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

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Abstract

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 or exogenous NO from NO donor compounds decreased sGC protein and activity. This 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 whilst 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.

Introduction

The nitric oxide (NO)-cGMP signaling pathway plays several important roles in physiology including cardiopulmonary homeostasis [1,2]. 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 guanosine triphosphate (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]. This results in potent vasodilatory, anti-proliferative and anti-thrombotic effects [5]. In disease, heme loss, appearance of NO-insensitive apo-sGC and impaired NO-cGMP signaling have been described [6,7].

In addition to sGC's acute activation, NO appears to have further roles in regulating sGC. During enzyme maturation, NO facilitates heme incorporation into sGC [8,9], and activation of sGC by NO is followed by an acute and rapid desensitization involving protein S-nitrosylation [10,11]. In addition, chronic exposure to NO donor drugs has been suggested to negatively affect sGC activity in a not fully reversible manner [12-14]. It is unclear, however, whether this effect pertains also 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 hypertension [15,16]. We investigate the effects of chronic exposure to both exogenous (from NO donor drugs) and endogenous NO on sGC protein and activity. 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, apo-sGC. As disease model, we use again a condition related to pulmonary hypertension and chronically elevated levels of NO, porcine acute respiratory disease syndrome (ARDS) [17-19].

64

65 ## Results

66 **NO chronically decreases vascular sGC protein and activity *in-vivo* and *in-vitro*.**

67 To analyze the chronic effects of NO at a mechanistic level, we studied PPAECs. Cells were
68 incubated for up to 72h in the presence of the NO synthase (NOS) inhibitor N^G-nitro L-arginine
69 methyl ester (L-NAME) and sGC expression and activity were measured. In the presence of L-
70 NAME to eliminate endogenous NO formation, protein levels of the heme-binding sGCβ_1 subunit
71 were increased (Fig. 1A). This was associated also with increased sGC activity (Fig. 1B). Next we
72 wanted to test the reverse, i.e. whether an increase of NO to supra-physiological concentrations [20-
73 22] by chronic exposure to the long-acting NO donor compound, DETA/NO, would downregulate
74 sGC. Indeed, pre-incubating cells with DETA/NO (100 µM) decreased both sGCα_1 and sGCβ_1
75 protein (Fig. 1C) and sGC activity (Fig. 1D). Thus, *in-vitro* in PPAECs, endogenous NO chronically
76 downregulates sGC protein and activity in an L-NAME reversible manner, which is further
77 aggravated by exogenous, pharmacologically applied NO in supra-physiological concentrations.

78 Next, we wanted to validate these *in-vitro* observations at an *in-vivo* level in eNOS knock-out
79 mice (eNOS$^{-/-}$), eliminating endogenous NO formation similar to the *in-vitro* L-NAME experiment,
80 and in a porcine lung disease model (ARDS) characterized by NO overproduction, mimicking the
81 exposure the supraphysiological NO concentration through the NO donor [17,19,23]. In line with our
82 observations in PPAECs, eNOS$^{-/-}$ mice showed increased protein levels of sGCα_1 and sGCβ_1 (Fig.
83 1E) and increased sGC-activity (Fig. 1F), and in the high-NO pulmonary disease model, sGCα_1
84 and sGCβ_1 protein levels (Fig. 1G) and sGC activity were decreased (Fig. 1H). Collectively, these
85 data suggest that both *in-vitro* and *in-vivo* lowering endogenous NO increases, and increasing
86 endogenous NO lowers sGC protein subunit levels and sGC activity (Fig. 1I).

87 **cGMP/PKG do not mediate the downregulation of sGC protein and activity by chronic NO.**

88 Next, we aimed to clarify the mechanisms underlying the downregulation of sGC protein and
89 activity by chronic NO. First, we tested whether cGMP/PKG signaling is involved as it had been
90 shown previously to decrease both sGC activity [24] and expression [25]. Of experimental importance,

91 cell passaging can cause downregulation of PKG and prevent the detection of PKG-dependent
92 signaling [26-29]. Hence, we therefore restricted our studies to low passage number cells and ensured
93 fully functional PKG signaling by validating the known autoregulation of PKG expression [30,31].
94 Indeed, in our PPAEC system, both the PKG activator, 8-Br-cGMP, and the NO-independent sGC
95 stimulator and PDE inhibitor, YC-1, [32] were able to reduce PKG expression (Supplementary Fig.
96 S1 online) confirming the presence of fully functional PKG. We then studied whether the observed
97 downregulation of sGC protein and activity by NO can be mimicked by cGMP or is prevented by
98 inhibiting PKG. When we exposed PPAECs, however, for 72h with different concentrations of the
99 sGC stimulator and PDE inhibitor, YC-1, to raise cGMP in an NO-independent manner, or to the
100 direct PKG activator, 8-Br-cGMP, neither sGC protein nor activity were lowered (*cf.* to Fig. 1). In
101 fact, we observed even a slight upregulation of sGC protein (Fig. 2, A and B). Consistent with this,
102 the NO induced downregulation of sGC could not be prevented by co-incubation with the PKG
103 inhibitor, Rp-8-Br-PET-cGMPS (Supplementary Fig. S2 online). To extend these *in-vitro* findings
104 also to the *in-vivo* level, we subsequently studied sGC expression and activity in PKG knock-out
105 mice ($PKG^{-/-}$) [33]. Consistent with our *in-vitro* data, sGC protein levels (Fig. 2D) and sGC activity
106 (Fig. 2E) were unchanged in $PKG^{-/-}$ as compared to wildtype mice. In conclusion, both our *in-vivo*
107 and *in-vitro* data suggested that the downregulation of sGC protein and activity by chronic NO is
108 cGMP- and PKG-independent and thus appeared to be due to a non-canonical mechanism (Fig.
109 2C). Two cGMP-independent effects on sGC have been reported, rapid desensitization [10,20,34] ,
110 which is reversible in a thiol-dependent manner [35,36], and oxidative heme-loss yielding the NO-
111 insensitive apo-form of (apo-sGC) [7,37]. These possibilities were tested in our two next sets of
112 experiments.

113 **NO-induced sGC downregulation is thiol-independent.**

114 It has been shown previously, that thiol-sensitive mechanisms are involved in sGC regulation such
115 as sGC maturation and airway pathologies such as asthma [20]. Therefore, we assessed whether
116 NO-posttranslational modification of free-thiol cysteines i.e. S-nitrosylation contributes to the
117 downregulation of sGC by high chronic NO incubation. For this approach, PPAECs were again
118 exposed for 72 h to DETA-NO (100μM) in absence or presence, over the full-time frame, of the

119 membrane-permeable thiol-reducing agent, N-acetyl-L-cysteine (NAC; 1mM). NAC is a membrane-
120 permeable de-nitrosylating agent and glutathione precursor that has been shown to protect from
121 sGC nitrosylation [38,39]. Although some studies had used higher concentration of NAC, 1 mM is
122 sufficient to induce de-nitrosylation [40,41]. The presence of NAC, however, neither affected sGC
123 protein levels (Fig. 3A) nor sGC activity (Fig. 3B). This set of experiments suggested that it is
124 unlikely that a thiol-reversible mechanism similar to the acute desensitization is involved in the
125 chronic NO-induced downregulation of sGC protein and activity. This left oxidative heme-loss
126 yielding the NO-insensitive apo-form of (apo-sGC) [7,37] as only known cGMP-independent effect on
127 sGC.

128 **NO-induced sGC downregulation generates NO-insensitive sGC.**

129 We therefore examined whether chronic NO converts sGC to the NO-insensitive, heme-free form
130 of sGC, i.e. apo-sGC. To assay for the presence of apo-sGC, we took advantage of the apo-sGC
131 activator drug, BAY 58-2667 (cinaciguat), which specifically binds to the empty heme binding
132 pocket of apo-sGC and re-activates cGMP formation in an NO-independent manner [42]. Indeed, up
133 to 72 h exposure of PPAECs to DETA-NO (100 μM) increased apo-sGC activity, measured as BAY
134 58-2667-induced cGMP formation (Fig. 3C), and reduced sGC activity (Fig. 1D). To validate this
135 mechanistic finding *in-vivo*, we re-examined the above already mentioned and utilized high-NO
136 porcine ARDS model in which we had observed lower sGC protein levels and activity (see Fig. 1G,
137 H). Consistent with our above *in-vitro* findings, apo-sGC activity was also increased in the high-NO
138 porcine ARDS model (Fig. 3D) and established apo-sGC formation as one possible chronic
139 mechanism of chronic NO in addition to total sGC protein loss (Fig. 3E).

140 **Discussion**

141 Our findings close important gaps in our understanding of NO-cGMP signaling, in particular on the
142 long-term effects of endogenously formed NO versus NO donors on sGC and the
143 pathophysiological relevance of chronic NO for sGC regulation. We thus expand the previously
144 observed notion that NO donors drugs can reduce sGC mRNA levels [43] to the protein level and
145 importantly from pharmacology to endogenous NO. Previously, sGC protein levels were not
146 consistently investigated or with antibodies of unclear specificity [12,44]. Moreover, the functional

147 consequences of PKG on cGMP levels were investigated only in some cases [43,45] or in relation to
148 cGMP metabolism rather than its formation [43,46,47].
149 Surprisingly, not only pathological high levels of NO, as in our porcine ARDS model, but already
150 low chronic endogenous NOS activity suppressed sGC protein and activity in an L-NAME
151 reversible manner. These findings establish a previously not recognized delicate steady state in
152 the interactions between NO and sGC, acutely stimulating and chronically limiting its expression
153 and activity. On a positive note, under conditions of diminished NO synthesis, this may in turn
154 rapidly upregulate sGC protein and activity as we have observed in the presence of the NOS
155 inhibitor, L-NAME and *in-vivo* in eNOS$^{-/-}$ mice. In this regard, previous data are controversial. For
156 example, sGC activity was increased in eNOS$^{-/-}$ mice [13,48] which agrees with our findings. However,
157 other studies showed that neither sGC expression nor activity were changed in eNOS$^{-/-}$ mice [49,50],
158 or upon treatment with high dose NO donors [51]. The reasons for this discrepancy are unclear.
159 Of therapeutic importance is also the previously not recognized risk of chronic use of NO donor
160 drugs as they will lead to a downregulation of both sGC protein and activity. Together with the
161 problematic pharmacokinetics and known, but entirely different phenomenon of pharmacokinetic
162 tolerance due to lack of NO donor drug conversion to NO [52-54], this adds to the limitations of this
163 widely used drug class. With the introduction of NO-independent sGC stimulators and cGMP
164 elevating agents into clinical practice [55] there is now an alternative. Indeed, we show that the
165 prototypic sGC stimulator and PDE inhibitor, YC-1, does not lead to sGC downregulation.
166 With respect to the underlying mechanisms, we initially considered two known mechanisms in
167 NO-cGMP physiology, *i.e.* cGMP/PKG and thiol modification [9,10]. Surprisingly, both could be
168 excluded, which was reminiscent of an earlier observation where long-term exposure to an
169 exogenous NO donor also reduced sGC activity in a manner that could not be recovered with thiol
170 treatment [14]. Instead, our findings suggest that endogenous and exogenous NO chronically induce
171 a net shift from sGC to apo-sGC and that this is not only a pathophysiological mechanism but
172 pertains to NO-cGMP physiology. This explains why apo-sGC activator-induced cGMP formation
173 and functional effects are enhanced in but not exclusive to disease conditions [7]. Nevertheless, the
174 availability of sGC activator compounds allows now to overcome such conditions in which sGC

175 protein and activity is diminished in favor of apo-sGC and still induce cGMP formation. As a
176 limitation, other potential underlying mechanisms such as decreased mRNA [43] or increased
177 degradation of sGC have not been addressed by our study [56] and cannot be excluded.
178 Our findings also add to our understanding of apo-sGC as a therapeutic target. Hitherto apo-
179 sGC has been mainly studied by using the heme oxidant, ODQ, or by expressing enzyme where
180 the proximal heme ligating histidine had been deleted [57]. The mechanisms by which apo-sGC
181 forms in pathophysiology were less clear. Now chronic exposure to (high) levels of NO can be
182 considered one of these conditions. Whether this involves additional interactions with for example
183 reactive oxygen species and from which source remains to be investigated. Certainly, intermediate
184 compounds such as peroxynitrite would be candidate molecule to potentiate NO's oxidative
185 potential [58]. Of note, the shift from sGC to apo-sGC is not 1-to-1. Some sGC appears to be lost due
186 to inactivation beyond recovery by apo-sGC activators, e.g. by channeling into the ubiquitylation-
187 proteasome pathway [59]. Nevertheless, an apparent net shift from sGC to apo-sGC as main source
188 of cGMP formation is a common denominator and has recently also been observed by us in
189 another high NO model of ischemic stroke [6] and by others in an asthma model [20]. In contrast to
190 other observations, in our settings chronic NO incubation for 72 h unlike others for overnight [20], did
191 have an effect on sGC β1 expression independent of S-nitrosylation.
192 In conclusion, our data suggest that both *in-vitro* and *in-vivo*, and both under physiological
193 conditions and in disease NO self-limits its ability to induce cGMP formation via a chemical redox
194 feedback which causes inactivation of sGC and an apparent net shift towards NO-insensitive apo-
195 sGC. Our findings are of direct therapeutic importance as a pathological sGC/apo-sGC ratio can
196 be treated with sGC activator compounds such as BAY58-2667 [59] thereby reinstalling cGMP
197 synthesis and PKG signaling [7,37]. Moreover, with respect to the long-established class of NO donor
198 drugs and the use of inhaled NO a cautionary note needs to be added. Not only do they cause
199 reversible tolerance, but, as we now find, also irreversible downregulation of sGC and apo-sGC
200 formation. This explains the superiority of the novel, NO-independent sGC stimulators, at least in
201 indications such as pulmonary hypertension [15].

202 **Materials and Methods**

203 **Chemicals**

204 Polyclonal antibodies specific for sGCα_1 and sGCβ_1 have been described elsewhere (30). Actin
205 monoclonal antibody (Oncogene Research Products, Boston, USA); collagenase type CLS II
206 (Merck, Netherlands); 8-bromo-cGMP (BIOLOG, Germany); L-NAME, DETA/NO, DEA/NO , IBMX
207 and GTP (Enzo Life Sciences, Netherlands); BAY 58-2667 was synthesized as described [60]. All
208 other chemicals were of the highest purity grade available and obtained from Sigma or Merck
209 (Netherlands). DETA/NO and DEA/NO were dissolved in 10 mM NaOH, BAY 58-2667 and YC-1 in
210 DMSO.

211 **Tissue isolation**

212 Tissues from i) 6- to 8-months old male PKG$^{-/-}$ and age-matched control mice were obtained from
213 Prof. Franz Hofmann, Department of Pharmacology and Toxicology at the Technical University
214 Munich (genetic background 129/Sv) [33], and ii) 6- to 8-months old male eNOS$^{-/-}$ mice and age-
215 matched control were obtained from the Department of Physiology at Heinrich-Heine-Universität
216 Düsseldorf (genetic background C57BL/6) [36]. Animals' care was in accordance with guidelines of
217 Technical University Munich and Heinrich-Heine-Universität Düsseldorf. Experimental protocols
218 were approved by the animal ethics committees of Technical University Munich and Heinrich-
219 Heine-Universität Düsseldorf.

220 **Preparation of pulmonary arteries from a porcine ARDS model**

221 Pulmonary arteries were removed immediately after death from an experimental porcine model of
222 ARDS, as previously described [35]. Pulmonary arteries were snap-frozen in liquid nitrogen and
223 stored at minus 80°C or otherwise processed immediately to tissue powder and subsequently
224 suspended in homogenization-buffer and homogenized in an Ultra Turrax at 4°C. These samples
225 were then used further for protein determination, protein immune blots and sGC activity assays.

226 **PPAECs**

227 Fresh porcine pulmonary arteries were obtained from a local slaughterhouse and maintained in
228 phosphate-buffered saline (PBS; 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 140mM NaCl, 2.7mM KCl, pH
229 7.4) at 37°C. PPAECs were isolated enzymatically by incubation of the aorta inner surface with

230 collagenase type CLS II (0.5 mg/mL for 10 min at room temperature) and then collected in HEPES-
231 buffered medium 199. After centrifugation (250 x g, 10 min) the pellet was re-suspended in growth
232 medium (medium 199 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL
233 streptomycin) and cells were propagated in coated plastic flasks and incubated (37°C, 6% CO_2).
234 Upon confluence, endothelial cell monolayers were sub-cultured in 35-mm (for Western blot) or 60-
235 mm (for cGMP determination) gelatin coated dishes. Confluent cell monolayers from the second
236 passage were used for experiments. The growth medium was replaced either every 12 or 24 hours
237 if applicable containing the indicated compounds. After incubation time cells were subsequently
238 used for sGC activity measurements or western blot analysis.

239 **Detection and quantification of sGC protein**

240 Western blotting procedures were described previously [61]. Briefly, cells were lysed in 250 µL Roti-
241 Load sample buffer (ROTH, Karlsruhe, Germany), preheated to 95°C and then boiled for additional
242 10 min prior loading on SDS gel electrophoresis. Primary antibodies were diluted 1:4000 for anti-
243 sGCα_1 and 1:2000 for anti-sGCβ_1 antibody in 3% dry milk in TBST and incubated with
244 nitrocellulose membranes at 4°C over-night following challenge of membranes with secondary goat
245 anti-rabbit antibody (1:2000 in 3% milk in TBST) conjugated to horseradish peroxidase (Dako A/S,
246 Denmark). Immuno-complexes were visualized using an enhanced chemiluminescence kit
247 (Amersham Pharmacia Biotech, Freiburg). Samples were quantified with a Kodak Imager Station
248 440 CF and with the NIH 1.6 software. All blots are standardized to ß-actin or GAPDH expression
249 that was not affected by the treatments. Representative western blot examples are shown in
250 Supplementary Fig. S3 online.

251 **Determination of sGC activity**

252 To measure sGC activity, cells were stimulated with 250 µM DEA/NO or 10 µM BAY 58-2667 for 3
253 min at 37°C. Thereafter, cells were immediately lysed in 80 % ethanol. Cells were scraped and,
254 after evaporation of ethanol, re-suspended in assay buffer and sonicated. Measurement of sGC
255 activity in crude homogenates of porcine tissue was performed as previously described [61]. Briefly,
256 all samples were measured as the formation of cGMP at 37 °C during 10 min in a total incubation
257 volume of 100 ml containing 50 mM triethanolamine-HCl (pH 7.4), 3 mM $MgCl_2$, 3 mM glutathione,

258 1mM IBMX, 100mM zaprinast, 5 mM creatine phosphate, 0.25 mg/ml creatine kinase and 1mM or
259 0.5 mM GTP. The reaction was started by simultaneous addition of the sample and either DEA/NO
260 or BAY 58-2667, respectively. After incubation of each sample for 10 min the reaction was stopped
261 by boiling for 10 min at 95°C. Thereafter the amount of cGMP was subsequently determined by a
262 commercial enzyme immunoassay kit (Enzo Life Sciences, Netherlands).

263 **Statistics**

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

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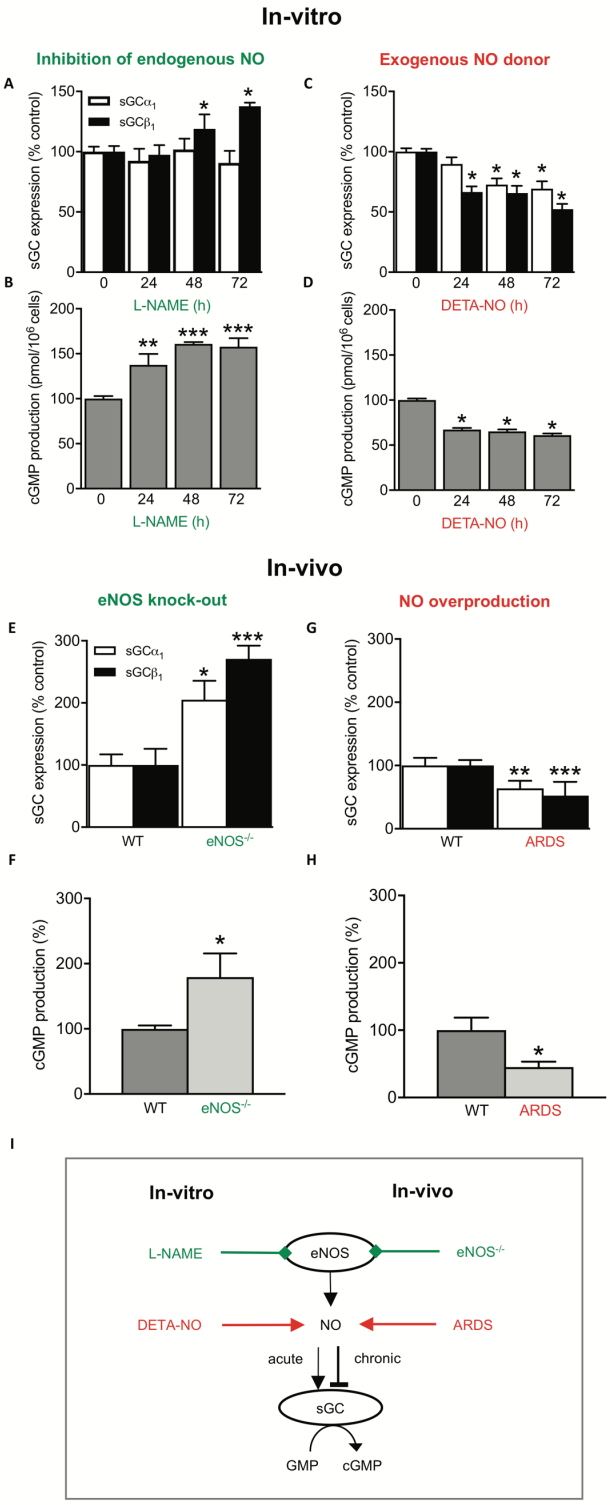
Data availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

450 **Figure and Figure Legends**

Figure 1


In-vitro
Inhibition of endogenous NO
Exogenous NO donor
A
B
C
D
sGC expression (% control)
cGMP production (pmol/10^6 cells)
sGCα1
sGCβ1
L-NAME (h)
DETA-NO (h)
In-vivo
eNOS knock-out
NO overproduction
E
F
G
H
cGMP production (%)
WT
eNOS-/-
ARDS
I
In-vitro
In-vivo
L-NAME
eNOS
eNOS-/-
DETA-NO
NO
ARDS
acute
chronic
sGC
GMP
cGMP

451

452
453 **Fig. 1. Chronic NO decreases vascular sGC protein and activity *in-vivo* and *in-vitro*.**

454 **(A)** Inhibiting basal NO formation in PPAEC by L-NAME (100 μM) for up to 72h increased sGCβ_1

455 expression (N=6). **(B)** This upregulation was associated with increased sGC activity (N=3).

456 Exposing cells to supra-physiological levels of NO by chronic exposure to the NO donor

457 compound, DETA/NO (100 μM), for up to 72h decreased both sGCα_1 and sGCβ_1 protein **(C)** (N=6)

458 and sGC activity **(D)** (N=5). *In-vivo* validation of the *in-vitro* observations showed in eNOS knock-

459 out mice (eNOS$^{-/-}$) mice increased sGC protein **(E)** and activity levels **(F)** (N=9), and in a porcine

460 lung disease model (ARDS) characterized by NO overproduction, decreased sGCα_1 and sGCβ_1

461 protein **(G)** (N=5) and sGC activity levels **(H)** (N=3). Data are expressed as mean ± SEM. *, **, ***:

462 $p < 0.05$, 0.01 or 0.001 *vs.* control, respectively. **(I)** Schematic summary showing that both *in-vitro*

463 (porcine lung endothelial cells) and *in-vivo* (the porcine lung disease model, ARDS) both

464 endogenous and exogenous NO downregulate sGC protein and activity. Representative full-length

465 blots are presented in Supplementary Figure S3.

466

Figure 2

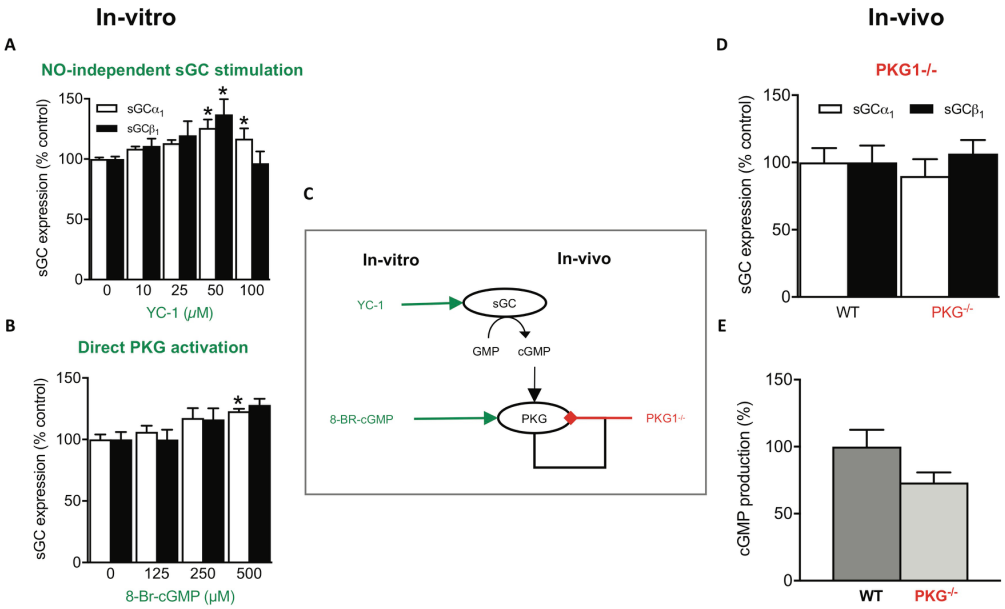


Fig. 2. PKG does not mediate the downregulation of sGC protein and activity by chronic NO. When PPAEC were incubated for 72 h in the absence or presence of increasing concentrations of **(A)** the NO-independent sGC stimulator, YC-1 (N=6), this did not cause a downregulation of $sGC\alpha_1$ and $sGC\beta_1$ expression but rather a small upregulation. Consistent with this, in **(B)** the direct PKG activator 8-Br-cGMP (N=6) lead to increased $sGC\alpha_1$ protein expression. **(C)** The scheme summarizes the *in-vivo* and *in-vitro* data suggesting 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. **(D)** sGC protein expression (N=4) and **(E)** activity (N=4) are not altered in $PKG^{-/-}$ as compared to wildtype mice. Data are expressed as mean ± SEM. *,**,***: $p < 0.05$, 0.01 or 0.001 *vs.* control, respectively. Representative full-length blots are presented in Supplementary Figure S3.

Figure 3

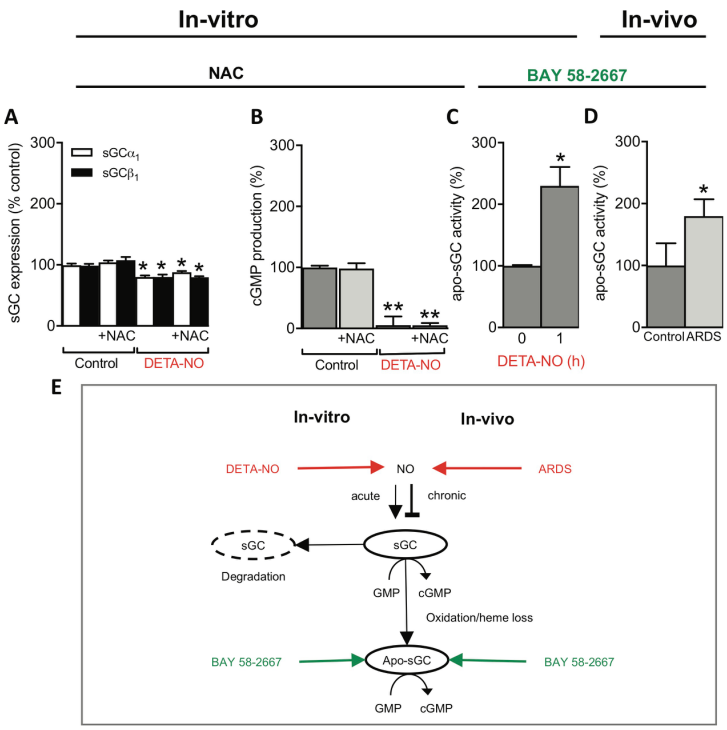


Fig. 3. NO-induced sGC downregulation is thiol-independent but involves sGC loss and a shift towards apo-sGC. When PPAECs were exposed for 72 h to DETA-NO (100μM) in the absence and presence of N-acetyl-L-cysteine (NAC; 1mM), NAC neither affected sGC protein levels (N=5) (**A**) nor activity (N=4) (**B**). Exposure of PPAECs for 72h 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 S3.