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

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Abstract (240 words)

Endothelial nitric oxide (NO) stimulates the heme protein, soluble guanylyl cyclase (sGC) to form vasoprotective cyclic GMP (cGMP). In different disease states such as pulmonary hypertension, NO-cGMP signaling is pharmacologically augmented, yet the pathomechanisms leading to its dysregulation are incompletely understood. Here we show in pulmonary artery endothelial cells that endogenous NO or NO donor compounds acutely stimulate sGC activity, but chronically down-regulate both sGC protein and cGMP formation. Surprisingly, this endogenous feedback mechanism was independent of canonical cGMP signaling via cGMP-dependent protein kinase. It did not involve thiol-dependent modulation, a process relevant for sGC maturation, either. Rather tonic NO exposure led to inactivation and degradation of NO-sGC and without affecting NO-insensitive apo-sGC levels. Apo-sGC could be re-activated pharmacologically by the heme mimetic class of so-called sGC activators. Importantly, this non-canonical feedback was also observed *in vivo*. Specifically, it was induced by pathological high levels of NO in acute respiratory distress syndrome in which a similar self-limiting redox shift from NO-sensitive sGC to NO-insensitive apo-sGC occurred. Thus, our data establish a bimodal mechanism by which NO acutely stimulates sGC and chronically decreases sGC levels as part of a physiological and pathological self-limiting feedback. Of therapeutic importance in disease, our findings i) caution against any chronic use of classical NO donor drugs and ii) suggest that high NO-induced apo-sGC can be functionally fully recovered by sGC activator drugs to re-establish cGMP formation.

Significance Statement (122 of 120)

Dysfunctional nitric oxide (NO) signaling via the cyclic GMP (cGMP) forming heme-protein soluble guanylate cyclase (sGC) is a key cardiopulmonary disease mechanism. However, in particular during chronic use, NO donor drugs display limited therapeutic benefit. Here we identify a previously unrecognized non-canonical chemical feedback mechanism, which self-limits cGMP formation in response to NO donor drugs and endogenous NO, both in health and disease. Whilst NO acutely stimulates sGC, we find that exposure of sGC to either chronic NO or pathological NO overproduction reduces sGC and generates heme-free apo-sGC which is insensitive to NO but sensitive to heme-mimetic sGC activators. Importantly, this chemical feedback explains the limited applicability of NO-donor drugs for chronic treatment and explain their mechanism-based indication in vascular disease conditions.

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Introduction

The NO-cGMP signaling pathway plays an important role in cardiopulmonary homeostasis (1, 2). The best-defined receptor and mediator of NO's actions is soluble guanylate cyclase (sGC), a heterodimeric heme protein. During enzyme maturation, NO facilitates heme incorporation into sGC (3, 4). In its Fe(II)heme-containing state, sGC binds NO and is thereby activated to convert guanosine triphosphate (GTP) to the second messenger, cGMP (5), which exerts its cardiopulmonary effects via cGMP-dependent protein kinase (6). This results in potent vasodilatory, anti-proliferative and anti-thrombotic effects (7). In disease, heme loss and appearance of an apo-sGC has been described (8) (9).

Activation of sGC by NO can cause acute and, importantly, reversible desensitization involving possibly protein S-nitrosylation (10). Chronic exposure to NO donor drugs has been suggested to negatively affect sGC more long-term and in a not fully reversible manner (11-13), but it is unclear how this effect is mediated and whether it also pertains to endogenously formed NO.

Here, we examine this important knowledge gap in porcine pulmonary artery endothelial cells (PPAECs) as this excellently relates to the clinical relevance of NO and cGMP modulating drugs in pulmonary hypertension (14, 15). We compare the effects of chronic exposure to exogenous NO donor drugs and endogenous NO generated by endothelial NO synthase both on sGC protein and cGMP formation. In addition, we investigate i) whether any of the underlying mechanisms involves canonical cGMP signalling, thiol modulation, or NO-insensitive apo-sGC and ii) whether this would be relevant in disease. For the latter, we use a well-established high-NO model of porcine acute respiratory disease syndrome (ARDS) related also to pulmonary hypertension (16).

Our findings provide important new understandings of NO-cGMP signaling. Pharmacologically, we identify i) previously not recognized risks of chronic use of NO donor drugs and ii) how chronically high-NO disease conditions lead to cGMP deficiency that can be recovered in a mechanism-based manner through heme mimetic, apo-sGC activator drugs.

Results

Endogenous NO down-regulates vascular sGC protein and activity *in vivo* and *in vitro*.

We first tested whether endogenous NO instead of only pharmacological NO donor compounds downregulate sGC upon chronic exposure. Previously, protein levels were non-consistently investigated or the antibodies used were of unclear specificity (11, 17). Moreover, the functional consequences of cGK-I on cGMP levels were investigated only in some cases (18, 19) and in these cases rather due to upregulated cGMP metabolism rather than an effect on sGC activity (18, 20, 21). We therefore, initially studied this using *in vivo* models, *i.e.* i) endothelial NO synthase wildtype or knock-out ($eNOS^{-/-}$) mice, which are characterized by physiological or decreased NO levels, respectively or ii) in the porcine lung disease model of lavage-induced acute respiratory distress symptom (ARDS) which is characterized by NO overproduction (22). In $eNOS^{-/-}$ mice, $sGC\alpha_1$ and $sGC\beta_1$ levels were up-regulated (Fig. 1A). This up-regulation of sGC protein subunits was associated with increased NO-stimulated sGC-activity (Fig. 1B). In ARDS, $sGC\alpha_1$ and $sGC\beta_1$ protein levels were modulated albeit, this time modulation occurred in the opposite direction (Fig. 1C) and resulted in decreased sGC-mediated cGMP production (Fig. 1D). Collectively, these data suggest that *in vivo* low NO led to compensatory up-regulation of sGC subunits and pathological high levels of endogenous NO effectively down-regulate sGC.

Next, we investigated whether PPAECs could be a suitable *in vitro* model to mechanistically characterize our *in vivo* observations. In line with the observations in $eNOS^{-/-}$ mice, 72h incubation of PPAECs in presence of the NO synthase (NOS) inhibitor N^G-nitro L-arginine methyl ester (L-NAME), increased $sGC\beta_1$ levels whilst $sGC\alpha_1$ levels remained unchanged (Fig.

2A). This up-regulation of sGCβ_1 protein was associated with increased sGC-activity (Fig. 2B). To test the converse, we increased PPAECs NO levels by incubating the cells with supra-physiological amounts of NO using the long-acting NO donor compound, DETA/NO (100 μM). Indeed, incubation with DETA/NO decreased sGCα_1 and sGCβ_1 protein levels (Fig 2C) and decreased DEA/NO-induced cGMP production (Fig. 2D). Thus, in PPAECs low levels of NO increase sGC expression and activity and high levels of NO down-regulate sGC.

cGK-I does not mediate the down-regulation of sGC expression and activity by chronic NO. Having established the effect of chronic/tonic NO on sGC protein and activity, we aimed to clarify the underlying mechanisms. Indeed, cGK-I, has been shown to reduce sGC mRNA levels (18). Cell passaging can cause downregulation of cGMP-dependent protein kinase-I (cGK-I), a critical component in the NO-cGMP pathway (23-26). Hence, we therefore restricted our studies to low passage number cells and ensured fully functional cGK-I signaling by validating that the NO donor, DETA-NO, the cGK-I activator, 8-Br-cGMP, or the NO-independent Fe(II)sGC stimulator were able to reduce cGK-I expression (Fig. 3A), an expressional regulation known to be cGMP/cGK-I-dependent (27).

sGK-I-mediated, cGMP-dependent cGK-I auto-regulation has been shown (28). Thus, having established functional cGK-I signaling in PPAECs, we studied whether a similar mechanism affects sGC expression by exposing PPAECs for 72h with different concentrations of YC-1 or 8-Br-cGMP. However, none of these compounds affected sGC protein levels like NO (*cf.* to Fig. 2). In fact, we observed an up-regulation of sGC (Fig. 3B/C). Consistent with this, DETA/NO induced down-regulation of sGC could not be reversed by the cGK-I inhibitor, RP-8-Br-PET (Fig. 3D).

We subsequently aimed to validate our *in vitro* findings *in vivo* using cGK-I knock-out (cGK-I$^{-/-}$) mice (29). Indeed, cGK-I$^{-/-}$ mice displayed normal sGC expression levels (Fig. 4A) and

unchanged sGC activity (Fig. 4B) as compared to wildtype controls. The apparent lower sGC activity level in cGK-I knock-out mice did not reach significance, but would be consistent with the up-regulation observed with cGK-I stimulators observed *in vitro*. In conclusion, our *in vivo* and *in vitro* data suggested that down-regulation of sGC expression by NO is both cGMP- and cGK-I-independent and thus non-canonical.

NO-induced sGC down-regulation is thiol-independent and generates NO-insensitive sGC.

Since our data suggested non-canonical sGC donw-regulation, we next considered two known cGMP-independent effects on Fe(II)sGC: rapid, reversible thiol-dependent desensitization (30, 31) or oxidative heme-loss. Importantly, both yield an NO-insensitive apo-sGC (8, 32).

To assess thiol-dependence of the sGC down-regulation we incubated PPAECs with DETA-NO (100μM for 72h) in absence or presence of the membrane-permeable thiol-reducing agent, N-acetyl-L-cysteine (NAC; 1mM for 72h). Presence of NAC did not affect sGCα_1 and sGCβ_1 protein expression (Fig. 5A), nor NO-stimulated sGC activity (Fig. 5B). These data suggested that it is unlikely that a thiol dependent desensitization-like mechanism (10, 33, 34) is involved in chronic NO-induced reduction in sGC expression/activity.

To examine whether chronic NO exposure causes formation of NO-insensitive apo-sGC we took advantage of one of the apo-sGC activator drugs (*i.e.* BAY 58-2667) which specifically bind to the empty heme binding pocket of apo-sGC and mimic heme sterically, with respect to its charge distribution, and regarding heme's effects on propionic acid substituents (35). Again, to simulate chronic NO exposure we incubated PPAECs with DETA-NO (100 μM for 1 to 72h) after which we assayed BAY 58-2667-stimulated (apo-)sGC activity. Indeed, NO incubation increased BAY 58-2667-induced apo-sGC activity within 1h and this increased stability remained stable

for at least 72h post-DETA-NO treatment (Fig. 6A). In parallel, DETA/NO incubation reduced NO-stimulated sGC activity within 1 h and this reduction was stable over time (Fig. 6A).

To validate this important mechanistic finding *in vivo*, we used the porcine ARDS model. Consistent with our *in vitro* findings, NO-stimulated sGC activity in ARDS animals was strongly reduced (Fig. 1D) while apo-sGC activity increased to a smaller extent (Fig. 6B). This was prominently evidenced by a dramatic shift in the cGMP forming capacity from NO-sensitive sGC towards apo-sGC (Fig. 6C). These data suggested that NO signaling by a self-limiting down-regulation of sGC is caused omission of NO-sensitive sGC so that NO-insensitive, but BAY 58-2667-sensitive apo-sGC remains. The higher protein levels in the presence of BAY 58-2667 can be explained by the fact that apo-sGC is prone to proteolytic degradation and stabilized by apo-sGC activator compounds (36, 37).

In conclusion, our data suggest that both *in vitro* and *in vivo*, both under physiological conditions and in disease NO appears to self-limit its ability to induce cyclic GMP formation via a chemical redox feedback which causes inactivation of sGC and subsequent generation, and degradation of NO-insensitive apo-sGC. Thus, our findings explain i) why cGMP synthesis is diminished in the context of chroming treatment with NO-donor drugs and ii) why apo-sGC activator compounds such as BAY 58-2667 should be considered as an alternative to NO-donors (4, 8).

Discussion

Here, we establish and characterize a previously unrecognized redox mechanism exerting an irreversible negative feedback within NO-cGMP signaling both under physiological and, more so, under pathological high NO conditions. NO not only facilitates sGC's maturation but also induces a small tonic degradation pressure generating also low levels of apo-sGC.

With respect to the underlying mechanism, we initially considered the two canonical mechanisms that are known to mediate or modulate NO-cGMP signaling, *i.e.* activation of cGK-I following an increase in cGMP, and short-term, reversible desensitization of sGC by thiol modification (4, 10). Surprisingly, both could be excluded. Instead, our finding indeed suggest a novel mechanism of sGC regulation reminiscent of an earlier observation where a longer term (20 h) exposure to an NO donor reduces sGC activity irreversibly and in a manner that could not be recovered by thiol treatment (13). The availability of sGC activator compounds now allowed us to fully elucidate the mechanism as related to a combination of sGC loss and apo-sGC formation. Our present study thereby fills an important knowledge gap in the pharmacology of sGC and establishes a third mechanism in sGC regulation by chronic NO. In health and in disease, this leads to a shift in the sGC redox ratio from NO-sensitive sGC to NO-insensitive apo-sGC.

These findings add both an important component to our understanding of this clinically highly relevant vasoprotective signaling pathway and explains its dysregulation in disease. Under disease conditions, the dysregulation of the sGC/apo-sGC ratio can apparently lead to fundamental changes in NO-cGMP signaling and this may be particularly relevant in conditions characterized by pathologically high NO levels. The latter include sepsis (38), (lipopolysaccharide-induced) lung injury (39) and a porcine model of acute respiratory distress syndrome (ARDS) in which several fold increased levels of endogenous NO are observed systemically (40), where we could now clarify the mechanism of the underlying dysfunctional cGMP signaling. We postulate that this will also explain why cGMP-signaling is impaired in other forms of high-NO conditions, such as stroke (41) and neurotrauma (42).

Our findings are also of direct therapeutic importance as a pathological sGC/apo-sGC ratio can be treated with sGC activator compounds such as BAY58-2667 (36) thereby reinstalling cGMP synthesis and cGK-I signaling (32, 43). However, with respect to the long-

established class of NO donor drugs a cautionary not needs to be added. Not only do they cause tolerance, but, as we now find, also irreversible downregulation of sGC and apo-sGC formation; this may explain the superiority of novel, NO-independent sGC modulation drugs in pulmonary hypertension, such as sGC stimulators (15).

Materials and Methods

Chemicals. Polyclonal antibodies specific for sGCα_1 and sGCβ_1 have been described elsewhere (30). Actin monoclonal antibody (Oncogene Research Products, Boston, USA); collagenase type CLS II (Biochrom, Berlin, Germany); 8-bromo-cGMP (BIOLOG, Bremen, Germany); L-NAME, DETA/NO and DEA/NO (Alexis, Lausen, Switzerland); IBMX and GTP (Enzo LifeSciences, Lörrach, Germany); YC-1 was a kind gift from Dr. Schönafinger, Aventis Pharma (Frankfurt, Germany). BAY 58-2667 was synthesized as described (44) . All other chemicals were of the highest purity grade available and obtained from either Sigma Chemicals (Deisenhofen, Germany) or Merck AG (Darmstadt, Germany). DETA/NO and DEA/NO were dissolved in 10 mM NaOH, BAY 58-2667 and YC-1 in DMSO.

Tissue isolation. Tissues from i) 6- to 8-months old male cGK-I$^{-/-}$ and age-matched control mice were obtained from the Department of Pharmacology and Toxicology at Technical University Munich (genetic background 129/Sv) (31) 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) (32).

Preparation of pulmonary arteries from a porcine ARDS model. Pulmonary arteries were removed immediately after death from an experimental porcine model of acute respiratory disease symptom (ARDS), as previously described (33). Pulmonary arteries were 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. These samples were then used further for protein determination, protein immune blots and sGC activity assays.

PPAECs. Fresh porcine pulmonary arteries were obtained from a local slaughterhouse and maintained in phosphate-buffered saline (PBS; 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 140mM NaCl, 2.7mM KCl, pH 7.4) at 37°C. PPAECs were isolated enzymatically by incubation of the aorta inner surface with collagenase type CLS II (0.5 mg/mL for 10 min at room temperature) and then collected in HEPES-buffered medium 199 (Sigma, Deisenhofen, Germany). After centrifugation (250 x g, 10 min) the pellet was re-suspended in growth medium (medium 199 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin) and cells were propagated in coated plastic flasks and incubated (37°C, 6% CO_2). Upon confluence, endothelial cell monolayers were sub-cultured in 35-mm (for Western blot) or 60-mm (for cGMP determination) gelatine coated dishes. Confluent cell monolayers from the second passage were used for experiments. The growth medium was replaced either every 12 or 24 hours if applicable containing the indicated compounds. After incubation time cells were subsequently used for sGC activity measurements or western blot analysis.

Detection and quantification of sGC protein. Western blotting procedures were described previously (45). Briefly, cells were lysed in 250 µL Roti-Load sample buffer (ROTH, Karlsruhe, Germany), preheated to 95°C and then boiled for additional 10 min prior loading on SDS gel electrophoresis. Primary antibodies were diluted 1:4000 for anti-sGCα_1 and 1:2000 for anti-sGCβ_1 antibody in 3% dry milk in TBST and incubated with nitrocellulose membranes at 4°C over-night 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 Imager Station 440 CF and with the NIH 1.6 software. All blots are standardized to ß-actin or GAPDH expression that was not

regulated by treatment. (Representative western blot examples are shown in Supportive information).

Determination of sGC activity. To measure sGC activity, cells were stimulated with 250 μM DEA/NO or 10 μM BAY 58-2667 for 3 min at 37°C. Thereafter, cells were immediately lysed in 80 % ethanol. Cells were scraped and, after evaporation of ethanol, re-suspended in assay buffer and sonicated. Measurement of sGC activity in crude homogenates of porcine tissue was performed as previously described (45). Briefly, all samples were measured as the formation of cGMP at 37 °C during 10 min in a total incubation volume of 100 ml containing 50 mM triethanolamine-HCl (pH 7.4), 3 mM MgCl, 3 mM glutathione (Carl Roth, Karlsruhe, Germany), 1mM IBMX, 100mM zaprinast, 5 mM creatine phosphate, 0.25 mg/ml creatine kinase and 1mM or 0.5 mM GTP. The reaction was started by 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, Lörrach, Germany or Biotrend, Cologne, Germany).

Statistics. For comparisons students´ 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.

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References

1. Ignarro LJ (2002) Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol* 53(4 Pt 1):503–514.

2. Bian K, Doursout M-F, Murad F (2008) Vascular system: role of nitric oxide in cardiovascular diseases. *J Clin Hypertens (Greenwich)* 10(4):304–310.

3. Ghosh A, Stasch J-P, Papapetropoulos A, Stuehr DJ (2014) Nitric oxide and heat shock protein 90 activate soluble guanylate cyclase by driving rapid change in its subunit interactions and heme content. *J Biol Chem* 289(22):15259–15271.

4. Ghosh A, Stuehr DJ (2012) Soluble guanylyl cyclase requires heat shock protein 90 for heme insertion during maturation of the NO-active enzyme. *Proc Natl Acad Sci USA* 109(32):12998–13003.

5. Cary SPL, Winger JA, Marletta MA (2005) Tonic and acute nitric oxide signaling through soluble guanylate cyclase is mediated by nonheme nitric oxide, ATP, and GTP. *Proc Natl Acad Sci USA* 102(37):13064–13069.

6. Tsai EJ, Kass DA (2009) Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. *Pharmacol Ther* 122(3):216–238.

7. Förstermann U, Xia N, Li H (2017) Roles of Vascular Oxidative Stress and Nitric Oxide in the Pathogenesis of Atherosclerosis. *Circ Res* 120(4):713–735.

8. Stasch J-P, et al. (2006) Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 116(9):2552–2561.

9. Langhauser F, et al. (2018) A diseasome cluster-based drug repurposing of soluble guanylate cyclase activators from smooth muscle relaxation to direct neuroprotection. *NPJ Syst Biol Appl* 4(1):8.

10. Beuve A (2016) Thiol-Based Redox Modulation of Soluble Guanylyl Cyclase, the Nitric Oxide Receptor. *Antioxid Redox Signal*:ars.2015.6591.

11. Brandes RP, et al. (2000) Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice: role of soluble guanylyl cyclase. *Hypertension* 35(1 Pt 2):231–236.

12. Faraci FM, Sigmund CD, Shesely EG, Maeda N, Heistad DD (1998) Responses of carotid artery in mice deficient in expression of the gene for endothelial NO synthase. *Am J Physiol* 274(2 Pt 2):H564–70.

13. Sardón T, Baltrons MA, García A (2004) Nitric oxide-dependent and independent down-regulation of NO-sensitive guanylyl cyclase in neural cells. *Toxicol Lett* 149(1-3):75–83.

14. Prasad S, Wilkinson J, Gatzoulis MA (2000) Sildenafil in primary pulmonary hypertension. *N Engl J Med* 343(18):1342–1342.

15. Auger WR, Jamieson SW, Pulmonary Thromboendarterectomy Program at University of California, San Diego (2013) Riociguat for pulmonary hypertension. *N Engl J Med*

369(23):2266–2268.

16. Lange M, Enkhbaatar P, Nakano Y, Traber DL (2009) Role of nitric oxide in shock: the large animal perspective. *Front Biosci (Landmark Ed)* 14:1979–1989.

17. Yuen PS, Doolittle LK, Garbers DL (1994) Dominant negative mutants of nitric oxide-sensitive guanylyl cyclase. *J Biol Chem* 269(2):791–793.

18. Ujiie K, et al. (1994) Homologous and heterologous desensitization of a guanylyl cyclase-linked nitric oxide receptor in cultured rat medullary interstitial cells. *J Pharmacol Exp Ther* 270(2):761–767.

19. Filippov G, Bloch DB, Bloch KD (1997) Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. *J Clin Invest* 100(4):942–948.

20. Ferrero R, Torres M (2002) Prolonged exposure of chromaffin cells to nitric oxide down-regulates the activity of soluble guanylyl cyclase and corresponding mRNA and protein levels. *BMC Biochem* 3(1):26.

21. Papapetropoulos A, Go CY, Murad F, Catravas JD (1996) Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. *Br J Pharmacol* 117(1):147–155.

22. Silva PL, et al. (2013) Effects of intravascular volume replacement on lung and kidney function and damage in nonseptic experimental lung injury. *Anesthesiology* 118(2):395–408.

23. Browner NC, Dey NB, Bloch KD, Lincoln TM (2004) Regulation of cGMP-dependent protein kinase expression by soluble guanylyl cyclase in vascular smooth muscle cells. *J Biol Chem* 279(45):46631–46636.

24. Draijer R, Atsma DE, van der Laarse A, van Hinsbergh VW (1995) cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ Res* 76(2):199–208.

25. Cornwell TL, Lincoln TM (1989) Regulation of intracellular Ca2+ levels in cultured vascular smooth muscle cells. Reduction of Ca2+ by atriopeptin and 8-bromo-cyclic GMP is mediated by cyclic GMP-dependent protein kinase. *J Biol Chem* 264(2):1146–1155.

26. Draijer R, et al. (1995) Expression of cGMP-dependent protein kinase I and phosphorylation of its substrate, vasodilator-stimulated phosphoprotein, in human endothelial cells of different origin. *Circ Res* 77(5):897–905.

27. Sellak H, Choi C-S, Dey NB, Lincoln TM (2013) Transcriptional and post-transcriptional regulation of cGMP-dependent protein kinase (PKG-I): pathophysiological significance. *Cardiovasc Res* 97(2):200–207.

28. Zhou Z, et al. (2008) Protein kinase G phosphorylates soluble guanylyl cyclase on serine 64 and inhibits its activity. *Arteriosclerosis, Thrombosis, and Vascular Biology* 28(10):1803–1810.

29. Pfeifer A, et al. (1998) Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J* 17(11):3045–3051.

30. Sayed N, et al. (2008) Nitroglycerin-induced S-nitrosylation and desensitization of soluble guanylyl cyclase contribute to nitrate tolerance. *Circ Res* 103(6):606–614.

31. Sayed N, Baskaran P, Ma X, van den Akker F, Beuve A (2007) Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation. *Proc Natl Acad Sci USA* 104(30):12312–12317.

32. Evgenov OV, et al. (2006) NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* 5(9):755–768.

33. Ghosh A, et al. (2016) Soluble guanylate cyclase as an alternative target for bronchodilator therapy in asthma. *Proc Natl Acad Sci USA* 113(17):E2355–62.

34. Fernhoff NB, Derbyshire ER, Underbakke ES, Marletta MA (2012) Heme-assisted S-nitrosation desensitizes ferric soluble guanylate cyclase to nitric oxide. *J Biol Chem* 287(51):43053–43062.

35. Schmidt P, Schramm M, Schröder H, Stasch J-P (2003) Mechanisms of nitric oxide independent activation of soluble guanylyl cyclase. *Eur J Pharmacol* 468(3):167–174.

36. Meurer S, et al. (2009) Nitric oxide-independent vasodilator rescues heme-oxidized soluble guanylate cyclase from proteasomal degradation. *Circ Res* 105(1):33–41.

37. Hoffmann LS, et al. (2009) Distinct molecular requirements for activation or stabilization of soluble guanylyl cyclase upon haem oxidation-induced degradation. *Br J Pharmacol* 157(5):781–795.

38. Fernandes D, et al. (2009) Late, but not early, inhibition of soluble guanylate cyclase decreases mortality in a rat sepsis model. *J Pharmacol Exp Ther* 328(3):991–999.

39. Glynos C, et al. (2007) Soluble guanylyl cyclase expression is reduced in LPS-induced lung injury. *Am J Physiol Regul Integr Comp Physiol* 292(4):R1448–55.

40. Mehta S (2005) The effects of nitric oxide in acute lung injury. *Vascul Pharmacol* 43(6):390–403.

41. Kleinschnitz C, et al. (2016) NOS knockout or inhibition but not disrupting PSD-95-NOS interaction protect against ischemic brain damage. *J Cereb Blood Flow Metab* 36(9):1508–1512.

42. Stover JF, et al. (2014) Nitric oxide synthase inhibition with the antipterin VAS203 improves outcome in moderate and severe traumatic brain injury: a placebo-controlled randomized Phase IIa trial (NOSTRA). *J Neurotrauma* 31(19):1599–1606.

43. Stasch J-P, et al. (2006) Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 116(9):2552–2561.

44. Stasch J-P, et al. (2002) NO- and haem-independent activation of soluble guanylyl

cyclase: molecular basis and cardiovascular implications of a new pharmacological principle. *Br J Pharmacol* 136(5):773–783.

45. Nedvetsky PI, Kleinschnitz C, Schmidt HHHW (2002) Regional distribution of protein and activity of the nitric oxide receptor, soluble guanylyl cyclase, in rat brain suggests multiple mechanisms of regulation. *Brain Res* 950(1-2):148–154.

Figure Legends

Fig. 1: *In vivo* sGC expression/activity is regulated by basal NO release and chronic high NO exposure. In eNOS$^{-/-}$ mice, sGC protein expression (A; N=4) and activity (B; N=9) are increased. In contrast, during porcine ARDS, a lung disease characterized by NO overproduction, sGC protein expression is decreased (C; N=3-5). Moreover, sGC activity, measured by acute incubation with DEA/NO (250 μM) was decreased (B; N=4-6). Data are expressed as mean ± SEM. *, **, ***: $p < 0.05$, 0.01 or 0.001 *vs.* control, respectively.

Fig. 2: sGC expression/activity patterns in PPAECs resemble *in vivo* findings. Eliminating PPAECs-mediated basal NO release by L-NAME (100 μM) incubation increases $sGC_{\beta1}$ expression (A; N=6) and, importantly, DEA-NO-induced sGC activity is increased after L-NAME incubation (B; N=3). In contrast, DETA/NO (100 μM) exposure decreases sGC protein expression (C; N=24-6) as well as sGC activity (D; N=5). Data are expressed as mean ± SEM. *, **, ***: $p < 0.05$, 0.01 or 0.001 *vs.* control, respectively.

Fig. 3: cGK-I does not mediate down-regulation of sGC expression/activity by chronic NO in PPAECs. PPAECs display functional cGK-I signaling as activating sGC by DETA-NO, 8-Br-cGMP or YC-1 exposure causes down-regulation of cGK-I expression (A; N=9-6). In PPAECs, 72h incubation with high concentrations of either the NO-independent sGC stimulator YC-1 (A; N=15-6), or the direct activator of cGMP-dependent protein kinase I (cGK-I) 8-Br-cGMP (B; N=9-6), caused an upregulation of sGC. However, down-regulation of sGC expression induced by 72h DETA-NO (100 μM) exposure was not prevented by Rp-8-cGMP (10 μM) (C; N=6). *, **, ***: $p < 0.05$, 0.01 or 0.001 *vs.* control, respectively.

Fig. 4: sGC expression/activity are not affected by cGK-I-deficiency. sGC protein expression (A; N=4) and activity (B; N=4) are not altered in cGK-I$^{-/-}$ mice.

Fig. 5: NO-induced sGC down-regulation is not thiol-dependent. A) 72h incubation of PPAECs with the DETA/NO (100μM), which generates supra-physiological amounts of NO, down-regulates both sGC protein subunits. Presence of N-acetyl cysteine (NAC; 1mM) did not affect basal sGC protein subunit expression or the DETA/NO-induced sGC subunit down-regulation (N=5-7). B) In PPAECs exposed to supra-physiological amounts of NO (DETA/NO 100 μM for 2h sGC protein activity, defined as cGMP production in response to acute incubation with the short-acting NO donor, DEA/NO (250 μM), was down-regulated irrespective of presence or absence of NAC (N=3-4). Data are expressed as mean ± SEM. *,**: $p < 0.05$ or 0.01 *vs.* control, respectively. $$: $p < 0,01$ *vs.* NAC.

Fig. 6: *In vivo* and *in vitro*, supra-physiological and pathological NO formation up-regulate apo-sGC activity. In PPAECs, NO incubation tended to increase BAY 58-2667-induced cGMP formation and decreased DEA/NO-induced cGMP formation (A; N=4-6). During procine ARDS, apo-sGC activity, measured by acute incubation with the apo-sGC activator BAY 58-2667 (10 μM), is increased (B; N=4-6) and the cGMP forming capacity sifts from sGC to apo-sGC as evidenced by the increased apo-sGC/sGC activity (C; N=4-6). Data are expressed as mean ± SEM. *: $p < 0.05$ *vs.* control.

Figure 1

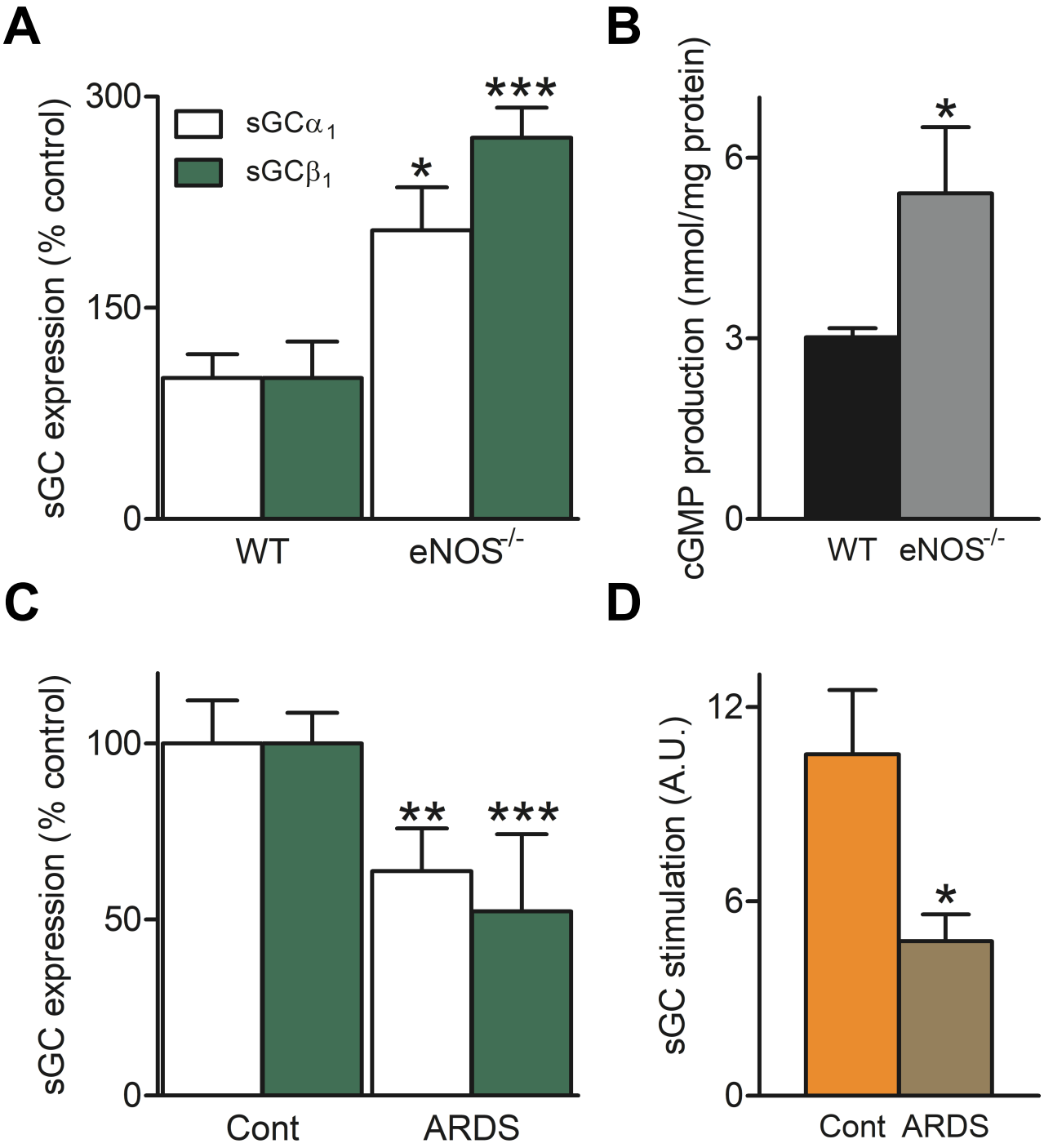

A
sGC expression (% control)
300
150
0
sGCα1
sGCβ1
*
***
WT
eNOS-/-
B
cGMP production (nmol/mg protein)
6
3
0
*
WT
eNOS-/-
C
sGC expression (% control)
100
50
0
**
***
Cont
ARDS
D
sGC stimulation (A.U.)
12
6
0
*
Cont
ARDS

Figure 2

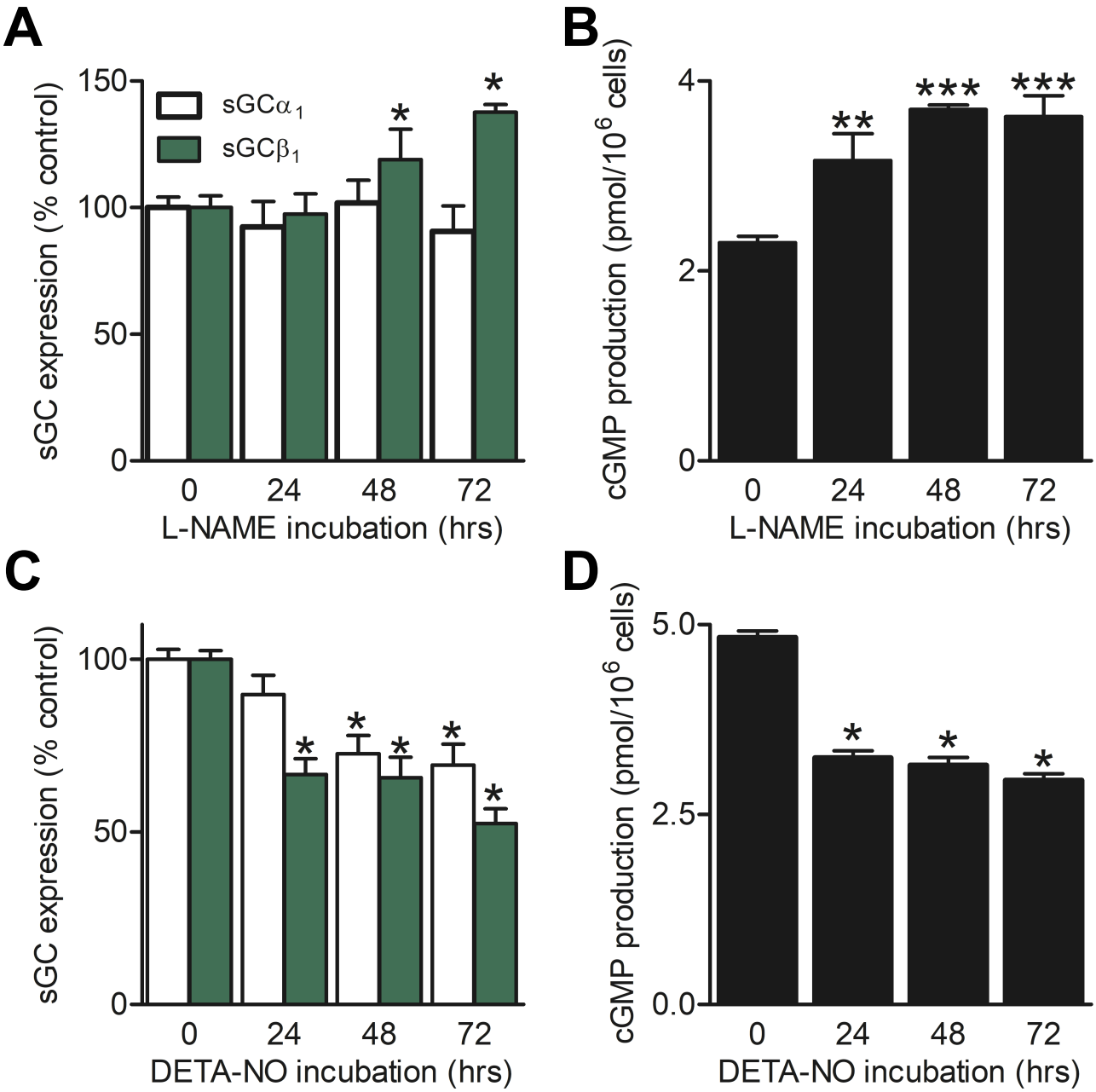

A
sGC expression (% control)
150
100
50
0
sGCα1
sGCβ1
*
*
0
24
48
72
L-NAME incubation (hrs)
B
cGMP production (pmol/10^6 cells)
4
2
0
**
***
***
0
24
48
72
L-NAME incubation (hrs)
C
sGC expression (% control)
100
50
0
*
*
*
*
*
0
24
48
72
DETA-NO incubation (hrs)
D
cGMP production (pmol/10^6 cells)
5.0
2.5
0.0
*
*
*
0
24
48
72
DETA-NO incubation (hrs)

Figure 3

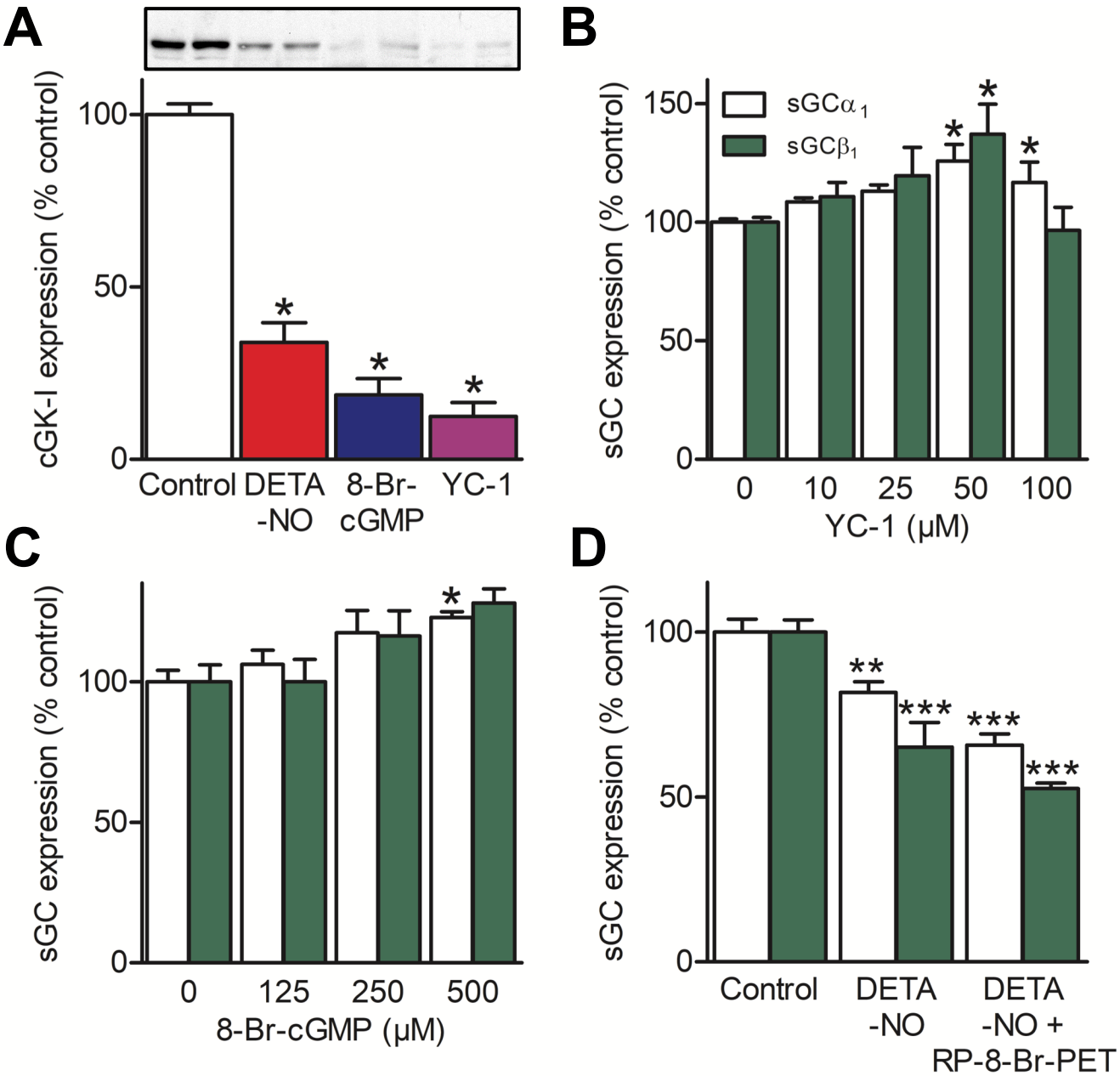

A
cGK-I expression (% control)
100
50
0
Control
DETA -NO
8-Br- cGMP
YC-1
B
sGC expression (% control)
150
100
50
0
sGCα1
sGCβ1
0
10
25
50
100
YC-1 (μM)
C
sGC expression (% control)
100
50
0
0
125
250
500
8-Br-cGMP (μM)
D
sGC expression (% control)
100
50
0
Control
DETA -NO
DETA -NO + RP-8-Br-PET

Figure 4

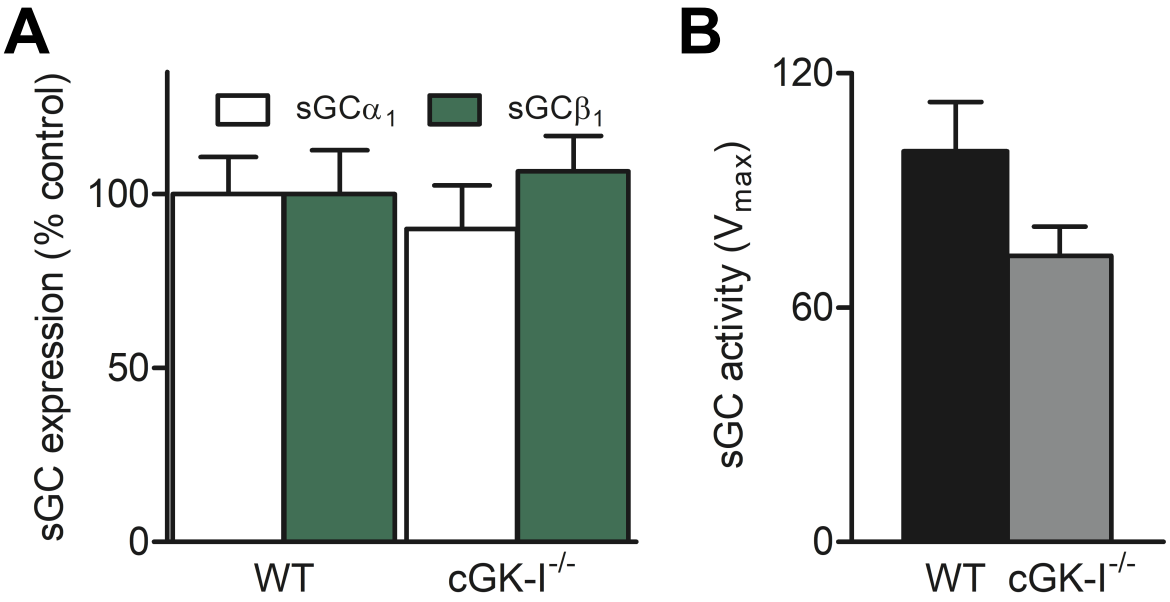

A
sGC expression (% control)
sGCα1
sGCβ1
100
50
0
WT
cGK-I-/-
B
sGC activity (Vmax)
120
60
0
WT
cGK-I-/-

Figure 5

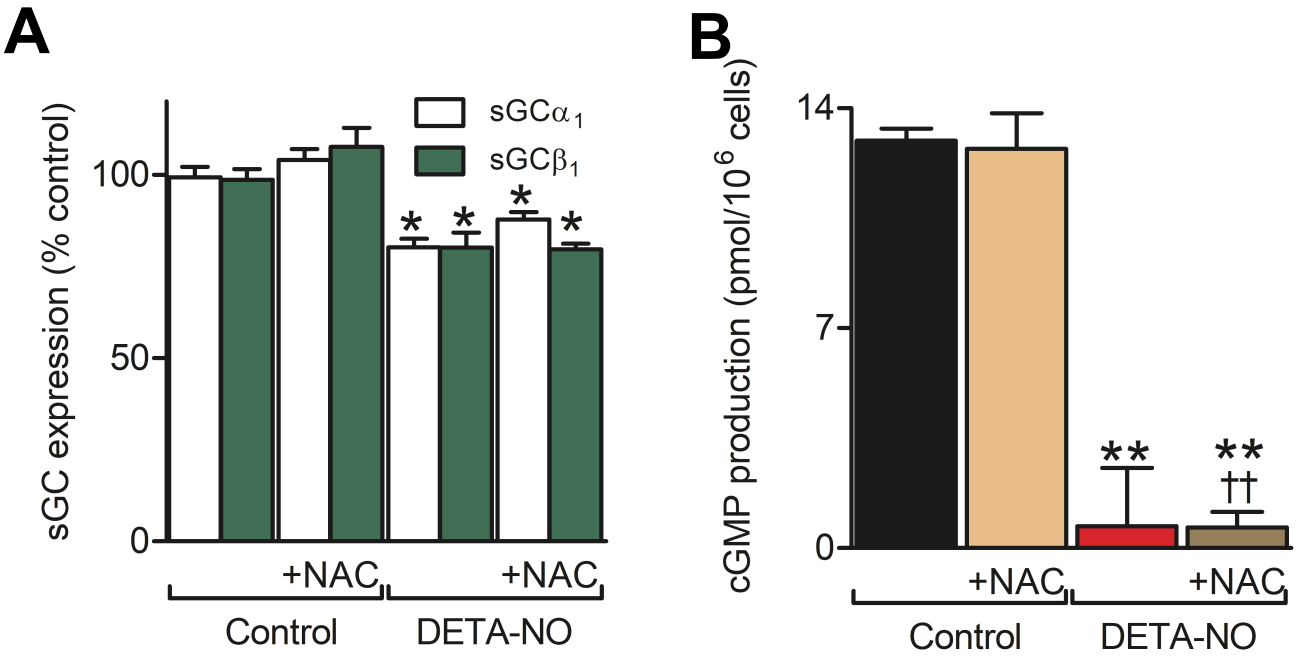
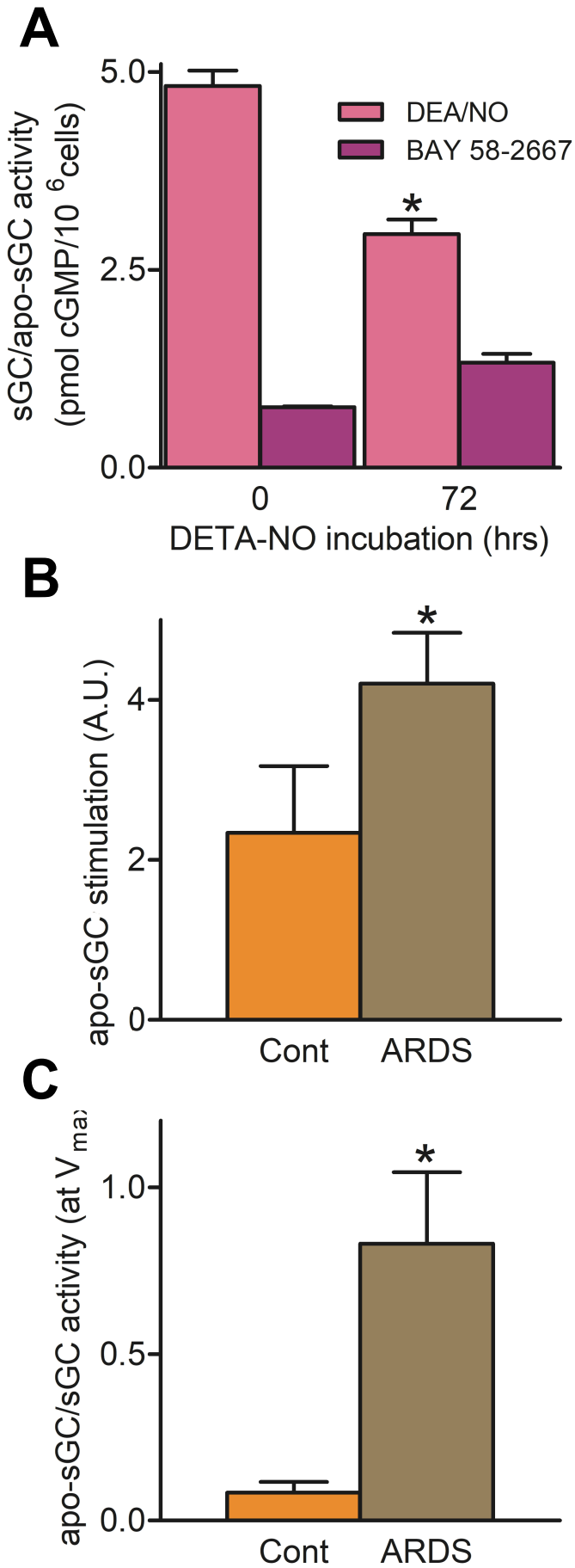

A
sGC expression (% control)
100
50
0
sGCα1
sGCβ1
*
*
*
*
+NAC
+NAC
Control
DETA-NO
B
cGMP production (pmol/10^6 cells)
14
7
0
**
**
††
+NAC
+NAC
Control
DETA-NO

Figure 6


A
5.0
2.5
0.0
sGC/apo-sGC activity
(pmol cGMP/10 6cells)
DEA/NO
BAY 58-2667
*
0
72
DETA-NO incubation (hrs)
B
*
4
2
0
apo-sGC stimulation (A.U.)
Cont
ARDS
C
*
1.0
0.5
0.0
apo-sGC/sGC activity (at V ma
Cont
ARDS