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Non-canonical chemical feedback self-limits nitric oxide-cyclic GMP signaling in health and disease

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Nitric oxide (NO)-cyclic GMP (cGMP) signaling is a vasoprotective pathway therapeutically targeted, for example, in pulmonary hypertension. Its dysregulation in disease is incompletely understood. Here we show in pulmonary artery endothelial cells that feedback inhibition by NO of the NO receptor, the cGMP forming soluble guanylate cyclase (sGC), may contribute to this. Both endogenous NO from endothelial NO synthase and exogenous NO from NO donor compounds decreased sGC protein and activity. This effect was not mediated by cGMP as the NO-independent sGC stimulator, or direct activation of cGMP-dependent protein kinase did not mimic it. Thiol-sensitive mechanisms were also not involved as the thiol-reducing agent N-acetyl-L-cysteine did not prevent this feedback. Instead, both in-vitro and in-vivo and in health and acute respiratory lung disease, chronically elevated NO led to the inactivation and degradation of sGC while leaving the heme-free isoform, apo-sGC, intact or even increasing its levels. Thus, NO regulates sGC in a bimodal manner, acutely stimulating and chronically inhibiting, as part of self-limiting direct feedback that is cGMP independent. In high NO disease conditions, this is aggravated but can be functionally recovered in a mechanism-based manner by apo-sGC activators that re-establish cGMP formation.

The nitric oxide (NO)-cGMP signaling pathway plays several essential roles in physiology, including cardio-pulmonary homeostasis1,2. The main receptor and mediator of NO’s actions is soluble guanylate cyclase (sGC), a heterodimeric heme protein. In its Fe(II)heme-containing state, sGC binds NO and is thereby activated to convert GTP to the second messenger, cGMP, whose steady-state levels are counter-regulated by different phosphodiesterases (PDEs)3. cGMP exerts its cardiopulmonary effects via activating cGMP-dependent protein kinase I (PKG)4. The latter results in protective vasodilation, anti-proliferation, and anti-thrombosis5. In disease, heme loss and the appearance of NO-insensitive apo-sGC impair NO-cGMP signaling6,7.

In addition to acutely activating sGC, NO appears to have further roles in sGC regulation. During enzyme maturation, NO facilitates heme incorporation into apo-sGC8,9, and activation of sGC by NO is followed by acute and rapid desensitization involving protein S-nitrosylation10,11. In addition, chronic exposure to NO donor drugs has been suggested to negatively affect sGC activity in a not fully reversible manner12–14. It is unclear, however, whether this pharmacological effect also pertains to endogenously formed NO and has pathophysiological relevance.

Here, we examine this important knowledge gap in the (patho)biology of NO. As model systems, we chose porcine pulmonary artery endothelial cells (PPAECs) as they relate to the clinical application of NO and cGMP-modulating drugs in pulmonary hypertension15,16. We investigate the effects of chronic exposure to exogenous (from NO donor drugs) and endogenous NO on sGC protein and activity in these cells. In addition, we investigate in health and disease, whether chronic effects of NO on sGC involve canonical cGMP signaling, thiol
modulation, or formation of heme-free sGC (apo-sGC). As disease model, we use again a condition related to pulmonary hypertension and chronically elevated levels of NO, i.e., porcine acute respiratory disease syndrome (ARDS)\(^{17,19}\).

**Results**

**NO chronically decreases vascular sGC protein and activity in vivo and in vitro.** For analyzing the chronic effects of NO at a mechanistic level, PPAECs were incubated for up to 72 h in the presence of the NO synthase (NOS) inhibitor, N\(^5\)-nitro-L-arginine methyl ester (L-NAME), and sGC expression and activity were measured. In the presence of L-NAME to eliminate endogenous NO formation, protein levels of the heme-binding sGC\(^{\alpha}\) subunit were increased (Fig. 1A), and this was also associated with increased sGC activity (Fig. 1B). Next, we tested the reverse, i.e., whether an increase of NO to supra-physiological concentrations\(^{20,22}\) by chronic exposure to the long-acting NO donor compound, DETA/NO, downregulates sGC. When establishing the concentration-dependence of DETA/NO on sGC expression, we found 100 µM to exert a maximal downregulation (Supplementary Fig. S1) without affecting cell viability. In line with this, DETA/NO was also used in previous studies at a concentration of 100 µM to mimic chronically high-NO disease conditions\(^{20,22}\). Therefore, in all subsequent experiments, cells were exposed to 100 µM DETA/NO, unless otherwise indicated. Pre-incubating PPAECs with DETA/NO (100 µM) decreased both sGC\(^{\alpha}\) and sGC\(^{\beta}\) protein (Fig. 1C) and sGC activity (Fig. 1D). Thus, *in-vitro* in PPAECs, endogenous NO chronically downregulates sGC protein and activity in an L-NAME-reversible manner, and this is further aggravated by exogenous, pharmacologically applied NO in supra-physiological concentrations.

Next, we wanted to validate these *in-vitro* observations at an *in vivo* level. To eliminate endogenous NO formation similar to the *in-vitro* L-NAME experiment, we chose eNOS knockout mice (eNOS\(^{-/-}\)); as a high-NO condition, the previously extensively validated porcine ARDS model\(^{17,19,23}\). In line with our observations in PPAECs, eNOS\(^{-/-}\) mice showed increased protein levels of sGC\(^{\alpha}\) and sGC\(^{\beta}\) (Fig. 1E) and increased sGC-activity (Fig. 1F). In the high-NO porcine ARDS model, sGC\(^{\alpha}\) and sGC\(^{\beta}\) protein levels (Fig. 1G) and sGC activity were decreased (Fig. 1H). These data collectively suggest in both *in-vitro* and *in vivo* that decreasing endogenous NO elevates, and increasing it lowers sGC protein subunit levels and sGC activity (Fig. 1I), respectively.

**cGMP/PKG does not mediate the downregulation of sGC protein and activity by chronic NO.** Next, we aimed to clarify the mechanisms underlying the downregulation of sGC protein and activity by chronic NO. First, we tested whether cGMP/PKG signaling is involved, as it had been shown previously to decrease both sGC activity\(^{26,29}\) and expression\(^{28,29}\). Of experimental importance, all passaging can cause downregulation of PKG and prevent the detection of its dependent signaling\(^{28,29}\). Hence, we, therefore, restricted our studies to low passage number cells and ensured fully functional PKG signaling by validating the known autoregulation of PKG expression\(^{26,29}\). Indeed, in our PPAEC system, both the PKG activator, 8-Br-cGMP, and the NO-independent sGC stimulator and PDE inhibitor, YC-1\(^{27}\), were able to reproduce the reduction of PKG expression (Supplementary Fig. S2) confirming the presence of a fully functional PKG. We then studied whether the observed downregulation of sGC protein and activity by NO can be mimicked by cGMP or is prevented by inhibiting PKG. When we exposed PPAECs, however, for 72 h to different concentrations of the sGC stimulator and PDE inhibitor, YC-1\(^{27}\), to raise cGMP in a NO-independent manner, or to the direct PKG activator, 8-Br-cGMP, neither sGC protein nor activity were lowered (cf. to Fig. 1). Unexpectedly, we observed even a slight upregulation of sGC protein (Fig. 2A,B). Consistent with this, the NO-induced downregulation of sGC could not be prevented by co-incubation with the PKG inhibitor, Rp-8-Br-PET-cGMPS (Supplementary Fig. S3). To extend these *in-vitro* findings to the *in vivo* level, we studied sGC expression and activity in PKG knockout mice (PKG\(^{-/-}\)). Consistent with our *in-vitro* data, sGC protein levels (Fig. 2D) and sGC activity (Fig. 2E) were unchanged in PKG\(^{-/-}\) compared to wildtype mice.

In conclusion, both our *in vivo* and *in vitro* data suggested that the downregulation of sGC protein and activity by chronic NO is cGMP- and PKG-independent and thus appeared to be due to a non-canonical mechanism (Fig. 2C). At least two cGMP-independent effects on sGC have been reported, rapid desensitization\(^{26,29}\), which is reversible in a thiol-dependent manner\(^{26,34}\), and oxidative heme-loss yielding the NO-insensitive apo-form of sGC (apo-sGC)\(^{7,37}\). These possibilities were tested in our two next sets of experiments.

**N-acetyl-L-cysteine does not prevent NO-induced sGC downregulation.** Thiol-sensitive mechanisms are involved in both sGC regulation, such as sGC maturation, and airway pathologies such as asthma\(^{20}\). Therefore, we assessed whether NO-posttranslational modification of free-thiol cysteines i.e., S-nitrosylation, contributes to the downregulation of sGC by high chronic NO incubation. For this approach, PPAECs were again exposed for 72 h to DETA-NO (100 µM) in absence or presence, over the full-time frame, of the membrane-permeable thiol-reducing agent, N-acetyl-L-cysteine (NAC; 1 mM). NAC is a membrane-permeable de-nitrosylating agent and glutathione precursor that has been extensively validated to protect sGC from nitrosylation\(^{32,33}\) down to concentrations as low as 1 mM\(^{40,41}\), which we, however, did not re-validate. NAC, however, neither affected sGC protein levels (Fig. 3A) nor sGC activity (Fig. 3B), suggesting that it is unlikely that a thiol-reversible mechanism similar to the acute desensitization is involved in the chronic NO-induced downregulation of sGC. These findings left oxidative heme-loss yielding apo-sGC\(^{7,37}\) as the most likely cGMP-independent effect on sGC.

**NO-induced sGC downregulation generates NO-insensitive sGC.** We, therefore, examined whether chronic NO converts sGC to apo-sGC. To assay for the presence of apo-sGC, we took advantage of the apo-sGC activator, BAY 58-2667 (cinaciguat), which specifically binds to the empty heme-binding pocket of apo-sGC and re-activates cGMP formation in a NO-independent manner\(^{42}\). Indeed, up to 72 h exposure of PPAECs to...
Figure 1. Chronic NO decreases vascular sGC protein and activity in-vivo and in-vitro. (A) Inhibiting basal NO formation in PPAECs by L-NAME (100 μM) for up to 72 h increased sGCβ1 expression (N = 6). (B) This upregulation was associated with increased sGC activity (N = 3). Exposing cells to supra-physiological levels of NO by chronic exposure to the NO donor compound, DETA/NO (100 μM), for up to 72 h decreased both sGCα1 and sGCβ1 protein (C) (N = 6) and sGC activity (D) (N = 5). In-vivo validation of the in-vitro observations showed in eNOS knockout mice (eNOS−/−) mice increased sGC protein (E) and activity levels (F) (N = 9), and in a porcine lung disease model (ARDS) characterized by NO overproduction, decreased sGCα1 and sGCβ1 protein (G) (N = 5) and sGC activity levels (H) (N = 3). Data are expressed as mean ± SEM. *,**,**,***p < 0.05, 0.01 or 0.001 vs. control, respectively. (I) A schematic summary showing that both in-vitro (porcine lung endothelial cells) and in-vivo (the porcine lung disease model, ARDS) both endogenous and exogenous NO downregulate sGC protein and activity. Representative full-length blots are presented in Supplementary Figure S4.
DETA-NO (100 µM) increased apo-sGC activity, measured as BAY 58-2667-induced cGMP formation (Fig. 3C), and reduced sGC activity (Fig. 1D). To validate this mechanistic finding in-vivo, we re-examined the high-NO porcine ARDS model and found indeed apo-sGC activity to be increased (Fig. 3D). Collectively, this established apo-sGC formation as one possible mechanism of NO-induced reduction in sGC activity in addition to the loss in sGC protein (Fig. 3E).

Discussion

Our findings close important gaps in our understanding of NO-cGMP signaling, in particular on the long-term effects of endogenously formed NO versus NO donors on sGC and the pathophysiological relevance of chronic NO for sGC regulation. Thus, we expand the previously observed notion that NO donor drugs can reduce sGC mRNA to the protein level and, importantly, from pharmacology to endogenous NO. Previously, sGC protein levels were not consistently investigated or with antibodies of unclear specificity. Moreover, only in some cases were the effects of PKG on cGMP levels investigated or in relation to cGMP metabolism rather than cGMP formation.

Surprisingly, not only pathological/high levels of NO, as in our porcine ARDS model but already low chronic endogenous NOS activity, suppressed sGC protein and activity in an L-NAME reversible manner. These findings establish a previously not recognized delicate steady state in the interactions between NO and sGC, acutely stimulating and chronically limiting its expression and activity. On a positive note, under conditions of diminished NO synthesis, this may, in turn, rapidly upregulate sGC protein and activity, as we have observed in the presence of the NOS inhibitor, L-NAME, and in-vivo in eNOS−/− mice. In this regard, previous data are controversial. For example, sGC activity was increased in eNOS−/− mice, which agrees with our findings, while others found neither sGC expression nor activity to be changed, neither in eNOS−/− mice nor upon treatment with high doses of NO donors. The reasons for this discrepancy are unclear. In our ARDS model, the pan-NOS inhibitor, L-NAME, could not differentiate between eNOS and other isoforms present, such as iNOS. Activation of the latter, e.g., by nuclear factor kappa B (NFκB) increases peroxynitrite leading to sGC oxidation and down-regulation of sGCβ. In endothelial cells and under physiological conditions, however, iNOS is not expressed or only at low levels and thus unlikely to be involved in the here observed non-canonical feedback.

Therapeutically relevant is the previously not recognized risk of chronic use of NO donor drugs as they will lead to a downregulation of both sGC protein and activity. Together with their problematic pharmacokinetic tolerance, this adds to the clinical limitations of this widely used drug class. With the introduction of NO-independent sGC stimulators and cGMP elevating agents into clinical practice, there is now an
alternative. Indeed, we show that the prototypic sGC stimulator and PDE inhibitor, YC-1, does not lead to sGC
downregulation.

Concerning the underlying mechanisms, we initially considered two known mechanisms in NO-cGMP physiology, i.e., cGMP/PKG and thiol modification. Surprisingly, both could be excluded, which was reminiscent of earlier observations where long-term exposure to an exogenous NO donor reduced sGC activity in a manner that could not be reversed or prevented with thiol treatment. Instead, our findings suggest that endogenous NO or pharmacological NO donor compounds that acutely stimulate sGC, chronically decreased both sGC protein and activity leading to inactivation of sGC and an apparent net shift towards NO-insensitive apo-sGC. Data are expressed as mean ± SEM. **p < 0.05 or 0.01 vs. control, respectively. Representative full-length blots are presented in Supplementary Figure S4.

**Figure 3.** NO-induced sGC downregulation is thiol-independent but involves sGC loss and a shift towards apo-sGC. When PPAECs were exposed for 72 h to DETA-NO (100 µM) in the absence and presence of N-acetyl-L-cysteine (NAC; 1 mM), NAC neither affected sGC protein levels (N = 5) (A) nor activity (N = 4) (B). Exposure of PPAECs for 72 h to DETA-NO (100 µM) increased apo-sGC activity, measured as BAY 58-2667-induced cGMP formation (BAY 58-2667, 10 µM) (N = 3) (C). Validation of the above in-vitro mechanistic findings in-vivo in the porcine high-NO ARDS model showing also increased apo-sGC activity (N = 3) (D). (E) A scheme summarizing both our in-vitro and in-vivo data that both endogenous NO or pharmacological NO donor compounds that acutely stimulate sGC, chronically decreased both sGC protein and activity leading to inactivation of sGC and an apparent net shift towards NO-insensitive apo-sGC. Data are expressed as mean ± SEM. **p < 0.05 or 0.01 vs. control, respectively. Representative full-length blots are presented in Supplementary Figure S4.

Our findings also add to our understanding of apo-sGC as a therapeutic target. Hitherto apo-sGC has been mainly studied by using the heme oxidant, ODQ, or by expressing enzyme where the proximal heme ligation histidine had been deleted. The mechanisms by which apo-sGC forms in pathophysiology were less clear. Now chronic exposure to (high) levels of NO can be considered as one of these conditions. There are at least three possible non-canonical mechanisms by which high levels of NO can induce the transition from sGC to apo-sGC. First, NO can interact with reactive oxygen species to form the potent oxidant, peroxynitrite. Second, NO can impair heme synthesis or activate heme oxygenase-1 (HO-1), which increases sGC heme degradation. However, this effect is controversial since high concentrations of NO donors (including DETA-NO) inhibit the
heme degradation in endothelial cells. Third, high NO can increase the association of sGCβ1 with heat shock protein 90 (Hsp90), but not with sGCα1, resulting in the formation of NO-insensitive sGC.

Of note, the shift from sGC to apo-sGC is not 1-to-1. Some sGC appears to be lost due to inactivation beyond recovery by apo-sGC activators, e.g., by channeling into the ubiquitylation-proteasome pathway. Nevertheless, an apparent net shift from sGC to apo-sGC as the primary source of cGMP formation is a common denominator and has recently been observed by us in another high NO model of ischemic stroke and others in an asthma model. In contrast to other observations, in our settings, chronic NO incubation for 72 h versus overnight, did affect sGCβ1 expression independent of S-nitrosylation.

In conclusion, our data suggest that both in-vitro and in-vivo, and both under physiological conditions and in disease, NO self-limits its ability to induce cGMP formation via chemical redox feedback, which inactivates sGC and causes an apparent net shift towards apo-sGC. Our findings are of direct therapeutic importance as a pathological sGC/apo-sGC ratio can be treated with sGC activator compounds, thereby reinstalling cGMP synthesis and PKG signaling. Moreover, concerning the long-established class of NO donor drugs and the use of inhaled NO, a cautionary note needs to be added. Not only do they cause reversible tolerance, but also, as we now find, irreversible downregulation of sGC and apo-sGC formation. Our data explain, thus, also the superiority of the novel NO-independent sGC stimulators, at least in indications such as pulmonary hypertension.

**Materials and Methods**

**Chemicals.** Polyclonal antibodies specific for sGCα1 and sGCβ1 have been described elsewhere. Actin monoclonal antibody (Oncogene Research Products, Boston, USA); collagenase type CLS II (Merck, Netherlands); 8-Bromo-cGMP (BIOLOG, Germany); L-NAME, DETA/NO, DEA/NO, IBMX and GTP (Enzo Life Sciences, Netherlands); BAY 58-2667 was synthesized as described. All other chemicals were of the highest purity grade available and obtained from Sigma or Merck (Netherlands). DETA/NO and DEA/NO were dissolved in 10 mM NaOH, BAY 58-2667 and YC-1 in DMSO.

**Tissue isolation.** Thoracic aortae from i) 6- to 8-months old male PKG−/− and age-matched control mice were obtained from Prof. Franz Hofmann, Department of Pharmacology and Toxicology at the Technical University Munich (genetic background 129/Sv), and ii) 6- to 8-months old male eNOS−/− mice and age-matched control were obtained from the Department of Physiology at Heinrich-Heine-Universität Düsseldorf (genetic background C57BL/6). Animals’ care was in accordance with guidelines of Technical University Munich and Heinrich-Heine-Universität Düsseldorf. Experimental protocols were approved by the animal ethics committees of Technical University Munich and Heinrich-Heine-Universität Düsseldorf. Thoracic aortae were grounded in a mortar to a powder that was used for protein determination, Western blots, or sGC activity assays. About 50 mg of tissue powder was suspended in homogenization buffer and boiled in 10 mM NaOH, BAY 58-2667 and YC-1 in DMSO.

**Preparation of pulmonary arteries from a porcine ARDS model.** Pigs were acclimated for at least 24 h before use in the study and handled carefully to avoid any stress. Body temperature was kept constant using a circulating-water heating pad and cage heating. The porcine ARDS model was induced as previously described. Briefly, pigs (30–35 kg) were pre-medicated with midazolam (1 mg/kg i.m.) and ketamine (10 mg/kg i.m.); intravenous anesthesia was induced and maintained with midazolam (bolus 0.5–1 mg/kg, followed by 1–2 mg/kg/h) and ketamine (bolus 3–4 mg/kg, followed by 10–18 mg/kg/h). Neuromuscular block was achieved with atracurium (bolus 3–4 mg/kg, followed by 1–2 mg/kg/h). Pigs were mechanically ventilated in a volume-controlled mode with the following settings: Tidal volume (Vt) of 8 ml/kg, positive end-expiratory pressure (PEEP) of 5 cm H2O, the fraction of inspired oxygen (FiO2) of 1.0, respiratory rate (RR) adjusted in accordance to an arterial partial pressure of carbon dioxide (PaCO2) between 35–45 mmHg and inspiration to expiration (I:E) of 1:1. The overall duration of mechanical ventilation was 10–11 h. After that, animals were sacrificed by intravenous boluses of 2 g thiopental and 50 ml KCl i M, and organs were snap-frozen in liquid nitrogen. Pulmonary arteries were removed immediately after death, snap-frozen in liquid nitrogen and stored at minus 80 °C or otherwise processed immediately to tissue powder and subsequently suspended in homogenization-buffer and homogenized in an Ultra Turrax at 4 °C. Samples were diluted with four volumes of Rotiload buffer (Roth, Germany) and boiled for 5 min. These samples were then used further for protein determination, protein immune blots, and sGC activity assays.
Detection and quantification of sGC protein. Western blotting procedures were described previously.

Briefly, cells were lysed in 250 μL Roti-Load sample buffer (ROTH, Karlsruhe, Germany), preheated to 95 °C and then boiled for an additional 10 min before loading on SDS gel electrophoresis. Primary antibodies were diluted 1:4000 for anti-sGCSR and 1:2000 for anti-sGCα, antibody in 3% dry milk in TBST and incubated with nitrocellulose membranes at 4 °C overnight following challenge of membranes with secondary goat anti-rabbit antibody (1:2000 in 3% milk in TBST) conjugated to horseradish peroxidase (Dako A/S, Denmark). Immuno-complexes were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Freiburg). Samples were quantified with a Kodak Image Station 440 CF and with the NIH 1.6 software, and all blots were standardized to β-actin or GAPDH expression that was not affected by the treatments. Representative western blot examples are shown in Supplementary Fig. S4.

Measurement of cGMP levels. For measuring cGMP levels, cells were pretreated for 30 min with the phosphodiesterase inhibitors84, IBMX (1 mM) and zaprinast (100 μM). Then, sGC was consistently stimulated with 250 μM DEA/NO or 10 μM BAY 58-2667 for 3 min at 37 °C. The high concentration of DEA/NO (250 μM) was chosen based on previous studies using endothelial cells, including PPAEC63,67,85. However, other studies showed that cGMP accumulation in endothelial cells in response to DEA/NO peaked at about 1 μM88. Of note, however, there are controversial data regarding the NO peak concentration released from DEA/NO. One study67 showed that 250 μM DEA/NO would give a peak NO concentration of about 30 μM while another study88 showed that 1 mM DEA/NO would release 2.87 μM/min of NO during 3 minutes. Therefore, 250 μM would release 0.71 μM/min of NO. Additional factors, such as medium components may be responsible for this broad range of apparent free concentrations of NO.

After sGC stimulation, cells were immediately lysed in 80% ethanol. Cells were scraped and, after evaporation of ethanol, resuspended in assay buffer and sonicated. Measurement of sGC activity in crude homogenates of porcine tissue was performed as previously described85. Briefly, all samples were measured as the formation of cGMP at 37 °C during 10 min in a total incubation volume of 100 μL containing 50 mM triethanolamine–HCl (pH 7.4), 3 mM MgCl2, 3 mM glutathione, 1 mM IBMX, 100 mM zaprinast, 5 mM creatine phosphate, 0.25 mg/mL creatine kinase and 1 mM or 0.5 mM GTP. The reaction was started by the simultaneous addition of the sample and either DEA/NO or BAY 58–2667, respectively. After incubation of each sample for 10 min, the reaction was stopped by boiling for 10 min at 95 °C. Thereafter the amount of cGMP was subsequently determined by a commercial enzyme immunoassay kit (Enzo Life Sciences, Netherlands).

Statistics. For comparisons, Student's t-test or multiple comparisons one-way analysis of variance (ANOVA) was followed by Bonferroni's test. Calculations were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, USA). All data are expressed as mean ± SEM. P-value <0.05 was considered significant.

Data availability
All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

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References
1. Bian, K., Dousset, M.-F. & Murad, F. Vascular system: role of nitric oxide in cardiovascular diseases. Journal of clinical hypertension (Greenwich, Conn.) 10, 304–310 (2008).
2. Ignarro, L. J. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. Journal of physiology and pharmacology: an official journal of the Polish Physiological Society 53, 503–514 (2002).
3. Cary, S. P L., Winger, J. A. & Marletta, M. A. Tonic and acute nitric oxide signaling through soluble guanylate cyclase is mediated by nonheme nitric oxide, ATP, and GTP. Proceedings of the National Academy of Sciences of the United States of America 102, 13064–13069, https://doi.org/10.1073/pnas.0506289102 (2005).
4. Tsai, E. J. & Kass, D. A. Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. Pharmacology & therapeutics 122, 216–238, https://doi.org/10.1016/j.pharmthera.2009.02.009 (2009).
5. Förstermann, U., Xia, N. & Li, H. Roles of Vascular Oxidative Stress and Nitric Oxide in the Pathogenesis of Atherosclerosis. Circulation research 120, 713–735, https://doi.org/10.1161/CIRCRESAHA.116.309326 (2017).
6. Langenhuaber, F. et al. A diseasome cluster-based drug repurposing of soluble guanylate cyclase activators from smooth muscle relaxation to direct neuroprotection. NPJ systems biology and applications 4, 8, https://doi.org/10.1038/s41540-017-0039-7 (2018).
7. Stasch, J.-P. et al. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. The Journal of clinical investigation 116, 2552–2561, https://doi.org/10.1172/JCI28371DS1 (2006).
8. Ghosh, A., Stasch, J.-P., Papapetropoulos, A. & Stuehr, D. J. Nitric oxide and heat shock protein 90 activate soluble guanylate cyclase by driving rapid change in its subunit interactions and heme content. The Journal of biological chemistry 289, 15259–15271, https://doi.org/10.1074/jbc.M114.559393 (2014).
9. Ghosh, A. & Stuehr, D. J. Soluble guanylyl cyclase requires heat shock protein 90 for heme insertion during maturation of the NO-active enzyme. Proceedings of the National Academy of Sciences of the United States of America 109, 12998–13003, https://doi.org/10.1073/pnas.1205854109 (2012).
10. Beuve, A. Thiol-Based Redox Modulation of Soluble Guanylyl Cyclase, the Nitric Oxide Receptor. Antioxidants &amp; redox signaling, ars.2015.6591, https://doi.org/10.1089/ars.2015.6591 (2016).
11. Mayer, B. et al. Inactivation of soluble guanylate cyclase by stoichiometric S-nitrosation. Mol Pharmacol 75, 886–891, https://doi.org/10.1124/mol.108.052142 (2009).
86. Schmidt, K., Schrammel, A., Koesling, D. & Mayer, B. Molecular mechanisms involved in the synergistic activation of soluble guanylyl cyclase by YC-1 and nitric oxide in endothelial cells. *Mol Pharmacol* 59, 220–224, https://doi.org/10.1124/mol.59.2.220 (2001).

87. Schmidt, K., Desch, W., Klatt, P., Kukovetz, W. R. & Mayer, B. Release of nitric oxide from donors with known half-life: a mathematical model for calculating nitric oxide concentrations in aerobic solutions. *Naunyn Schmiedebergs Arch Pharmacol* 355, 457–462, https://doi.org/10.1007/pl00004969 (1997).

88. Zou, M. H. & Ullrich, V. Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett* 382, 101–104, https://doi.org/10.1016/0014-5793(96)00160-3 (1996).

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**Author contributions**

H.H.H.W.S. designed the research; V.T.D., M.D., P.I.N., A.Gü., C.I.A. performed research; A.Gü. and A.Gö. contributed to new reagents/analytic tools; V.T.D., M.E., M.D., P.I.N., C.I.A. and H.H.H.W.S. analyzed data; and V.T.D., M.E., A.Gö. and H.H.H.W.S. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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