**NO rapidly mobilizes cellular heme to trigger assembly of its own receptor**

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Nitric oxide (NO) signaling in biology relies on its activating cyclic guanosine monophosphate (cGMP) production by the NO receptor soluble guanylyl cyclase (sGC). sGC must obtain heme and form a heterodimer to become functional, but paradoxically often exists as an immature heme-free form in cells and tissues. Based on our previous finding that NO can drive sGC maturation, we investigated its basis by utilizing a fluorescent sGC construct whose heme level can be monitored in living cells. We found that NO generated at physiologic levels quickly triggered cells to mobilize heme to immature sGC. This occurred when NO was generated within cells or by neighboring cells, began within seconds of NO exposure, and led cells to construct sGC heterodimers and thus increase their active sGC level by several-fold. The NO-triggered heme deployment involved cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–heme complexes and required the chaperone hsp90, and the newly formed sGC heterodimers remained functional long after NO generation had ceased. We conclude that NO at physiologic levels triggers assembly of its own receptor by causing a rapid deployment of cellular heme. Redirecting cellular heme in response to NO is a way for cells and tissues to modulate their cGMP signaling and to more generally tune their hemeprotein activities wherever NO biosynthesis takes place.

\[\text{cGMP} \mid \text{soluble guanylyl cyclase} \mid \text{heme trafficking} \mid \text{hemeprotein}\]

**Nitric oxide (NO)** has long been known to impact iron and heme metabolism and to alter functions of proteins that utilize these cofactors for their activity. When made in excessive amounts, NO can down-regulate its own production by NO synthases (NOs) (1, 2) and can also inhibit other hemeproteins including cytochrome P450s, catalase, and hemoglobin (1, 3, 4). Conversely, NO activates its natural receptor, the enzyme soluble guanylyl cyclase (sGC), by binding to a heme cofactor within sGC (5). Indeed, many of NO’s biological functions manifest through its activating cyclic guanosine monophosphate (cGMP) production by sGC (6, 7), including vasorelaxation (8), peristalsis (9), immune surveillance (10), reproductive processes (11), and neural functions (12).

Conventional sGC activation proceeds through NO binding to a ferrous heme located within the sGCβ subunit of a mature sGC α–β heterodimer (13, 14). This causes protein structural changes that activate cGMP production (13–16). The essential role that heme plays in sensing NO underscores the importance of the heme delivery and insertion steps that enable sGC to mature in cells (17). Our recent studies have illuminated parts of the sGC maturation process: After translation, the immature sGCβ subunit is heme-free and accumulates in cells in the form of a complex with the cell chaperone hsp90 (18, 19). Mitochondrial-derived heme is provided to this species by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hsp90 then drives heme insertion into the apo-sGCβ and dissociates from the complex (20). This allows the heme-replete sGCβ subunit to bind with a partner sGCA subunit to form a mature sGC heterodimer that can respond to NO for cGMP signaling (17, 20).

Over the last decades, pharmacologic agonists have been developed that can activate sGC independent of NO. These fall into two groups: those that only activate the heme-containing, NO-responsive, mature sGC, as exemplified by BAY 41-2272 (BAY41) (21), and those that only activate the heme-free, NO-unresponsive sGC, as exemplified by BAY 58-2667 (BAY58) (22). Studies with these compounds and related biochemical data have indicated that cells and tissues typically contain a significant level of immature heme-free (apo) sGC even in normal healthy conditions (17, 23), with estimates indicating apo-sGCβ represents from 40 to 80% of the total sGC. Why cells and tissues maintain such high levels of heme-free NO-insensitive sGC is puzzling, and how they might convert their apo-sGCβ to an active sGC is currently unclear, but answering these questions would improve our understanding of NO–sGC–cGMP signaling in biology. For example, if the existing pool of immature apo-sGCβ could be stimulated toward maturation, it would provide cells and tissues with an alternative means to significantly increase their functional sGC activity independent of any gene activation event or new protein synthesis.

Given this, we sought to better understand the nature of the apo-sGCβ subpopulation in cells and how it may become mobilized. We previously discovered that NO can stimulate sGC maturation in cells (17) but the basis remained untested. Since then, we have studied sGC maturation using an sGC reporter construct originally developed by Hoffmann et al. (24), which has a tetra-Cys–containing sequence inserted near the heme binding site in sGCβ (TC-sGCβ). When the TC sequence is labeled with the arsensical dye FlAsH (25), the TC-sGCβ protein becomes fluorescent and its emission intensity is inversely proportional to the heme level and can be monitored in living cells. We found that NO generation at physiologic levels triggers a rapid redployment of intracellular heme, an iron-containing cofactor, and we show that this drives the assembly of the natural NO receptor protein, soluble guanylyl cyclase, which is needed for NO to perform its biological signaling functions. Our study uncovers a way that NO can shape biological signaling processes and a way that cells may use NO to control their hemeprotein activities through deployment of the heme cofactor. These concepts broaden our understanding of NO function in biology and medicine.

**Significance**

Nitric oxide (NO) performs many biological functions, but how it operates at the molecular and cellular levels is not fully understood. We discovered that NO generation at physiologic levels triggers a rapid redployment of intracellular heme, an iron-containing cofactor, and we show that this drives the assembly of the natural NO receptor protein, soluble guanylyl cyclase, which is needed for NO to perform its biological signaling functions. Our study uncovers a way that NO can shape biological signaling processes and a way that cells may use NO to control their hemeprotein activities through deployment of the heme cofactor. These concepts broaden our understanding of NO function in biology and medicine.

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proportional to the protein’s bound heme content. This provides a way to measure the sGC heme level that has advantages over other methods that are less direct and have inherently poor time resolution (i.e., measuring sGC catalytic activity, heterodimer level, or level of 14C[hemee]). We have recently utilized FlAsH-labeled TC-sGCβ to monitor real-time changes to its heme content in living cells, and in doing so have discovered that GAPDH is involved in sGC heme delivery (20), uncovered molecular details of the hsp90-driven sGC heme insertion reaction (17, 19), and defined how sGC inactivation impacts its heme content in living cells (23). In our current study, we utilized FlAsH–TC-sGCβ to study how physiologic levels of NO might impact its heme content and maturation. Our findings reveal that NO triggers living cells to quickly redistribute heme to apo-TC-sGCβ. Remarkably, the redistribution is triggered when the same or neighboring cells generate immunologic or signaling levels of NO and causes the cells to increase their level of mature, functional sGC by several-fold within minutes. Overall, our work uncovers a role for NO in promoting the assembly of its own biological receptor through its causing a rapid reallocation of cellular heme. This presents an additional way that NO can influence cGMP signaling in biology and provides a suitable population of apo-TC-sGCβ to test how NO may impact its heme level.

The kinetic traces in Fig. 1 track the FlAsH fluorescence emission of live cell cultures after NOC12 addition. For cells expressing FlAsH-labeled TC-sGCβ the fluorescence intensity remained steady for ~40 s and then began to decrease over the rest of the 5-min recording period. In comparison, there was no change in the fluorescence emission intensities in replica cultures that received the media vehicle without NOC12, or for cultures that received NOC12 but expressed a FlAsH-labeled TC-sGCβ variant (Y135A R139A) that is missing critical heme-binding amino acid side chains and therefore cannot bind heme either in its purified form or when it is expressed in cells (TC-sGCβ HD) (20, 27) (Fig. 1C). In experiments where we collected fluorescence readings over a 1-h period, the decrease in cell FlAsH–TC-sGCβ fluorescence continued well beyond 5 min and ultimately reached an equilibrium value after 30 min (Fig. 1D). Thus, NO released from NOC12 triggered the cells to reallocate their heme to the apo-TC-sGCβ population in a process that began within 1 min and continued for about 30 min until a new equilibrium heme level was achieved.

Because NO is slowly released from NOC12 and the release only begins when the alkaline stock solution is diluted into the

Results

NO Triggers Cell Heme Reallocation to Apo-sGCβ. We utilized the FlAsH-labeled TC-sGCβ reporter construct whose incorporation or loss of heme is indicated by a corresponding quenching or increase in its FlAsH fluorescence emission, respectively (Fig. 1A) (24). We first tested how the slow-release chemical NO donor 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC12) (Fig. 1B), whose half-life is 6 h (26), would impact the heme level in FlAsH-labeled TC-sGCβ that was expressed in HEK293 cells under normal culture conditions. Under this circumstance, it is known that a significant proportion (≥50%) of sGCβ when either expressed naturally or via transfection, or when TC-sGCβ is expressed via transfection, accumulates in heme-free (apo) forms in cells (17–19). This provides a suitable population of apo-TC-sGCβ to test how NO may impact its heme level.

The kinetic traces in Fig. 1C track the FlAsH fluorescence emission of live cell cultures after NOC12 addition. For cells expressing FlAsH-labeled TC-sGCβ the fluorescence intensity remained steady for ~40 s and then began to decrease over the rest of the 5-min recording period. In comparison, there was no change in the fluorescence emission intensities in replica cultures that received the media vehicle without NOC12, or for cultures that received NOC12 but expressed a FlAsH-labeled TC-sGCβ variant (Y135A R139A) that is missing critical heme-binding amino acid side chains and therefore cannot bind heme either in its purified form or when it is expressed in cells (TC-sGCβ HD) (20, 27) (Fig. 1C). In experiments where we collected fluorescence readings over a 1-h period, the decrease in cell FlAsH–TC-sGCβ fluorescence continued well beyond 5 min and ultimately reached an equilibrium value after 30 min (Fig. 1D). Thus, NO released from NOC12 triggered the cells to reallocate their heme to the apo-TC-sGCβ population in a process that began within 1 min and continued for about 30 min until a new equilibrium heme level was achieved.

Because NO is slowly released from NOC12 and the release only begins when the alkaline stock solution is diluted into the
cell-culture fluid, it takes several minutes for the cultures to reach a steady-state NO concentration (28, 29). We therefore calculated how the NO concentration would build over time after we added NOC12 to our cultures, using the measured rate of NO release under our conditions (80 nM NO release per minute at 0.5 mM NOC12 in culture media) and the known second-order rate of NO oxidation in solution, as previously done by others (28, 30). The traces indicating the calculated NO concentration in the cell-culture solution versus time (blue lines in Fig. 1 C and D) predict that the NO concentration increased after NOC12 addition for about 15 min and then achieved a steady-state NO concentration of ~800 nM, consistent with published NO electrode measures made under similar experimental circumstances (29, 31). To eliminate this pre–steady-state complexity, we repeated our experiments but in this case initiated an immediate NO exposure by giving the cells a media solution of 0.5 mM NOC12 that had been equilibrated for 20 min before use in order for it to achieve a steady-state NO concentration (calculated to be ~800 nM). Under this circumstance, we no longer observed any delay in the fluorescence decrease upon NOC12 addition for about 15 min and then achieved a steady-state NO concentration of ~800 nM, consistent with published NO electrode measures made under similar experimental circumstances (29, 31).

Fig. 2. NO-driven heme allocation to apo-sGC results in functional sGC heterodimer formation. HEK293 cells expressing sGCβ (or sGCβ HD) and sGCα were given NOC12 and cell supernatants were prepared at the indicated times. Supernatants were subjected to pull-down assay by an anti-sGC antibody to determine levels of associated Hsp90 and sGCα proteins, or had their sGC activities measured in response to BAY41 or BAY60. (A and D) Representative Western analyses indicating the relative levels of sGCα and Hsp90 associated with sGCβ or sGCβ HD at the times after NOC12 addition. (B and E) Quantification of results from A and D. (C and F) cGMP production activities in response to BAY41 or BAY60 of cell supernatants prepared at the indicated times after cells received NOC12. Each data point is the mean ± SD; n = 3 from one of three independent experiments. IB, immunoblotting; IP, immunoprecipitation; wt, wild type.

Fig. 3. Cell heme reallocation and functional sGC formation are stable after a transient NO exposure. HEK293 cells expressing FlAeH-labeled TC-sGCβ and sGCα underwent a 5-min NOC12 exposure followed by washout of NOC12. In some cases, cells were harvested and supernatants were prepared at various times during the experiment. (A) Fluorescence emission of live cell cultures versus time during and following the NOC12 exposure. (A, Inset) Western blots showing the expression levels of sGCα and TC-sGCβ throughout the experiment in supernatants of replica cell cultures. (B) sGC activation profiles versus time after NOC12 for the cell supernatants in response to BAY41 and BAY58. Each data point is the mean ± SD; n = 3 from one of two to four independent experiments.
addition to cells expressing FlAsH–TC-sGCβ (Fig. 1E). Thus, exposing the cells to a solution of ∼800 nM NO immediately triggered them to begin realloca ting heme to their FlAsH-labeled apo-TC-sGCβ population within the time resolution of mixing (∼10 s elapsed between the addition of equilibrated NOC12 media solution and the start of fluorescence data acquisition). This indicates that NO at nanomolar concentrations triggered cells to support a near-instantaneous heme reallocation to their apo-TC-sGCβ.

**NO-Driven Heme Allocation Generates a Functional sGC Heterodimer.** We next determined if the NO-driven heme reallocation would change the cell’s level of functional sGC. Our previous studies showed that heme insertion into the apo-sGCβ subunit is required for bound hsp90 to dissociate and be replaced by an sGCα partner subunit to form a functional sGC heterodimer (32). Fig. 2A and B show that adding NOC12 to HEK293 cells expressing both sGCα and sGCβ led them to convert their existing pool of apo-sGCβ into sGC heterodimers, as indicated by Western analyses that showed sGCβ subunits lost hsp90 and gained in sGCα association. This exchange in sGCβ protein partners was accompanied by a change in the sGC activation response profile, such that the cell supernatant sGC activity diminished fivefold in response to BAY58, which can only activate the sGCβ HD variant along with sGCα, it did not undergo any changes in its protein partners or activation profile, consistent with sGCβ HD being unable to bind heme, and thus being unable to form a functional sGC heterodimer in response to NOC12 (Fig. 2 D–F). These findings show that the NOC12-triggered heme reallocation to apo-sGCβ led cells to increase their functional sGC level by several-fold within 15 min.

**NO-Driven Heme Reallocation and sGC Heterodimer Formation Are Long-Lasting.** We next examined if continuous NO exposure was needed for cells to maintain the newly formed sGC heterodimer. HEK293 cells expressing FlAsH-labeled TC-sGCβ and sGCα were given NOC12 for 5 min followed by washout. The fluorescence emission traces in Fig. 3A show that the 5-min NOC12 exposure triggered heme insertion into the apo-TC-sGCβ population as expected. After NOC12 removal the cell fluorescence emission continued to decrease somewhat to a lower value that remained steady through 6 h of continued culture. Expression levels of sGCα and TC-sGCβ proteins remained constant throughout the period (Fig. 3A, Inset). Fig. 3B shows that the heme allocation to apo-TC-sGCβ triggered by the NOC12 exposure was accompanied by a shift in the sGC activation response profile consistent with coincident transition of apo-sGCβ to a functional heterodimer. The cell heterodimer level after NOC12 removal remained steady through 6 h. Thus, NO-driven heme reallocation to apo-TC-sGCβ and its transformation to a functional sGC heterodimer did not reverse after NO exposure had ceased.

**NO Generated by Immunostimulated Cells or by Cells in Signaling Amounts Triggers Heme Reallocation and sGC Maturation.** We next explored if cell-generated NO would stimulate heme reallocation to apo-TC-sGCβ and its maturation to a heterodimer. Experiments with cell-generated NO were performed under three different circumstances: 1) a coculture system in which Transwell inserts that contain NO-generating RAW264.7 macrophage cells expressing inducible NOS (iNOS) were placed above monolayers of HEK293 cells expressing FlAsH-labeled TC-sGCβ and sGCα (33) (Fig. 4A); 2) a coculture system in
which HEK293 cells that stably express neuronal NOS (nNOS) (34) were cultured along with neighboring HEK293 cells expressing FLA-h labeled TC-sGCβ and sGCa. For the experiments involving nNOS, their cell NO production was activated by adding the Ca\(^{2+}\) ionophore A23187, which causes a rapid increase in intracellular Ca\(^{2+}\) that drives calmodulin binding to the nNOS and thus activates its NO synthesis (35). This mimics the Ca\(^{2+}\)-initiated NO generation by nNOS that occurs during biological signal transduction (36).

Fig. 5. Calcium-triggered NO synthesis in one cell group causes neighboring cells to reallocate heme to apo-TC-sGCβ. (A) HEK293 cells expressing nNOS were added to cultures of HEK293 cells expressing TC-sGCβ or TC-sGCa or TC-sGCβ HD, sGC heterodimer formation, sGC activation response profile, and nitrite accumulation were monitored versus time. Cultures were given media alone (vehicle) or given media with the Ca\(^{2+}\) ionophore A23187 to initiate nNOS NO synthesis at time 0, and L-NAME was in some cases added to block NO synthesis. (B and C) Fluorescence emission of cell cultures expressing FLA-h-TC-sGCβ or TC-sGCβ HD versus time under the indicated coculture conditions. (D) Representative Western blots of immunoprecipitations indicating the change in sGCβ association with sGCa and hsp90 versus time after A23187 addition. (G) Culture media nitrite concentration versus time after A23187 addition. Each data point is the mean ± SD; n = 3 from one of three independent experiments.

Fig. 6. NO-triggered cell heme reallocation to apo-TC-sGCβ requires the hsp90 chaperone. HEK293 cells expressing FLA-h labeled TC-sGCβ or TC-sGCa HD proteins were given radicicol or vehicle and then given NOC12 and their fluorescence emission was followed versus time. Each data point is the mean ± SD; n = 3 from one of two independent experiments.
activated macrophage cells diffused from the Transwell inserts through the culture to stimulate HEK293 cells to reallocate heme to apo-sGCβ, which in turn led to functional sGC heterodimer formation. Thus, NO generated naturally by these cells acted identically as did NO released from NOC12 in driving cell heme reallocation and formation of functional sGC.

In our second approach, we seeded at a 3:1 ratio HEK293 cells that stably express nNOS into cultures that contained HEK293 cells expressing FiaSH-labeled TC-sGCβ. After 3 h, we added the Ca²⁺ ionophore A23187 to activate nNOS NO synthesis and then monitored the cell-culture FiaSH fluorescence over time (Fig. 5A). A steady decrease in the FiaSH–TC-sGCβ fluorescence took place after A23187 addition which leveled off at around 2 h (Fig. 5B), whereas no fluorescence decreases occurred in replica cultures that received a NOS inhibitor along with the A23187, did not receive A23187 (vehicle), or received A23187 but contained HEK293 cells expressing the heme binding-defective TC-sGCβ HD variant (Fig. 5B and C). We then repeated these experiments but used HEK293 cells coexpressing sGCβ and sGCα. The cells were harvested at different times after adding A23187 and their supernatants were subjected to antibody pull-down assays and Western analyses and to measurements of the sGC drug response profile. These showed that activating NO synthesis by nNOS in the one HEK293 cell population drove sGC maturation in the neighboring cell population, as indicated by a gradual shift in sGCβ protein partners to increase the sGC heterodimer population (Fig. 5D and E) and by a shift in the sGC drug response to favor cGMP production toward BAY41 and disfavor production toward BAY58 (Fig. 5F). Nitrite accumulation in the culture media increased from 1 to 3 μM after A23187 addition (Fig. 5G), confirming that it activated nNOS to generate a low level of NO in the cultures.

In our third approach, we performed experiments as noted above but used HEK293 cells that had been transiently transfected to express the nNOS, TC-sGCβ, and sGCα proteins all together in the same cells rather than in two different cell groups. This yielded results (SI Appendix, Fig. S1) that were highly similar to what we observed when the nNOS and sGC proteins were expressed in separate HEK293 cell groups and then combined. Together, the results with HEK293 cells show that activating low-level NO generation by nNOS in either neighboring cells or in the same cells was enough to trigger heme reallocation to apo-sGCβ and form functional sGC heterodimers.

**NO-Driven Heme Allocation Relies on the Actions of hsp90 and GAPDH.** The heme delivery and insertion steps that occur during sGC maturation involve GAPDH and hsp90, respectively (18–20). To investigate if hsp90 is also needed for the NO-driven heme allocation to apo-sGCβ, we added radicicol to block hsp90 function in HEK293 cells expressing FiaSH-labeled TC-sGCβ just prior to adding NOC12. Fig. 6 depicts fluorescence emission traces recorded for cell cultures after receiving NOC12. In cells that received NOC12 alone there was significant heme reallocation to apo-TC-sGCβ as expected. Radicicol reduced the NOC12-driven heme reallocation to a level that matched what was observed for cells expressing the TC-sGCβ HD variant that cannot bind heme. The results indicate that cell hsp90 function is required for NO-driven heme reallocation to apo-TC-sGCβ.

To check for GAPDH involvement, we employed our previously established small interfering RNA (siRNA) knockdown and rescue strategies (20, 37). We first performed a targeted siRNA knockdown of GAPDH expression in HEK293 cells, and then combined. Together, the results with HEK293 cells show that activating low-level NO generation by nNOS in either neighboring cells or in the same cells was enough to trigger heme reallocation to apo-sGCβ and form functional sGC heterodimers.
consistent with our previous results (20, 37). The level of total GAPDH expression was restored when either of the HA-tagged GAPDH proteins was expressed in the knockdown cells, and the level of TC-sGCβ expression was unaffected by the different treatments (Fig. 7A). The fluorescence emission traces in Fig. 7B show that the knockdown of GAPDH expression greatly diminished the extent of NOC12-triggered heme reallocation to apo-TC-sGCβ, whereas cells that had been treated with a scrambled siRNA maintained their GAPDH expression level (Fig. 7A) and showed a normal rate and extent of NOC12-induced heme reallocation to apo-TC-sGCβ (Fig. 7B). The expression of wild-type HA-tagged GAPDH in the knockdown cells restored their NOC12-induced heme reallocation to a normal level (Fig. 7C), whereas expression of the HA-tagged GAPDH-H53A mutant in the knockdown cells did not. These results show that NO-induced heme reallocation to apo-TC-sGCβ depends on the cell GAPDH expression level and on the ability of GAPDH to bind intracellular heme.

Discussion

NO signaling relies on a functional sGC being present to detect NO and generate cGMP as a second-messenger molecule, which performs a myriad of biological functions (12, 14, 36, 38). Surprisingly, we found that NO can trigger assembly of its own receptor in living cells by causing them to reallocate heme to apo-sGCβ. This response was remarkably sensitive and fast: It could be triggered by NO made in neighboring cells at either immunologic or signaling levels, and could commence as soon as cells became exposed to NO or their NO synthesis was initiated. Importantly, the NO-driven heme allocation caused sGCβ to change its protein partners and generate an sGC heterodimer, which ultimately increased the cell’s functional sGC level by three- to fivefold within minutes. Overall, this uncovers an additional way that NO can shape the dynamics of NO-sGC-cGMP signaling in biology.

The concept that ligands can drive the maturation of their own receptors was initially controversial (39) but has since been demonstrated for growth factor receptors whose inactive monomers dimerize into functional form after binding their ligands (40, 41). Heme can act in a similar manner. For example, hsp90-driven heme incorporation into monomeric NOS subunits drives their conversion into functional homodimers (18, 42–44), and heme binding within NADPH oxidase 5 promotes its active oligomer formation in cell membranes (45). Heme binding also causes protein partner exchange for a gain in function, as shown here and previously for sGC (32) and also for myoglobin (46), hemoglobin β and γ (47), BACH1 (48), and REV-ERBβ (49). In this context, the ability of NO to drive cell heme reallocation presents an additional layer of regulation: NO promotes the binding of the ligand (heme) to its inactive receptor (apo-sGCβ), which in turn undergoes a protein partner exchange (loss of hsp90 and replacement by sGCα) that creates a functional receptor (the sGC heterodimer). Indeed, our work establishes how NO can operate in cells and tissues by controlling heme binding in proteins. This role is consistent with NO causing heme remobilization into the cytosol (50) and its capacity to promote heme transfer between purified proteins (51).

How NO impacts living systems depends heavily on the NO concentration achieved in the system and the duration of NO exposure (52). We found that NO at nanomolar concentrations was sufficient to trigger cells to reallocate heme to apo-sGCβ in a process that could begin within seconds. This places cell heme reallocation into a range of physiologic NO responses where it also can induce angiogenesis (53) and osteogenesis (54), stabilize HIF1α (55), and activate sGC itself. Indeed, our coculture experiments in which NO was generated at immunologic or signaling levels by neighboring cells showed that it can trigger cell heme reallocation in a paracrine manner at low nanomolar NO concentrations. This implies the process can occur naturally in biological systems as a dynamic response to low-level NO generation or transient exposure. Indeed, we speculate that the basal NO generation that goes on in tissues of many multicellular organisms (for example, in the human circulatory system, lung airway, and nasal sinuses) provides a tonic level of NO that influences cell heme allocation and

![Diagram of NO signaling](https://example.com/diagram.png)
ensures that enough functional sGC is present in these tissues. This concept is consistent with cells and tissues containing variable levels of heme-free sGCβ and also implies that if basal NO production is diminished due to inflammatory disease, advanced age, or genetic predisposition, sGC signaling functions may become compromised due to a deficit in NO-driven heme allocation.

Our study sheds light on the cellular mechanisms that enable NO-driven heme reallocation to apo-sGCβ. For example, we found that GAPDH, which binds apo-sGCβ and provides it with mitochondrially generated heme during normal sGC maturation (19, 20), was also involved in the NO-triggered heme reallocation to apo-sGCβ. Likewise, we found that hsp90, which must bind with apo-sGCβ in cells to enable its heme insertion during maturation (17–19), was also needed for the NO-driven process. This implies that NO acts on the same machinery that cells employ to deliver and insert heme during normal sGC maturation (19, 20), and for normal maturation of NOS enzymes (45) and globins (56). Whether NO acts through a common mechanism to reallocate heme to these or other heme proteins is an exciting possibility that can now be explored.

Based on the speed in which NO initiated heme allocation to apo-TC-sGCβ (within seconds), the mechanism is not likely to involve mitochondrial heme biosynthesis or release, because when these processes are stimulated it requires tens of minutes before there is a rise in the heme level of apo-TC-sGCβ (19). Rather, NO is likely to act on the heme transfer process itself. In the initial phase of NO-induced heme reallocation to apo-sGCβ, it is possible that NO acts on GAPDH–heme complexes that are already associated with the apo-sGCβ–hsp90 complex and thus are poised for an immediate heme transfer. Given that the heme with GAPDH has an open axial coordination site (57) and that NO is a strong heme iron ligand (58), NO could coordinate immediately to the GAPDH heme and thus influence the heme transfer process. Because the ferrous heme–NO complex of sGCβ is highly stable (14, 59), such NO–heme binding would create a positive thermodynamic driving force that supports a heme–NO species transfer from GAPDH to apo-sGCβ. Indeed, NO can promote a heme transfer from purified myoglobin to apo-sGC (51, 60), and how thermodynamic gradients might govern heme transfers within cells has been discussed (61). Our current study shows that heme transfer between proteins can quickly take place in living cells in response to low concentrations of NO, indicating that the process is biologically relevant. A cartoon that illustrates our findings and a possible mechanism for the NO-driven heme transfer to apo-sGCβ is presented in Fig. 8.

NO-driven heme allocation to apo-sGCβ may portend a general role for heme–NO in regulating cell heme allocations. Indeed, the molecular heme–NO complex is stable in cell culture and can enter cells to become quickly incorporated into apo-NADPH oxidase 5 (45). Heme–NO complexes have also been proposed to help drive NO bioactivity (62). Our findings and a possible mechanism for the NO-driven heme transfer to apo-sGCβ implies that it promotes a transfer within a protein–protein complex. Our findings also raise larger questions about how NO generated at normal or pathophysiological levels might impact an organism’s ability to traffic heme, utilize it for signaling, or regulate heme protein functions. These questions can now be explored.

Materials and Methods

Reagents. The TC-FlAsH In-Cell Tetracysteine Tag Detection Kit was obtained from Invitrogen. BAY41, the sGC activator BAY58, and the A23187 calcium ionophore were purchased from Sigma-Aldrich. All other reagents and materials were obtained from sources reported elsewhere (20, 23).

Molecular Biology. pCMV mammalian expression plasmids containing rat sGCα(1–690), sGCβ(1–619), or sGCα(1–619) Y135A R139A with a tetracysteine motif (CCPGC) at residues 239 to 244 have been reported previously (19). pDNA3.1 plasmids encoding HA-tagged human GAPDH wild type or H53A that are resistant to GAPDH siRNA (Dharmacon) have been reported previously (20).

Cell Culture. HEK293 cells and RAW264.7 cells were cultured as described previously (4, 20) and in SI Appendix.

Detection of cGMP. cGMP production by sGC in the HEK293 cell supernatants was measured by a previously described method (20). Briefly, guanosine triphosphate (0.25 mM), MgCl2 (0.5 mM), phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mM), sGC simulator BAY41 (10 μM), or sGC activator BAY58 (10 μM) was mixed with cell supernatant samples and incubated for 30 min at 37 °C. The cGMP produced was quantified using a cGMP ELISA Kit (Cell Signaling Technology). Absorbance values at 450 nm were read on a FlexStation 3 plate reader ( Molecular Devices), cGMP production values were normalized according to the total protein amounts in the cell supernatants.

In-Cell FlAsH Labeling of TC-sGCβ(1–619). TC-sGCβ(1–619) expressed in HEK293 cells was labeled with FlAsH using the method described previously (20, 24). Briefly, HEK293 cells expressing TC-sGCβ(1–619) grown in fluorescence plates were washed three times with phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) containing 1 g/L glucose. Cells were then incubated with a mixture of FlAsH and 1,2-ethanedithiol made in Opti-MEM (final concentrations 0.5 and 12.5 μM, respectively) for 30 min at 37 °C. Afterward, cells were washed three times with 250 μM, 1,2-ethanedithiol in phenol red-free DMEM with 10% fetal bovine serum (FBS) and then given phenol red-free DMEM with 10% FBS and 15 mM Hepes prior to measurements.

Monitoring the Heme Content of TC-sGCβ in Cells. Heme content of the FlAsH–TC-sGCβ(1–619) expressed in HEK293 cells was monitored using a method reported previously (20). Briefly, after labeling the TC-sGCβ in HEK293 cells with FlAsH, the fluorescence of FlAsH–TC-sGCβ in cells was monitored using a FlexStation 3 plate reader over time at 37 °C using excitation at 508 nm and emission at 528 nm.

Data Availability. All study data are included in the article and/or SI Appendix.

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