Nitric Oxide and Heat Shock Protein 90 Activate Soluble Guanylate Cyclase by Driving Rapid Change in Its Subunit Interactions and Heme Content*  

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Background: Heme insertion into soluble guanylate cyclase (sGC) enables it to bind nitric oxide (NO) for cell signaling.

Results: NO triggered a rapid, reversible, and hsp90-dependent heme insertion into sGC-β1 and an association with sGC-α1 subunit. sGC activator BAY 60-2770 did the same.

Conclusion: NO dynamically impacts the maturation and stability of active sGC heterodimer.

Significance: The data uncover new mechanisms that regulate cellular NO signaling cascades.

The chaperone heat shock protein 90 (hsp90) associates with signaling proteins in cells including soluble guanylate cyclase (sGC). hsps90 associates with the heme-free (apo) sGC-β1 subunit and helps to drive heme insertion during maturation of sGC to its NO-responsive active form. Here, we found that NO caused apo-sGC-β1 to rapidly and transiently dissociate from hsp90 and associate with sGC-α1 in cells. This NO response (i) required that hsp90 be active and that cellular heme be available and be capable of inserting into apo-sGC-β1; (ii) was associated with an increase in sGC-β1 heme content; (iii) could be mimicked by the heme-independent sGC activator BAY 60-2770; and (iv) was followed by desensitization of sGC toward NO, sGC-α1 dissociation, and reassociation with hsp90. Thus, NO promoted a rapid, transient, and hsp90-dependent heme insertion into the apo-sGC-β1 subpopulation in cells, which enabled it to combine with the sGC-α1 subunit to form the mature enzyme. The driving mechanism likely involves conformational changes near the heme site in sGC-β1 that can be mimicked by the pharmacologic sGC activator. Such dynamic interplay between hsp90, apo-sGC-β1, and sGC-α1 in response to NO is unprecedented and represent new steps by which cells can modulate the heme content and activity of sGC for signaling cascades.

Soluble guanylyl cyclase (sGC) is an intracellular enzyme that plays a primary role in sensing NO and transducing its multiple signaling effects in mammals (1, 2). The active mammalian sGC is a heterodimer made up of slightly dissimilar α and β subunits that each contain a N-terminal regulatory, mid-dle dimerization, and C-terminal catalytic domains (3–6). A metal cofactor (iron protoporphyrin IX, heme) binds only in the regulatory domain of the β subunit and is critical for sGC function because it enables NO to bind and activate the enzyme (3–6). Although the sGC heme normally functions in its reduced (ferrous) oxidation state, an increase in cell oxidant stress that can occur under many inflammatory conditions (7) can cause oxidation and loss of the sGC heme, thus creating a population of heme-free (apo) sGC that is insensitive to NO (8, 9). This led to development of novel drug candidates that can activate sGC independent of NO or its heme (10, 11) and has piqued interest in the cellular mechanisms that control the heme content, protein associations, and activity of sGC.

hsps90 is a ubiquitously expressed, ATP-dependent chaperone that helps to fold, stabilize, or modify the functions of select client proteins (12, 13). We recently found that hsp90 drives heme insertion into sGC during its maturation in cells (14). Hsp90 is bound primarily to the heme-free sGC-β1 subunit in cells, drives heme insertion into the apo-sGC-β1 in an ATP-dependent process, and then dissociates afterward. In the same study, we saw that the hsp90 association with apo-sGC-β1 fell off rapidly when we added an NO donor to cells to activate their sGC. This was surprising because it suggested that NO might play additional roles in addition to simply activating the heme-replete, mature sGC. Our current study explores the basis for the NO effect and revealed that NO triggers dynamic and transient rearrangements between hsp90, sGC-β1, and sGC-α1 in conjunction with a rapid heme insertion into apo-sGC-β1 in the cells.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma or Thermo Fisher Scientific. Succinyl acetone (SA), ascorbic acid, hemoglobin, radicicol, and phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine were purchased from Sigma. BAY 60-2770 and BAY 41-2272 were obtained from Bayer, NO donors, 5-nitroso-N-acetyl-D,L-penicillamine (SNAP), 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-12) and sodium nitroprusside were purchased from Sigma, and Lipo-
fectamine was purchased from Invitrogen. cDNAs for sGC α1, β1, and sGC-β1\textsuperscript{H105F} mutant were gifts from Dr. Andreas Papa-
metopoulos (University of Patras, Patras, Greece). Green African
monkey kidney cells COS-7, rat fetal lung fibroblast (RFL-6) cells, and bovine aortic endothelial cells were pur-
chased from ATCC. cGMP ELISA assay kit was obtained from
Cell Signaling Technology.

**Antibodies**—Rabbit polyclonal sGC-β1 and sGC-α1 antibodies
were obtained from Cayman Chemicals and Novus Biologicals,
respectively, whereas mouse monoclonal and goat polyclonal
sGC-β1 antibodies were purchased from Santa Cruz Biotechnology.
Rabbit polyclonal hsp90 and monoclonal Myc tag antibodies were purchased from Cell Signaling Technology,
epitope-tagged anti-V5 antibody was purchased from Invitro-
Biosciences) for 1 h at 4 °C, beads were pelleted, and the super-
natants were then loaded in SDS-PAGE gels before being harvested. For NO scavenging experiments,
sGC activator from varying time points between 0–45 min
were treated with cycloheximide (10 μM) or NOC-12 (35 μM) for 2 h prior to transfection or to activation of sGC (14). In such cases, the sGC activity in RFL-6 supernatants was determined by
passing the supernatants through the desalting PD-spin trap
G-25 columns (GE Healthcare) prior to addition of the above
column fractions was determined by adding 500 μM GTP and
20 μM of sGC activators BAY 41-2272 or BAY 60-2770 and
incubating for 10 min at 37 °C. Reactions were quenched by
addition of 10 mM Na₂CO₃ and Zn(CH₃COO)₂. In certain other
cases, sGC activity in RFL-6 supernatants was determined by
passing the supernatants through the desalting PD-spin trap
G-25 columns (GE Healthcare) prior to addition of the above
constituents. The cGMP concentration was then determined
by ELISA.

**Measurement of NO Release Rate**—The NO-mediated con-
version of oxy-hemoglobin to methemoglobin was used to
determine the rate of NO release from sGC or NOC-12 at
25 °C, following procedures as described previously (17).

**Biotin Switch Assay**—The biotin switch assay was performed
to determine S-nitrosated proteins as described previously (18),
and the presence of the S-nitrosated target protein was assayed
by immunoblotting with specific antibodies.

**RESULTS**

**NO Transiently Alters the hsp90-sGC Association**—We studied
how a brief NO exposure influenced the hsp90-sGC protein
association by transiently expressing a V5-tagged sGC-β1 in
COS-7 cells, adding the NO donor SNAP (in the presence of a
phosphodiesterase inhibitor) and assessing subsequent hsp90
protein association and the cellular cGMP content at three time
points within the first 30 min. As shown in Fig. 1, A and B, there was detectable hsp90-sGC-β1 association in the resting cells
that fell with SNAP treatment by the 5th minute and then gradu-
ally recovered by the 30th minute. Measures of cell cGMP
accumulation imply that the sGC was active only during the
first 5 min of SNAP treatment (Fig. 1C), despite continuous NO
release from SNAP occurring over the 30-min period, which we
measured to be 144 nM NO per min from 50 μM SNAP (Fig. 1D).
Similar or identical changes in the hsp90-sGC-β1 association
dynamics were observed using the alternative NO donors
sodium nitroprusside or NOC-12 (Fig. 1, E and F), which
released NO at a similar rate to SNAP (Fig. 1D).

We studied the NO effect on the hsp90-sGC-β1 association in
two other cell types that constitutively express the sGC
enzyme (bovine aortic endothelial and RFL-6 cells). The NO
donor again caused a quick loss and gradual return in hsp90 association with sGC-β1 in both cell types (Fig. 2, A and C), and the endogenous sGC was only active during the first 5 min of the SNAP treatment (Fig. 2, B and D). When we performed a follow-up study with RFL-6 cells in a smaller time window, we found that the hsp90-sGC-β1 association was entirely lost even within the first 2 min after SNAP addition, whereas steady sGC activity continued through the 6th min (Fig. 2, E and F).

We saw that the reassociation of sGC-β1 with hsp90 during longer NO exposure was associated with an increase in sGC-β1 S-nitrosation levels (Fig. 1, G and H). The sGC-β1 reassociation was significantly diminished if hemoglobin and ascorbic acid

FIGURE 1. Changes in hsp90-sGC interaction during and after NO-dependent activation. COS-7 cells that were transfected with a V5-tagged sGC-β1 construct for 42 h, or RFL-6 cells expressing endogenous sGC, were treated with SNAP (50 μM), sodium nitroprusside (SNP) (50 μM) or NOC-12 (35 μM) and cell supernatants generated between 0-30 min as indicated. Supernatant aliquots (equal protein) were analyzed for cGMP level by ELISA and were immunoprecipitated with an anti-V5 antibody followed by SDS-PAGE and Western analysis with anti-hsp90 and V5 antibodies. Likewise, supernatants harvested from RFL-6 cultures were subjected to biotin switch assays. A, E, and F, immunoprecipitation showing bound hsp90 and sGC-β1 (input 20%) retained on the beads. B, densitometric quantification of bound hsp90 with sGC-β1 in SNAP-treated COS-7 cells (n=3). C, cell supernatant cGMP concentrations. Values depicted are mean ± S.D. of three independent experiments (*, p < 0.05, by one-way ANOVA). D, rates of NO release from SNAP and NOC-12 under culture conditions as determined by the oxyhemoglobin assay. G (left and right), supernatants and eluates from NOC-12-treated cells that underwent biotin switch assays were Western blotted with sGC-β1 and GAPDH antibodies. H, densitometric quantification of S-nitroso-sGC-β1 levels as revealed by corresponding panel in G (upper right). Values depicted are mean ± S.D. of three independent experiments. IB, immunoblot.
FIGURE 2. Hsp90 interaction with endogenous sGC is dynamic following activation by NO and further NO exposure. Confluent cultures of RFL-6 or bovine aortic endothelial cells (BAEC) had their endogenous sGC activated by SNAP (50 μM), and supernatants were prepared at indicated time points. Parallel experiments added hemoglobin (Hb, 3 μM) and ascorbic acid (AA, 1 mM) to RFL-6 cultures after 3 min of SNAP addition to scavenge the excess NO, and cells were harvested at indicated time points. Aliquots (equal protein) of cell supernatants were subjected to immunoprecipitation and assayed for cGMP concentration by ELISA. A, C, E, and G, immunoprecipitation showing bound hsp90 and sGC-β1 (input 20%) retained on the beads. B, D, and F, cGMP concentrations in the corresponding supernatants made at the indicated time points. Values are mean ± S.D. of three independent experiments. IB, immunoblot.

NO Triggers Heme Insertion and Heterodimerization of sGC
were added to RFL-6 cultures after 3 min of SNAP activation to scavenge NO (Fig. 2G), suggesting that the continuous and/or excess exposure to NO may be driving the process. Together, our results show that the hsp90-sGC-β1 association is dynamic and quickly falls off during NO-dependent sGC activation, but under continued NO exposure returns with time and after the sGC becomes insensitive toward NO activation.

NO Transiently Alters sGC-β1 Distribution in Cells—We went on to examine the apparent mass distributions of sGC-β1, sGC-α1, and hsp90 in the RFL-6 cell supernatants, to see whether their distributions became altered during the SNAP treatment. Cultures of RFL-6 cells were treated with vehicle or SNAP for 5 or 30 min, supernatants were prepared at 4 °C and then run on a high performance size exclusion column at 4 °C, with column fractions undergoing Western analysis using anti-sGC-β1, sGC-α1, or hsp90 antibodies, and also undergoing sGC activity measurements. Figs. 3, A and D, show that resting cells (-SNAP) contained two main molecular mass populations of sGC-β1, whose apparent molecular weight (M) ranges (see M, scale in B) suggest that the sGC-β1 was primarily in complex with other cell proteins, possibly including hsp90 and sGC-α1, which co-eluted in fractions that contained sGC-β1 (Fig. 4, A and B). Interestingly, the predominant form of sGC-α1 expressed in the RFL-6 cells was a truncated splice variant of 52 kDa, probably similar to the Δ1-239 splice variant that is expressed in BE2 neuroblastoma cells and in lung tissue, which is missing a portion of the α subunit N-terminal regulatory domain (19, 20). The mass distribution of Δ1-239 sGC-α1 in the cell supernatant was broad and similar to that of the sGC-β1 subunit, consistent with the truncated sGC-α1 subunit and the sGC-β1 subunit still being able to combine and form an active heterodimer in cells (19, 20).

Activity measurements on the supernatant column fractions prepared from resting cells showed that the two M, populations of sGC-β1 both displayed a blend of heme-dependent and heme-independent enzyme activity, as judged from the nearly equivalent cGMP production measured in reactions that contained either a heme-dependent or heme-independent sGC activator (BAY 41-2272 and BAY 60-2770, respectively) (Fig. 3, F and H, left three bars). These activity data confirm that both heme-free and heme-replete forms of sGC-β1 were present in the resting RFL-6 cells and show that these two forms were distributed fairly similarly among the higher and lower M, populations in the cell supernatant.

In supernatant prepared from RFL-6 cells that had been given SNAP for 5 min, the M, distribution patterns shifted for sGC-β1 and hsp90 proteins (+SNAP, 5 min, Fig. 3, B and E). In particular, there emerged a lower M, subpopulation of sGC-β1 that clearly had no hsp90 associated with it (lanes 23-26). This subpopulation existed only transiently in the cells because it was no longer present in cells that underwent a longer SNAP exposure for 30 min (Fig. 3C). This transient low M, sGC-β1 subpopulation could be activated by the heme-dependent activator BAY 41-2272 (Fig. 3G), but the heme-independent activator BAY 60-2770 no longer activated, thus identifying this low M, sGC-β1 subpopulation as exclusively heme-replete. These fractions also contained sGC-α1 (Fig. 4, A and B), consistent with their having enzymatic activity.

Because most of the column fractions from the cells that were given 5-min SNAP treatment appeared to have greater BAY 41-2272 activities and lower BAY 60-2770 activities relative to the replica column fractions that were created from the resting cell supernatant (compare Fig. 3, F and G), this suggested that the 5-min SNAP treatment may have increased the heme content of the sGC-β1. We examined this in a semi-quantitative way by summing the total heme-dependent (BAY 41-2272) activities measured for the two sets of column fractions that were derived from equal amounts of supernatant protein from RFL-6 cells that either had or had not received the 5-min SNAP treatment (i.e. the data from the graphs depicted in Fig. 3, F and G). The total cGMP production in response to BAY 41-2772 for the control cells was 1049 ± 19 nm, compared with 1752 ± 29 nm for the cells treated with SNAP for 5 min. This 67% increase is consistent with transformation of apo-sGC-β1 into heme-containing sGC-β1 in response to the 5-min NO treatment. This was consistent with our finding that the unfraccionated cell supernatant prepared from the 5 min SNAP-treated cells had an increased BAY 41-2272 activity and a decreased BAY 60-2770 activity relative to the resting cell supernatant (Fig. 3H). This change appeared to be time-sensitive because the supernatant prepared from cells given 30-min SNAP treatment had lower sGC activity toward both BAY 41-2272 and BAY 60-2770 compared with resting cells.

Together, our findings suggest a dynamic change in the hsp90-apo-sGCβ1 association occurs upon NO exposure, irrespective of cell type or whether the sGC is endogenously or transiently expressed. The process creates a larger population of heme-containing, active sGC that within a short time becomes inactive and ultimately reassociates with hsp90 and possibly other proteins into higher M, complexes.

NO Causes an Increase in sGCα1-β1 Heterodimer Association—To investigate whether NO may alter interaction of sGC-α1 with the apo-sGCβ1 in our system, we transiently transfected sGC-α1 (Myc-tagged) and β1 (v5-tagged) constructs into COS-7 cells, and 42 h post transfection, the cells were either given SNAP or the NO donor NOC-12 for various times. Because the NO release rate of N0C-12 (210 nM/min) was faster than SNAP (144 nM/min, Fig. 1D) we adjusted the concentration of NOC-12 to 35 μM to give an equivalent NO flux in the cultures. Cell supernatants prepared at various time points were subjected to immunoprecipitation with anti-v5 antibody and immunoblotted with anti-Myc, hsp90, and anti-v5 antibodies. As shown in Fig. 4, C and D, both NO donors caused a transient decay in the sGC-β1 association with hsp90, followed by a recovery by the 30th min, as we saw earlier. In the same cells, the sGC-β1 association with sGCα1 was initially low, was greater at the 5- and 15-min time points, and then decayed by the 30th min, the exact inverse of what we observed for the hsp90 association. This implied that interactions of sGC-β1 with hsp90 and sGC-α1 may be mutually exclusive and can change quickly and reversibly during the NO exposure (Fig. 4E). Taken together, our data suggest that NO induced apo-sGC-β1 to incorporate heme and shift its association from hsp90 to sGC-α1 to form an active sGC heterodimer, but more prolonged NO exposure reversed this process.
FIGURE 3. NO alters Mr distributions of sGC-β1 and hsp90 in RFL-6 cells. RFL-6 cells were given vehicle or SNAP for 5 or 30 min, and then supernatants were prepared. Equal protein amounts of supernatants were fractionated on a Superdex 200 gel filtration column. The fractions were analyzed by Western blotting with sGC-α1/β1 or hsp90 antibodies and for sGC activity. A–C, sGC-β1 and hsp90 protein levels in the column fractions under various conditions as indicated. B also has an Mr scale for the eluted proteins based on injected protein Mr standards. D and E, densitometric quantification of sGC-β1 and hsp90 levels in column fractions as shown in A and B, respectively. F and G, cGMP concentrations achieved in reactions containing column fractions from A and B in response to sGC activators BAY 41-2272 or BAY 60-2770 (n = 3). H, supernatants generated from control or 5 or 30 min SNAP-treated RFL-6 cells were buffer-exchanged (PD-25, spin trap), and sGC activity in the eluants was assayed in response to BAY 41-2272 or BAY 60-2770 (n = 3). Values are mean ± S.D. of three independent experiments (*, p < 0.05, by one-way ANOVA; ns, not statistically significant).
Heme Must Be Available and Be Insertable into Apo-sGC-β1 for NO to Alter the hsp90 Association—We sought to define how NO diminishes the hsp90-apo-sGC-β1 association. To test a role for cell heme, we used RFL-6 and COS-7 cells that had been made heme-deficient and thus could constitutively or transiently express only the apo-sGC-β1, respectively (14). Fig. 5, A and B, shows that a 5-min SNAP treatment did not alter the hsp90-apo-sGC-β1 association in this circumstance and did not activate sGC catalysis in either cell type (Fig. 5 C). This implied the NO effect requires heme to be generally available in cells for insertion into apo-sGC-β1. We also found that the 5-min SNAP treatment did not diminish hsp90 association with a V5-tagged sGC-β1 mutant that is defective in heme binding (sGC-β1H105F), even when the cells that expressed the mutant were heme-replete (Fig. 5 D). This suggested that actual heme insertion into sGC-β1 must take place to diminish the hsp90 binding in response to NO. Heme insertion into apo-sGC-β1 also requires that hsp90 have an intact ATPase activity (14). Here, we found that NO did not diminish the hsp90-apo-sGC-β1 association if the hsp90 ATPase activity was inhibited with radicicol, and under this circumstance, less cGMP accumulated in the cells in response to the 5-min SNAP treatment (Fig. 5, E and F). Thus, the NO effect on hsp90 dissociation required two features that enable heme insertion into apo-sGC-β1, namely, an active hsp90 ATPase activity, and a functional heme binding site within the sGC-β1. This further supports the concept that NO caused its effects by stimulating heme insertion into sGC-β1.

Dissecting the Importance of Heme Site Occupancy—To further probe the mechanism, we utilized the apo-sGC activator BAY 60-2770. This molecule binds in the heme pocket of the sGC-β1 homolog Nostoc H-NOX domain (21) and is thought to activate sGC by triggering protein conformational changes in the sGC-β1 subunit that mimic those caused by NO binding to the sGC-β1 heme (21). When BAY 60-2770 was given to cells that transiently expressed the heme-free mutant sGC-β1H105F, or to heme-deficient RFL-6 cells that expressed endogenous apo-sGC-β1, it caused rapid dissociation of the hsp90-apo-sGC-β1 complex in both cases (Fig. 6, A and B, respectively), followed by a much weaker and more gradual hsp90 reassociation than what was observed in our previous experiments using NO donors (see Fig. 1). This behavior correlated with BAY 60-2770 stimulating a much longer-lived activation of sGC in the cells (Fig. 6 C) compared with the NO donors. Gel filtration analysis of V5-based immunoprecipitations showing bound hsp90, sGC-α1, and sGC-β1 (input 20%) retained on the beads. E, band intensities versus time of NO treatment for the hsp90 and sGC-α1 bound to sGC-β1 as determined from D. Data are representative of three replica experiments.
60-2770 treatment (data not shown), consistent with their containing some apo-sGC-β1 under normal culture conditions. Thus, pharmacore occupancy of the sGC-β1 heme binding site was also able to mimic NO in causing hsp90 dissociation and the M_r redistribution of apo-sGC-β1. Importantly, BAY 60-2770 did so even in heme-deficient cells. This distinguishes it from NO, which only diminished the hsp90 interaction and altered the apparent M_r distribution of sGC-β1 when cellular heme was available for insertion into apo-sGC-β1.

To further examine the role of heme site occupancy, we added hemin to RFL-6 cultures to promote heme insertion into the subpopulation of apo-sGC-β1. We previously reported that adding hemin to cells enabled heme insertion into apo-sGC-β1 and resulted in its dissociation from hsp90 (14). Here, we assessed how hemin treatment with or without a subsequent exposure to SNAP would impact the apparent M_r distribution of the sGC-β1. As expected, giving cells hemin for 2 h increased the proportion of heme-replete sGC-β1, as judged by the increased sGC activation in response to the heme-dependent drug (BAY 41-2272) and the decreased response to the heme-independent drug (BAY 60-2770) (Fig. 7A). However, the hemin treatment only caused a relatively modest shift in the apparent M_r distribution of sGC-β1 and created a relatively small subpopulation of the hsp90-free, low M_r, heme-replete sGC-β1, as judged by the elution profile and drug response profile of the column fractions to BAY 41-2272 versus BAY 60-2770 (Fig. 7, B, fraction D, and D). This low M_r sGC-β1 subpopulation formed to a much greater extent if the cells that received hemin also received a subsequent dose of SNAP for 5 min (Fig. 7, B, fraction E, and E), and in cells given hemin the low
Mr sGC-β1 population partly persisted even after the 30-min SNAP exposure (Fig. 7, B, fractions F, and F). This persistence suggested that the added hemin stabilized the heme-replete sGC-β1 species in the continued presence of NO. Taken together, our data suggest that incorporating heme into apo-sGC-β1, despite its causing hsp90 dissociation (14), did not promote extensive Mr redistribution of sGC-β1 in the cells, unless NO was subsequently added.

**DISCUSSION**

We found that NO triggers a dynamic change in association among hsp90, apo-sGC-β1, and sGC-α1 in cells. NO quickly diminished apo-sGC-β1 association with hsp90 and caused a concomitant increase in its association with sGC-α1 that was independent of cell type or whether the sGC was transiently or naturally expressed. These NO effects were transient and reversed with further NO exposure and after sGC became desensitized toward NO and its catalysis had stopped.

Possible Mechanism of Action—One reason that hsp90 associates with apo-sGC-β1 in cells is to drive heme insertion into the enzyme, and hsp90 dissociates from sGC-β1 after heme insertion takes place (14). Our observing an hsp90-sGC-β1 and altered its apparent Mr distribution. We conclude that sGC activation per se does not impact these parameters unless it occurs through a mechanism that directly involves the sGC-β1 subunit.

**FIGURE 6.** Hsp90-sGC interaction dynamics in response to heme-dependent versus heme-independent sGC activators. COS-7 cells expressing a V5-tagged heme-free mutant sGC-β1H105F or heme-deficient (SA-pretreated) RFL-6 cells expressing endogenous apo-sGC were given the heme-independent activator BAY 60-2770 (10 μM), and supernatants were made at 0, 15, 30, and 45 min. Parallel experiments utilized the heme-dependent activator BAY 41-2272 (10 μM). A and B, gel and Western analysis of immunoprecipitations with anti-V5 and sGC-β1 antibodies showing hsp90 associated with sGC-β1H105F or apo-sGC-β1, respectively (input 20%). C, cGMP concentrations in supernatants as indicated. D, hsp90 associated with sGC-β1 (input 20%) after cell treatment with BAY 41-2272. E, gel filtration fractions of supernatant from SA-pretreated RFL-6 cultures given BAY 60-2770 for 30 min, analyzed by Western blotting using sGC-β1 or hsp90 antibodies. Scale indicates Mr range of column fractions determined with protein Mr standards. Values in the bar graph are mean ± S.D. of three independent experiments. IB, immunoblot.

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complex in all the cell types used in our study implies that cells contain a mixture of apo-sGC-β1 and holo-sGC-β1 under normal culture conditions. This concept is supported by our observing a strong sGC activation to the heme-independent sGC activator BAY 60-2770 in the various cell types, and by the BAY 60-2770 response becoming muted (and the corresponding response to BAY 41-2272 increasing) when the cells were incubated with hemin to increase the sGC-β1 heme content.
Thus, we can surmise that NO caused hsp90 to quickly dissociate from the apo-sGC-β1 subpopulation that was present in cells. But how might this occur?

In principle, NO could weaken the hsp90 association with apo-sGC-β1 by several ways. We saw that the heme-independent sGC activator BAY 60-2770 could mimic the effect of NO in promoting hsp90 dissociation, whereas the heme-dependent sGC activator BAY 41-2272 could not. The ability of BAY 60-2770 to do so is perhaps the best indicator that the mechanism of NO action does not necessarily require any NO-based protein modifications such as protein S-nitrosation or tyrosine nitration, which can otherwise occur in hsp90 and sGC proteins when cells are exposed to NO (23–25). Rather, our results suggest a mechanism of action that involves fundamental changes in the apo-sGC-β1 subunit.

A model that is consistent with the data is illustrated in Fig. 8. It has NO-stimulating heme insertion into the subpopulation of apo-sGC-β1, leading to dissociation of hsp90 and to association of sGC-α1 to form the active hetrodimeric enzyme. This model is consistent with the following: (i) NO boosting sGC activation by the heme-dependent activator BAY 41-2272 and causing a concurrent loss in sGC activation by the heme-independent activator BAY-60-2770, as should occur when heme incorporates into apo-sGC-β1; (ii) NO being unable to diminish the hsp90 association if the cells are heme-deficient and thus lacking available heme for insertion, or if the sGC-β1 contains a mutation that impairs its ability to incorporate heme, because heme incorporation causes hsp90 dissociation (14); (iii) NO having no effect if the cell hsp90 ATPase activity is inhibited because the ATPase activity of hsp90 is needed for heme insertion to occur (14), (iv) BAY 60-2770 triggering hsp90 dissociation on its own because the drug is thought to bind in sGC-β1 as a structural cognate of the heme-NOX complex itself (21). Thus, hsp90 dissociation likely indicates that NO-driven heme insertion (or insertion of BAY 60-2770 in place of heme) has occurred in the apo-sGC-β1 subunit. Because the NO effect occurred within the first 2 min, it would seem that a pool of cellular heme exists that can rapidly insert into the apo-sGC-β1. Alternatively, NO might speed up the normal heme insertion process, which otherwise occurs over tens of minutes in the absence of added NO (14). That NO can quickly shift the equilibrium between apo- and holo-sGC-β1 in cells is remarkable and should be further investigated.

Structural Insights—The crystal structures of the Nostoc H-NOX domain are regarded to be good models of the mammalian sGC-β1 regulatory domain structure (21, 26), whose structure remains to be solved. In comparing the structures of a H-NOX domain containing bound BAY 58-2667 or BAY 60-2770 with that of the drug free, heme-containing form, the authors (21, 26) identified some specific structural changes that occur with drug binding, that mainly involve the α-F helix and flanking residues that are located proximal to the bound heme. Although these structural changes help to show how sGC-β1 may achieve a catalytically-active state in response to NO binding to the sGC-β1 heme, these particular structural changes are unlikely to be the ones that weaken the apo-sGC-β1 interaction with hsp90 because we know that heme insertion into apo-sGC-β1 alone, without any NO or sGC activation, is sufficient to weaken its hsp90 association (14). However, the same subset of structural changes (21, 26) is possibly associated with the process that led to a M↓ redistribution of sGC-β1 in the cells. Thus, BAY 60-2770 may promote extensive protein conformational changes within apo-sGC-β1 that lead to hsp90 dissociation, sGC-β1 redistribution in cells, and activation of sGC enzy-
motic activity. Although it is remarkable that BAY 60-2770 binding can mimic the effects of heme incorporation plus heme-NO binding in sGC-B1, this behavior is completely consistent with BAY 60-2770 binding in the heme site in Nostoc H-NOX to adopt a porphyrin-like structure (21).

In comparison, BAY 41-2272 showed no ability to diminish hsp90 interaction with sGC-B1, despite its activating sGC to a similar extent as did BAY 60-2770 in the RFL-6 cells. BAY 41-2272 is thought to bind to the sGC-α1 subunit near amino acid residues 236–290 (22, 27). This region would still be present in the N-terminally truncated sGC-α1 form that we found is predominantly expressed in RFL-6 cells and is consistent with BAY 41-2272 being able to activate sGC catalysis in the RFL-6 cells. Several studies show that BAY 41-2272 binding to the sGC-α1 subunit can measurably alter the structure and properties of the heme site in the partner sGC-B1 subunit of a heterodimer (28–30). However, our study suggests that sGC-α1 does not bind to apo-sGC-B1 and so cannot transduce any effects under this circumstance. Alternatively, if it did bind to apo-sGC-B1, perhaps the structural changes induced by BAY 41-2272 binding to sGC-α1 are unable to alter hsp90 binding or may not occur at all if heme is absent in the sGC-B1 subunit. Exploring these possibilities may help improve our understanding of the fundamental mechanisms of sGC activation.

 Redistribution of sGC-B1 in the Cell—We found that NO and BAY 60-2770 drove a reorganization of the sGC-B1 protein in cells that was manifested by the appearance of a lower Mr sGC-B1 subpopulation. In contrast, we found that the Mr distribution profile of sGC-α1 was largely unaltered by NO (Fig. 4, A and B). Such changes in sGC enzyme distribution have not been noted previously or appreciated. We surmise that the intracellular redistribution of sGC-B1 involves distinct structural changes that occur when NO stimulates heme incorporation into apo-sGC-B1 and binds to its heme, or alternatively when BAY 60-2770 binds in the apo-sGC-B1, that are related to the specific structural changes that were identified in the Nostoc H-NOX protein when BAY 60-2770 binds (21), as discussed above. In any case, it is interesting that activating sGC through its β subunit (either by NO or BAY 60-2770) can cause a temporary Mr redistribution of sGC-B1 within the cell cytosol, which likely reflects changes in sGC-B1 protein-protein interactions and/or intracellular compartmentalization. The mechanisms involved and the relationship to cellular sGC activity and biological function deserve further study.

 Hemeprotein Maturation Shows a Complex Response to NO—Our current study suggests that NO can have a more nuanced impact on heme protein maturation and function than was previously appreciated. NO appears to impact sGC maturation at three levels. (i) It can promote rapid heme insertion into apo-sGC-B1, as described in the present study. (ii) Prolonged NO exposure generally blocks cellular heme insertion into apo-heme proteins (17) through a mechanism involving build-up of S-nitrosated proteins in cells (31). (iii) Prolonged NO exposure can also promote oxidation and loss of heme from sGC-B1, due to increased oxidative stress (8, 9, 16, 32). There are likely to be important and fascinating distinctions among these three types of NO responses with regard to timing, concentration response, mechanism, and when they come into play in biology.

 sGC Reassociation with hsp90—The transient nature of the NO effects and their relationship to sGC activation were striking. Specifically, we found that sGC-B1 reassociated with hsp90 in cells during a longer (10–30 min) exposure to the NO donor. The reassociation depended on NO, was affiliated with desensitization of sGC toward NO activation and its consequent loss of activity, and correlated with sGC-B1 dissociation from sGC-α1 and its cytosol Mr redistribution back toward the pattern seen in resting cells. The fact that sGC-B1 reassociation with hsp90 was greatly reduced when BAY 60-2770 was used in place of NO donor suggests that the hsp90 reassociation may be a consequence of an NO-based event like sGC desensitization. Indeed, biotin switch assays showed that sGC-B1 became S-nitrosated in the cells over time with exposure to NOC-12 (Fig. 1, G and H). Previous studies showed there are two different time frames for sGC desensitization. Cell sGC activity can deflect very quickly (within seconds) after being activated by NO (33). NO and its derived oxidants can also desensitize sGC over a longer time frame of minutes, either through S-nitrosation of specific Cys residues in sGC-B1 (16, 34) or by causing oxidation or loss of heme from sGC-B1 (32). It will be important to examine whether these events help to drive the sGC-B1 reassociation with hsp90. Whether hsp90 reassociation is a natural process that helps to protect sGC-B1 from degradation (35) and whether there are accompanied changes in sGC-B1 heme site occupancy (36, 37) are interesting possibilities to be addressed.

 Implications—That NO triggers dynamic and reversible change in sGC-B1 heme content, protein interactions, and apparent Mr distribution is unexpected and provides new insight on the cellular mechanisms that may activate or desensitize sGC in response to NO. The key role of hsp90 in determining sGC outcomes has important implications for cancer drug development programs that target hsp90 (38, 39). It is important to emphasize that the activation pathway we describe involves the heme-free sGC-B1 subpopulation that is present to various extents in healthy cells but is likely present to a greater extent under inflammatory or diseased states due to greater oxidant stress (8, 14, 40, 41). The ability of BAY 60-2770 to trigger all of the same changes in apo-sGC-B1 that we saw with NO, while bypassing the requirements for active hsp90 and cellular heme, suggests that BAY 60-2770 could activate cGMP-based signal cascades in disease states that may compromise hsp90 activity or cellular heme or that lead to accumulation of desensitized sGC-B1.

REFERENCES
1. Lucas, K. A., Pitari G. M., Kazeronian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P., and Waldman, S. A. (2000) Guanylyl cyclases and signaling by cyclic GMP. Pharmacol. Rev. 52, 375–414
2. Garthwaite, J., and Boulton, C. L. (1995) Nitric oxide signaling in the central nervous system. Annu. Rev. Physiol. 57, 683–706
3. Gilles-Gonzalez, M. A., and Gonzalez, G. I. (2005) Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. J. Inorg. Biochem. 99, 1–22
4. Fritz, B. G., Roberts, S. A., Ahmed, A., Breij, L., Li, W., Weichsel, A., Brailey, J. L., Wysocki, V. H., Tama, F., and Montfort, W. R. (2013) Molecular model of a soluble guanylyl cyclase fragment determined by small-angle X-ray scattering and chemical cross-linking. Biochemistry 52,
