutions of the cell culture complete medium. No significant change in activity was observed (data not shown).

To further characterize this activating effect, we used cytosolic fractions of COS-7 cells, because they are easier to grow than HUVEC and do not express detectable levels of sGC or nitric-oxide synthase that could interfere with our assay, yet they seem to contain a strong sGC-activating component.

The sGC-activating Effect Is Dependent on Concentration of the Extract—Increasing concentrations of cytosol were added to the sGC reaction buffer, and activity of the purified sGC was measured in basal conditions and in the presence of submaximal concentration (1 μM) of the NO donor, SNAP (Fig. 2). In both basal and NO-stimulated conditions, increasing concentrations of COS-7 cytosol promoted increasing production of cGMP. In contrast to the initial screening, cytosols at the highest concentration induced a slightly larger increase in NO-stimulated GC activity than in basal GC activity, probably because the NO donor is used at submaximal concentration (1 μM versus 100 μM in the initial experiment). A previous study showed that BSA at 0.19 mg/ml induced an ~30% increase in sGC activity, probably by stabilization of the enzyme over the time of reaction (19). Thus, we repeated the same experiments in the presence of serial dilutions of BSA that roughly corresponded to the concentration of COS-7 cytosol used. We measured a slight increase of sGC activity (1.4-fold) that was essentially unchanged over a range of 0.0025–0.5 mg of BSA/ml and similar in basal and NO-stimulated conditions (Fig. 2).

Protease and Temperature Sensitivity—The cellular extract was subjected to digestion by proteinase K at 50 °C, as described under “Experimental Procedures.” We measured the sGC-activating effect of digested cytosol on basal and NO-stimulated activity of sGC. The proteinase K treatment abolished the cGMP-promoting effect (less than 1.5 ± 0.3% activity remaining), suggesting that the cytosolic factor has a protein component. We assessed the stability of the endogenous factor by subjecting the COS-7 cytosol to various temperatures for 15 min prior to the addition to the sGC assay mix (Table I). 50 °C treatment of the cytosolic extract did not significantly affect the cGMP-promoting effect. At 70 °C, 58 and 60% of the cGMP-promoting effect was retained in basal and NO-stimulated conditions, respectively. Treatment at 100 °C virtually eliminated the activating effect, indicating that the factor is heat-labile.

The sGC-activating Effect Is Independent of NO—To date, the only known endogenous activators of sGC are NO and CO. We wanted to exclude the possibility that the effect seen was due to an increase in basal activity of the sGC by atmospheric traces of NO. The NO scavenger, cPTIO, was added to the GC assay buffer, and the cytosol-activating effect was assessed in basal condition, as described under “Experimental Procedures.” In the presence of 450 μM cPTIO, the cytosolic extract was still able to stimulate, at a lower but significant level (p < 0.05), the basal GC activity (about 3-fold; Table I). 450 μM cPTIO completely scavenges 1 μM SNAP.

Because the sGC-activating effect was attenuated in the presence of a NO scavenger that removes potential traces of NO, we concluded that the factor is NO-labile.
Characterization of the cytosolic factor

In all experiments, NO donor SNAP was used at 1 μM. -Fold stimulation corresponded to the effect of 10 μg of cytosol on sGC activity. For temperature sensitivity, NO scavenger effect, and cGMP production measured by RIA, results are the mean ± S.E. of three experiments with each measurement done in duplicate; for Ca2+-independence, results are the mean ± S.E. of two experiments with each measurement done in duplicate. Variations seen in sGC activity and cytosol effects are probably due to the fact that different purified sGC preparations and several cytosolic batches were used. ND, not detectable.

| Temperature sensitivity | Percentage of activation remaining | % | % |
|-------------------------|-----------------------------------|---|---|
| Basal                  | NO-stimulated                     | ---| ---|
| 50 °C                  | 108.1 ± 7.0                      | 97.9 ± 2.1 |
| 70 °C                  | 58.6 ± 4.3                       | 60.0 ± 1.0 |
| 100 °C                 | 4.1 ± 1.1                        | 5.2 ± 0.5 |

| NO scavenger effect | Basal activity | -fold |
|---------------------|----------------|-------|
| sGC                 | 77 ± 8         |
| sGC + 10 μg of cytosol | 361 ± 73 |
| sGC + 450 μM cPTIO  | 75 ± 3         |
| sGC + 10 μg of cytosol + 450 μM cPTIO | 201 ± 19 |
| sGC + 450 μM cPTIO + 1 μM SNAP | 85 ± 7 |

| Ca2+-independence | Stimulation | -fold |
|--------------------|-------------|-------|
| Basal              | NO-stimulated | ---| ---|
| No EGTA            | 4.0 ± 0.3    | 4.7 ± 01 |
| 5 mM EGTA          | 4.2 ± 0.1    | 5.1 ± 0.4 |

| cGMP production measured by RIA | nmol cGMP min⁻¹ mg⁻¹ |
|---------------------------------|----------------------|
| Basal                           | NO-stimulated        | ---| ---|
| sGC                             | 96 ± 6               | 1270 ± 90 |
| sGC + 10 μg of cytosol          | 442 ± 28             | 4340 ± 301 |
| 10 μg of cytosol                | ND                   | ND |

NO (361 versus 201 nmol of cGMP min⁻¹ mg⁻¹; Table I), we asked whether one mechanism of activation of the cytosolic factor could be to potentiate the effect of NO. We conducted NO concentration-response curves in the absence or presence of 10 μg of COS-7 cytosol (see “Experimental Procedures”). As shown on Fig. 3, 10 μg of cytosol remarkably increased NO-stimulated GC activity and shifted the NO response curve to the left. This was reflected by a decrease in EC₅₀ for activation by NO (from 3.03 to 1.79 μM in the presence of 10 μg of cytosol), but this decrease did not reach significance (p = 0.067). The cytosol was further able to raise the plateau of maximal velocity of the NO-stimulated sGC (obtained at saturating concentration of SNAP) by more than 2-fold (p < 0.05).

Taken together, these results indicated that the cytosolic factor can increase basal sGC activity independently of NO and, in addition, can potentiate NO stimulation.

The sGC-activating Effect Is Independent of Calcium—To determine the potential calcium dependence of the effect, we measured the -fold stimulation of the cytosolic extract on the basal and NO-stimulated sGC activity in the presence of EGTA (while keeping constant the free magnesium concentration at 4 mM). There was no significant change (p > 0.5) in the activating effect in the presence of 5 mM EGTA (Table I).

The Activating Effect Is Not Due to Depletion of the Product of the Reaction cGMP or, Conversely, to Inhibition of Phosphodiesterase Activity—The velocity of an enzymatic reaction can be increased due to depletion of its product. In our GC assay, we measured the conversion of [α-32P]GTP to [α-32P]cGMP in the presence of an excess of cold GTP; thus, we needed to assess whether the depletion of cold cGMP was a factor. The addition of 250 μM IBMX, a nonspecific inhibitor of cyclic nucleotide phosphodiesterases, to the reaction buffer did not modify the activating effect of the cytosolic extracts. Because some phosphodiesterases might not be inhibited by IBMX, we also added increasing concentrations of cGMP to the reaction buffer (from 1 to 300 μM); the stimulating effect was not affected by any of the tested concentrations of cGMP (data not shown).

We also used RIA to test whether the observed effect was due to an unidentified artifact of the 32P assay. We measured the amount of cGMP produced by sGC under basal and stimulated conditions in the absence or presence of 10 μg of cytosolic fractions (see “Experimental Procedures”). The production of cGMP measured by RIA was remarkably similar to the amount of cGMP obtained with the 32P assay, confirming that the effect of the cytosolic fraction was to stimulate the production of cGMP by the sGC (Table I).

Although our experiments are carried out at saturated substrate concentration (0.5 mM GTP, 5 mM MgCl₂), we assessed whether GTP depletion could be a factor, and repeated the previous experiments in the presence of a regenerating system, as described under “Experimental Procedures.” There was no difference in the presence or absence of a GTP-regenerating system (data not shown).

The Major Peaks of Activity Elute between 40 and 70 kDa—To estimate the size of the endogenous activator, 2 ml of cytosolic fraction (1 mg/ml) was passed through a Sepharyl S-100 HR column (Fig. 4). We detected two major peaks of activity that eluted between BSA (66 kDa) and ovalbumin (45 kDa) and a minor peak of activity that eluted between ovalbumin and myoglobin (17.5 kDa). This estimation of the apparent molecular mass (between 40 and 70 kDa) is based on the assumption that the activating factor has a proteic component.

Partial Purification of the Endogenous Activator—Results of a three-step purification are summarized in Table II. The cytosol was first desalted with a PD-10 column (Sephadex G25). After desalting, COS-7 cytosolic fractions were applied to DEAE-Sephadex. Fractions containing the activity were pooled, dialyzed, and subjected to ion exchange chromatography on a MonoQ FPLC column. The activity was eluted with a NaCl gradient, recovered in one peak at a salt concentration of 350–375 mM (fractions 19–21), and desalted. From this final step, we calculated that the sGC-activating effect was purified

![Graph](http://www.jbc.org/)
44.5- and 71.6-fold for basal and NO-stimulated conditions, respectively. The reason for the -fold purification difference between the basal and NO-stimulated conditions is unknown. One possible explanation is that the cGMP-promoting effect is due to more than one endogenous factor and that one of these factors may act in combination with NO. Another possibility is that there is only one endogenous activator but it displays heme-dependent and heme-independent activities, similar to the benzylindazole activator YC-1 (20).

Characterization of Some Mechanisms of Activation of the Partially Purified Factor—
Mechanisms of activation were determined using pooled and desalted MonoQ fractions from the final step of purification.

**YC-1 and the Endogenous Factor Activate sGC by a Distinct Mechanism**—The basal and NO-stimulated sGC activity was assessed in the absence and presence of the partially purified factor (0.5 μg) and in combination with 10 μM YC-1 (Fig. 5). As previously reported, YC-1 increased both basal and NO-stimulated activity. The desalted MonoQ fraction induced a 4-fold increase in basal activity and synergistically activated the NO-stimulated GC activity (5-fold). The combination of YC-1 and the purified fraction had a synergistic effect on the basal activity and an additive effect on NO-stimulated activity, respectively. These results suggest that the cellular factor has a mechanism of activation different from that of YC-1 and NO. These synergistic and additive effects on basal and NO-stimulated GC activity were also observed when YC-1 was combined with dialyzed cytosolic extract (data not shown). The synergistic effect on basal activity suggests that YC-1 and the endogenous activating factor can potentiate each other’s effect. However, only an additive effect was observed in the presence of

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**TABLE II**

Partial purification of the endogenous activator

All fractions were assessed for their ability to increase the activity of 50 ng of purified sGC in the absence (basal) or presence of 1 μM SNAP. One unit is defined as the amount of material that increases sGC activity by 1 nmol of cGMP/min/mg. Results are representative of four different purifications with similar results. The different steps of purification are described under “Experimental Procedures.”

| Fraction                | Protein recovered (mg) | Total activity (units) | Percentage recovery (%) | Specific activity (units/μg) | Purification (Basal/SNAP) |
|-------------------------|------------------------|------------------------|-------------------------|-----------------------------|--------------------------|
| Cytosol                 | 13.30                  | 96,599                 | 100                     | 7.3                         | 1                        |
| After desalting         | 6.48                   | 64,399                 | 66.7                    | 9.9                         | 1.4/1.6                  |
| After DEAE-Sephadex     | 0.24                   | 21,960                 | 22.7                    | 91.5                        | 12.5/21.0                |
| After MonoQ (desalted)  | 0.05                   | 16,250                 | 16.8                    | 325.0                       | 44.5/71.6                |

44.5- and 71.6-fold for basal and NO-stimulated conditions, respectively. The reason for the -fold purification difference between the basal and NO-stimulated conditions is unknown.

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**FIG. 5.** The endogenous factor and YC-1 have different modes of activation. The partially purified factor (~0.5 μg) was assessed for its effect on basal (left panel) and NO-stimulated (right panel) GC activity in combination with 10 μM YC-1. SNAP was used at 1 μM. Results are the mean ± S.E. of three independent experiments, with each measurement done in duplicate.
terminus of the sGC and the candidate activators. Since the carboxyl by investigating the potential protein-protein interaction be-

FIG. 6. The endogenous factor increases CO-stimulated GC activity. The effect of the endogenous factor was assessed in the presence of CO, as described under “Experimental Procedures.” The sGC activity was assayed in the presence of saturated concentration of CO, MonoQ fraction (0.9 μg), and both. As a control, sGC activity was also assayed in the presence of 10 μM YC-1 and a saturated concentration of CO. Results are the mean ± S.E. of two experiments, done in duplicate.

NO, as if NO could block the synergy between YC-1 and the endogenous activator.

The Endogenous Factor Increases CO-stimulated GC Activity—CO, by itself, poorly increases the catalytic activity of sGC (less than 5-fold). However, a combination of YC-1 and CO is known to synergistically increase catalytic activity (13). We wanted to determine whether the endogenous factor had a similar effect on CO-stimulated GC activity. As shown on Fig. 6, the purified fraction further increased the maximally CO-stimulated GC activity (~3-fold), but this effect was not as drastic as YC-1 effect (~10-fold).

Identification of Candidate Activators in the MonoQ Fraction—To determine the identity of the factor(s), the final step of purification was subjected to MALDI MS analysis, as described under “Experimental Procedures.” In the range of 40–70 kDa, seven bands were detected, and four of them were identified by MALDI-TOF analysis as Hsp70, protein-disulfide isomerase (PDI), and β and γ actin (Fig. 7).

Hsp70 Directly Interacts with sGC in Vitro—We proceeded by investigating the potential protein-protein interaction between the sGC and the candidate activators. Since the carboxyl terminus of the β subunit of the semipurified sGC contains a His tag, we used cobalt resin to create a sGC affinity matrix (see “Experimental Procedures”). Washes were done at high stringency (150 and 500 mM NaCl) to ensure specificity of the interaction between the potential candidates and sGC. Immunoblot analysis showed that Hsp70 from COS-7 cytosol specifically interacts with sGC bound to the cobalt resin, since no Hsp70 was detected in the presence of resin but absence of sGC (Fig. 8A, panel 1). The other candidate activators, PDI and β-γ actin, did not seem to specifically interact with sGC (Fig. 8A, panels 2 and 3). In parallel, we assessed the activity of the eluates from the sGC affinity matrix. We measured the basal and NO-stimulated GC activity in fractions eluted from resin-bound sGC mixed with buffer only or with cytosolic extract, as described under “Experimental Procedures.” Eluates from sGC mixed with cytosolic extract exhibited 3- and 5-fold higher basal and NO-stimulated activity, respectively, than eluates without cytosol (Fig. 8, panel 5). To determine whether Hsp70 interacts directly with the sGC and not through intermediate proteins present in the cytosol, we repeated the same sGC affinity experiment using pure Hsp70. As shown by immunoblot analysis of the eluates (Fig. 8B), Hsp70 is specifically retained by the sGC affinity matrix, whereas no Hsp70 can be detected in the absence of sGC bound to the resin.

Co-immunoprecipitation in Lung Tissues and Co-localization in Smooth Muscle Cells Suggest That Hsp70 and sGC Interact in Vivo—To determine whether this association between Hsp70 and sGC exists in tissues, we conducted co-immunoprecipitation experiments in rat lung tissues (see “Experimental Procedures”). Rat lungs were used because they express a high amount of the NO-cGMP pathway components. Immunoprecipitates of Hsp70 from lung lysates contained sGC, whereas immunoprecipitates of nonimmune antisera did not, as shown in Fig. 9A. This result suggests that sGC and Hsp70 are capable of forming a complex in lung tissues. To determine whether the sGC-Hsp70 interaction could be of physiological relevance, we assessed the localization and distribution of sGC and Hsp70 in the smooth muscle cell line CRL-2018, using confocal microscopy. Immunostaining of sGC and Hsp70 was more intense at the edge of cells and at cell-cell contacts (Fig. 9B). At higher magnification, these stronger signals seemed to localize at the membrane (bottom panels). More importantly, these signals coincided (merge panels), confirming the co-localization of sGC and Hsp70, primarily at the membrane.

Hsp70 Is Partly Responsible for the sGC-activating Effect—First, we assessed whether Hsp70 co-purifies with the sGC-activating effect by immunoblot analysis of the various fractions obtained during the purification procedure. As shown in Fig. 10A, Hsp70 was detected in the fractions that contained the sGC-activating effect throughout the purification. Moreover, Hsp70 was absent from the MonoQ fractions that did not have an sGC-activating effect (fractions 18 and 22) but was detected in two of the fractions that constituted the peak of activity (19–21) after MonoQ fractionation (Fig. 10A). Hsp70 was also detected in the fraction corresponding to the high molecular weight peak of activity previously obtained by gel filtration chromatography (Sephacryl). We then assessed the effect of pure Hsp70 on sGC activity in our in vitro assay (see “Experimental Procedures”). Among various conditions tested, we observed a slight increase (~20%) in sGC activity when 50
ng of semipurified sGC was mixed with 50 ng of pure Hsp70 in the presence of 1 μM SNAP and 100 μM ATP (n = 2 experiments, done in duplicate). We did not see any significant change in sGC activity in the other conditions. Besides the possibility that our conditions are not optimal, one probable explanation for the weak enhancement of sGC activity is the fact that Hsp70 requires additional factors or co-chaperones to exert its activity (21). Thus, we examined whether immunodepletion of Hsp70 (and potential associated factors) from the cytosol could diminish the sGC-activating effect. Precleared COS-7 cytosol (100 μg) was incubated with serial dilutions of anti-Hsp70 (1:5 to 1:100) or nonimmune antisera (Fig. 10B). After immunoprecipitation, the effect of supernatants on NO-stimulated sGC activity was assessed as described under “Experimental Procedures” and are expressed in nmol of cGMP min⁻¹ mg⁻¹ of eluate. Results are the mean ± S.E. of two experiments, with each measurement performed in duplicate. N.D., not detectable. B, pure Hsp70 binds directly to the semipurified sGC. In an experiment similar to that in A, 2 μg of purified Hsp70 (input) was passed through the resin column in the presence or absence of sGC (1 μg). Hsp70 is specifically retained by the sGC resin and is not detectable in the eluates from the resin that lacks sGC. Eluates were analyzed by immunoblot with anti-Hsp70. WB, Western blot.

**DISCUSSION**

Recently, in vivo and structural evidence led to the hypothesis that sGC can be regulated independently of NO (7, 22–24). In the search for such endogenous modulators of sGC, we successfully isolated a cGMP-promoting activity from COS-7 cells.

Initial characterization determined that this sGC-activating effect was not due to phosphodiesterase or nitric-oxide synthase activities and was calcium-independent. The sGC-activating effect was maintained in the presence of a saturating concentration of the NO donor SNAP.
The cytosolic factor activity was sensitive to proteinase K and was destroyed by boiling, suggesting that it contains a protein component. Size exclusion chromatography revealed two major peaks of activity between 40 and 70 kDa. After partial purification by ion exchange chromatography, the mechanism of activation of this factor was further characterized. The partially purified factor increases by severalfold sGC basal activity and synergistically activates the NO-stimulated activity. In addition, we showed that the partially purified factor has a mechanism of activation different than that of YC-1, an allosteric activator of sGC.

MALDI MS analysis of the MonoQ fraction (the final step of purification) identified four candidate activators in the 40–70-kDa range: Hsp70, PDI, and actin. All of these candidates are known to interact with NO. PDI, an oxido-reductase protein that catalyzes thio-disulfide exchange reactions, was shown to catalyze the transfer of NO inside cells (25). Hsp70 and β- and γ-actin are among the 12 proteins known to be physiological targets of S-nitrosylation by endogenous nitric-oxide synthase (26, 27). In addition, all of these candidates are components of β- and γ-actin or involved in reorganization of the cytoskeleton (28–30).

Nonetheless, we were concerned that these four candidates are also highly expressed in a variety of cell types, a fact that could explain their identification by MALDI MS analysis. Thus, we assessed whether a direct interaction between sGC and these candidates existed. Using an sGC affinity matrix system, we showed that Hsp70 interacts specifically and directly with sGC and that the affinity matrix eluates retained the sGC-activating effect. By co-immunoprecipitation experiments, we confirmed that endogenous Hsp70 and sGC are bound in rat lung tissues. These findings were supported by confocal microscopy analysis in smooth muscle cells showing co-localization of Hsp70 and sGC at the membrane, in particular in the region involved in cell-cell contacts or cell extension. This is particu-
particularly interesting, since it was recently proposed that the sGC can be dynamically translocated to the membranes and was found to be associated with NO synthase-containing caveolar fractions in rat lung endothelial cells (31). These data imply that factors have to associate with the normally cytosolic sGC to target it to the membrane. As a molecular chaperone involved in trafficking, it is tempting to view Hsp70 as a candidate that could assist the translocation of sGC. Studies are now under way to determine the physiological relevance of the sGC-Hsp70 interaction.

Despite evidence of direct interaction in vitro and in vivo between sGC and Hsp70 and potential physiological relevance of their association, the question remained whether Hsp70 was responsible for the enhancement of sGC activity. We showed that Hsp70 co-purifies with the sGC-activating effect throughout the fractionation procedure, yet the addition of pure Hsp70 to sGC only induced a marginal increase in the sGC activity. This was not surprising, since the known activities of Hsp70 are dependent on the presence of other cofactors and co-chaperones (32). In fact, immunodepletion of Hsp70 was paralleled by a remarkable attenuation of the cytosol sGC-activating effect. Again the sGC-activating effect was not rescued by the addition of pure Hsp70 to the depleted cytosol. This may indicate that anti-Hsp70 antibody precipitated not only Hsp70 but also associated factors. On the other hand, the effect of Hsp70 can be dependent upon covalent modifications, such as phosphorylation, that would occur in the cytosol (and absent from the commercial preparation), as previously reported (33, 34).

So far, Hsp70 has not been described as a “classical” activator. Rather, it is defined as a chaperone that helps in folding and allows subsequent activation of a signaling pathway by association with other chaperones and cofactors (35–37). In our in vitro system, we measure the enhancement of activity of a semipurified sGC, which is likely to be already correctly folded. It is possible that Hsp70 activates sGC, not by directly assisting the folding but by “rearranging” the heterodimer into a more catalytically active conformation, as is the case for modulation of the homologous adenylyl cyclase activity, although not by molecular chaperones. This chaperone activity may be responsible for the stimulation of sGC activity observed in basal condition. Another potential mechanism of activation by Hsp70 and associated factors could be to increase affinity of NO for the heme and/or efficacy of NO stimulation, as does YC-1 (12, 38). Indeed, our results showed that the cytosolic fraction increases maximal sGC activity at saturating concentration of NO (increase in efficacy of NO stimulation). It is tempting to speculate that the lack of synergy on NO-stimulated activity seen in the presence of both YC-1 and the partially purified factor is due to a partial redundancy in the mechanism of activation of the NO-stimulated enzyme by these two activators. In addition, a recent study showed that another molecular chaperone, Hsp90, is required for heme binding and activation of the neuronal nitric-oxide synthase (39). By analogy, this may be a mechanism by which Hsp70 regulates sGC activity facilitating or stabilizing insertion of the heme.

It is known that Hsp70 and Hsp90 form a chaperone machinery for the correct folding and trafficking of proteins involved in various signal transduction pathways (40). Interestingly, a membrane form of GC, GC-A, was shown to be associated with both Hsp90 and Hsp70 (41). Also recently, an interaction between sGC and Hsp90 was documented in aortic endothelial cells (42). Because Hsp90 appears to interact with endothelial nitric-oxide synthase (43), it may be interesting to investigate whether the NO-sGC signaling pathway could be modulated by the Hsp70-Hsp90 chaperone machinery.

During the preparation of this manuscript, two new sGC-interacting proteins, in addition to the postsynaptic density protein 95 (44) and probably Hsp90 (42), were identified.

[Image: Fig. 10. Immunoblot analysis of fractionation and immunodepletion of Hsp70 in COS-7 cytosol. A, Hsp70 co-purifies with sGC-activating effect. The various fractions of purification procedure were analyzed by Western blot (WB) with anti-Hsp70. Because of low protein content, 35 μl of DEAE-Sephadex fraction and of MonoQ fractions were loaded and subjected to electrophoresis together with 3 or 1.5 μg of cytosol desalted or not, 100 ng of Hsp70, and 35 μl of the fraction with the highest activity from the gel filtration chromatography procedure (Sephacryl). B, 100 μg of desalted COS-7 cytosol was precleared and then immunoprecipitated with serial dilutions of anti-Hsp70 (1:5 to 1:100) or with nonimmune mouse IgG, as described under “Experimental Procedures.” 20 μl of supernatants of antibody-treated samples or of precleared cytosol (12 μg) were subjected to SDS-PAGE electrophoresis and immunoblotted with anti-Hsp70. 20 μl of the supernatants of antibody-treated samples or of precleared cytosol (input) were added to the sGC assay buffer and assessed for their effect on NO-stimulated GC activity. For measurements of GC activity, SNAP was used at 1 μM final concentration. Activities are expressed in nmol of cGMP min⁻¹ mg⁻¹. Results are the mean ± S.E. of three experiments, with each measurement done in duplicate.
of them is also a chaperonin (45) that participates with Hsp70 in protein folding, and the other one is AGAP1 (46), a member of the Arf-GAP family known to be involved in cytoskeleton organization.

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