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

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One-sentence summary: Nitric oxide ubiquitously signals via cyclic GMP, but its feedback regulation is cGMP-independent following a non-canonical redox mechanism that is aggravated under disease conditions.

Abstract

Signaling from endothelial nitric oxide (NO) to the heme protein, soluble guanylyl cyclase (sGC), forms vasoprotective cyclic GMP (cGMP). In different disease states such as pulmonary hypertension, this pathway is targeted therapeutically but the underlying pathomechanism leading to its dysfunction is incompletely understood. Here we show in pulmonary artery endothelial cells that this dysfunction is due at least in part to tonic autoinhibition involving NO but not cGMP signaling. Both endogenous NO or pharmacological NO donor compounds that acutely stimulate sGC, chronically decreased both sGC protein and activity. This apparent endogenous feedback was surprisingly independent of canonical cGMP signaling and cGMP-dependent protein kinase. Thiol-sensitive mechanisms known to be relevant in sGC maturation were also not involved. Rather, in vitro and in vivo and in health and disease, tonic NO exposure led to inactivation and degradation of sGC but not of the heme-free apo-sGC isoform, which in some cases increased. Thus, our data establish a bimodal mechanism by which NO regulates sGC, acutely stimulating, chronically inhibiting as part of self-limiting chemical feedback. Of therapeutic importance in disease, our findings caution thus against chronic use of NO donor drugs and suggest that apo-sGC induced by pathological high NO can be recovered in a mechanism-based manner by using apo-sGC activator drugs to re-establish cGMP formation and signaling.

Introduction

The NO-cGMP signaling pathway plays an important role in 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 (*3*), which 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 and appearance of an apo-sGC has been described (*6, 7*).

In addition to acute activation, NO appears to have further roles in sGC regulation. During enzyme maturation, NO facilitates heme incorporation into sGC (*8, 9*), and activation of sGC by NO is followed by an acute and rapidly reversible desensitization involving possibly protein S-nitrosylation (*10*). In addition, chronic exposure to NO donor drugs has been suggested to negatively affect sGC long-term and in a not fully reversible manner (*11-13*). 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 (*14, 15*). We investigate the effects of chronic exposure to both exogenous NO donor drugs and endogenous NO on sGC protein and activity. In addition, we investigate whether such chronic mechanisms involve canonical cGMP signaling, possible thiol modulation, or formation of heme-free, apo-sGC in health and disease. For the latter, we use again an experimental model related to pulmonary hypertension and chronically elevated levels of NO, porcine acute respiratory disease syndrome (ARDS) (*16-18*).

Results

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

To analyse the chronic effects of NO at a mechanistic level, we studied PPAECs. When cells were incubated for up to 72h in the presence of the NO synthase (NOS) inhibitor N^G-nitro L-arginine

methyl ester (L-NAME) to examine the effects of endogenously formed NO, protein levels of the heme-binding sGCβ_1 subunit were increased (Fig. 1A). This up-regulation was associated also with increased sGC activity (Fig. 1B). Next we wanted to test whether a further increase of NO to supra-physiological levels by chronic exposure to the long-acting NO donor compound, DETA/NO, aggravated this effect. Indeed, pre-incubating cells with DETA/NO (100 µM) decreased both sGCα_1 and sGCβ_1 protein (Fig. 1C) and sGC activity (Fig. 1D). Thus, *in-vitro* in PPAECs endogenous NO tonically downregulate sGC protein and activity, which is further aggravated by exogenous, pharmacologically applied NO in supra-physiological concentrations (Fig. 1E).

Next, we wanted to validate these *in-vitro* observations at an *in-vivo* level in eNOS knock-out mice (eNOS$^{-/-}$) and in a porcine lung disease model (ARDS) characterized by NO overproduction (*16, 18, 19*). In line with our observations in PPAECs, eNOS$^{-/-}$ mice showed increased protein levels of sGCα_1 and sGCβ_1 (Fig. 1F) and increased sGC-activity (Fig. 1H). In the high-NO pulmonary disease model, sGCα_1 and sGCβ_1 protein levels (Fig. 1G) and sGC activity were decreased (Fig. 1I). Collectively, these data suggest that both *in-vitro* and *in-vivo* lowering endogenous NO increases, and increasing endogenous NO lowers sGC protein subunit and sGC activity (Fig. 1E).

PKG does not mediate the down-regulation of sGC protein and activity by chronic NO. Next, we aimed to clarify the mechanisms underyling the downregulation of sGC protein and activity by chronic/tonic NO. First, we wanted to test whether this was due to canonical cGMP signaling via PKG. Of experimental importance, cell passaging can cause downregulation of PKG and just preclude the detection of PKG-dependent signaling (*20-23*). 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 (*24, 25*). Indeed, in our PPAEC system, both the PKG activator, 8-Br-cGMP, and the NO-independent sGC stimulator, YC-1, were able to reduce PKG expression (Fig. S1) confirming the presence of 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 72h with different concentrations of the sGC stimulator, YC-1, to raise cGMP in an NO-independent manner, or to the direct PKG activator, 8-Br-cGMP, neither sGC protein nor activity were lowered (*cf.* to Fig. 1). In fact, we observed even a slight up-regulation of sGC protein (Fig. 2, A and B). Consistent with this, the NO induced down-regulation of sGC could not be prevented by co-incubation with the PKG inhibitor, Rp-8-Br-PET-cGMPS (Fig. S2). To extend these *in-vitro* findings also to the *in-vivo* level, we subsequently studied sGC expression and activity in PKG knock-out mice ($PKG^{-/-}$) (*26*). Consistent with our *in-vitro* data, sGC protein levels (Fig. 2D) and sGC activity (Fig. 2E) were unchanged in $PKG^{-/-}$ as compared to wildtype mice. In conclusion, both our *in-vivo* and *in-vitro* data suggested that the down-regulation 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). Two cGMP-independent effects on sGC have been reported, rapid desensitization (*10, 27, 28*), which is reversible in a thiol-dependent manner (*29, 30*), and oxidative heme-loss yielding the NO-insensitive apo-form of (apo-sGC) (*7, 31*).

NO-induced sGC down-regulation is thiol-independent.

We first assessed the possible effect of thiols on the sGC down-regulation observed with chronic NO exposure. PPAECs were again exposed for 72 h to DETA-NO (100µM) in absence or presence, over the full time frame, of the membrane-permeable thiol-reducing agent, N-acetyl-L-cysteine (NAC; 1mM). Presence of NAC, however, did neither affect sGC protein levels (Fig. 3A) nor activity (Fig. 3B). These data suggested that it is unlikely that a thiol-reversible mechanism similar to the acute desensitisation is involved in the chronic NO-induced downregulation of sGC protein and activity.

NO-induced sGC down-regulation generates NO-insensitive sGC.

As second possible non-canonical mechanism, we examined whether chronic NO converts sGC to NO-insensitive apo-sGC. To assay for the presence of apo-sGC, we took advantage of the apo-sGC

activator drug, BAY 58-2667 (cinaciguat), which specifically binds to the empty heme binding pocket of apo-sGC, which re-activates cGMP formation (*32*). Indeed, up to 72 h exposure of PPAECs to 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 porcine high-NO ARDS model in which we had already observed lower sGC protein levels and activity (see Fig. 1I). Consistent with our above *in-vitro* findings, apo-sGC activity was also here increased (Fig. 3D).

Discussion

It is unclear, however, whether this effect pertains also to endogenously formed NO and has pathophysiological relevance. Our findings provide important new understandings of NO-cGMP signaling. We expand the previously observed notion that NO donors drugs can reduce sGC mRNA levels (*33*) to the protein level and importantly to endogenous NO. Previously, sGC protein levels were non-consistently investigated or with antibodies of unclear specificity (*11, 34*). Moreover, the functional consequences of PKG on cGMP levels were investigated only in some cases (*33, 35*) or in relation to cGMP metabolism rather than its formation (*33, 36, 37*).

Surprisingly, not only pathological high levels of NO, as in our porcine ARDS model, but already low tonic NOS activity suppressed sGC protein and activity. These findings establish a previously not recognized delicate steady state in the interactions between NO and sGC, acutely stimulating and chronically limiting its enzyme activity. On a positive note, under conditions of diminished NO synthesis, this may in turn swiftly de-inhibit and upregulate sGC protein and activity as we have observed in the presence of the NOS inhibitor, L-NAME.

Of therapeutic importance is also the previously not recognized risk of chronic use of NO donor drugs as they will downregulate both sGC protein and activity. Together with the problematic pharmacokinetics and related tolerance, this adds to the limitations of this widely used drug class. With the introduction of NO-independent sGC stimulators into clinical practice (*38*) there is now an

alternative. Indeed, we show that the prototypic sGC stimulator, YC-1, does not lead to sGC downregulation.

With respect to the underlying mechanisms, we initially considered two known mechanisms in NO-cGMP physiology, *i.e.* PKG and thiol modification (*9, 10*). Surprisingly, both could be excluded, which was reminiscent of an earlier observation where long-term exposure to an exogenous NO donor also reduced sGC activity in a manner that could not be recovered with thiol treatment (*13*). Instead, our findings suggest that endogenous and exogenous NO chronically induce a net shift from sGC to apo-sGC and that this is not only a pathophysiological mechanism but pertains to NO-cGMP physiology. This explains why apo-sGC activator-induced cGMP formation and functional effects are enhanced in but not exclusive to disease conditions (*7*). Nevertheless, the availability of sGC activator compounds allows now to overcome such conditions in which sGC protein and activity is diminished in favour of apo-sGC and still induce cGMP formation.

Our findings thus 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 ligating histidine had been deleted (*39*). The mechanisms by which apo-sGC forms in pathophysiology were less clear. Now chronic exppsure to (high) levels of NO can be considered one of these conditions. Whether this involves additional interactions with for example reactive oxygen species and from which source remains to be investigated. Certainly, intermediate compounds such as peroxynitrite would be candidate molecule to potentiate NO's oxidative potential (*40*). 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 ubiquitinylation-proteasome pathway (*41*). Nevertheless, an apparent net shift from sGC to apo-sGC as main source of cGMP formation is a common denominator and has recently also been observed by us in another high NO model of ischemic stroke (*6*).

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 a chemical redox

feedback which causes inactivation of sGC and an apparent net shift towards NO-insensitive apo-sGC. Our findings are of direct therapeutic importance as a pathological sGC/apo-sGC ratio can be treated with sGC activator compounds such as BAY58-2667 (*41*) thereby reinstalling cGMP synthesis and PKG signaling (*7, 31*). Moreover, with respect to 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 tolerance, but, as we now find, also irreversible downregulation of sGC and apo-sGC formation. This explains the superiority of the novel, NO-independent sGC stimulators, at least in indicatioins such as pulmonary hypertension (*14*).

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 (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 (*42*). 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

Tissues from i) 6- to 8-months old male cGK-I$^{-/-}$ 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) (*26*), 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 Du□sseldorf (genetic background C57BL/6) (*30*). Animals' care was in accordance with guidelines of Technical University Munich and Heinrich-Heine-Universität Du□sseldorf.

Preparation of pulmonary arteries from a porcine ARDS model

Pulmonary arteries were removed immediately after death from an experimental porcine model of ARDS, as previously described (*29*). 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. 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) gelatin 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 (*43*). 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 affected by the treatments. Representative western blot examples are shown in Fig. S3.

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 (*43*).

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, 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, Netherlands).

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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Competing interests: The authors declare that they have no competing interests.

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

Supplementary Materials

Fig. S1. Treatment of PPAECs with PKG activator and sGC stimulator

Fig. S2. Treatment of PPAECs with PKG inhibitor

Fig. S3. Representative western blots

Figure Legends

Figure 1

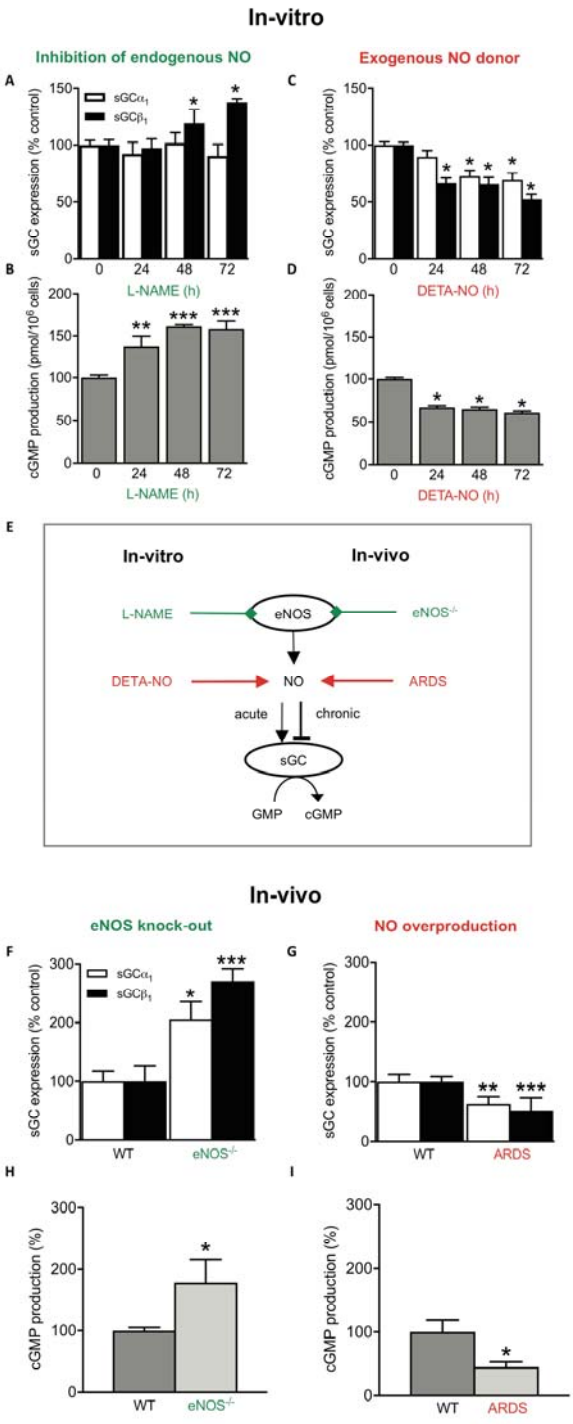

Fig. 1. Chronic NO decreases vascular sGC protein and activity *in-vivo* and *in-vitro*.

(A) Inhibiting basal NO formation in PPAEC by L-NAME (100 μM) for up to 72h increased $sGC\beta_1$ expression (N=6). **(B)** This up-regulation 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 72h decreased both $sGC\alpha_1$ and $sGC\beta_1$ protein **(C)** (N=6) and sGC activity **(D)** (N=5). **(E)** 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. *In-vivo* validation of the *in-vitro* observations showed in eNOS knock-out mice ($eNOS^{-/-}$) mice increased sGC protein **(F)** and activity levels **(G)** (N=9), and in a porcine lung disease model (ARDS) characterized by NO overproduction, decreased $sGC\alpha_1$ and $sGC\beta_1$ protein **(H)** (N=5) and sGC activity levels **(I)** (N=3). Data are expressed as mean ± SEM. *, **, ***: $p < 0.05$, 0.01 or 0.001 *vs.* control, respectively.

Figure 2

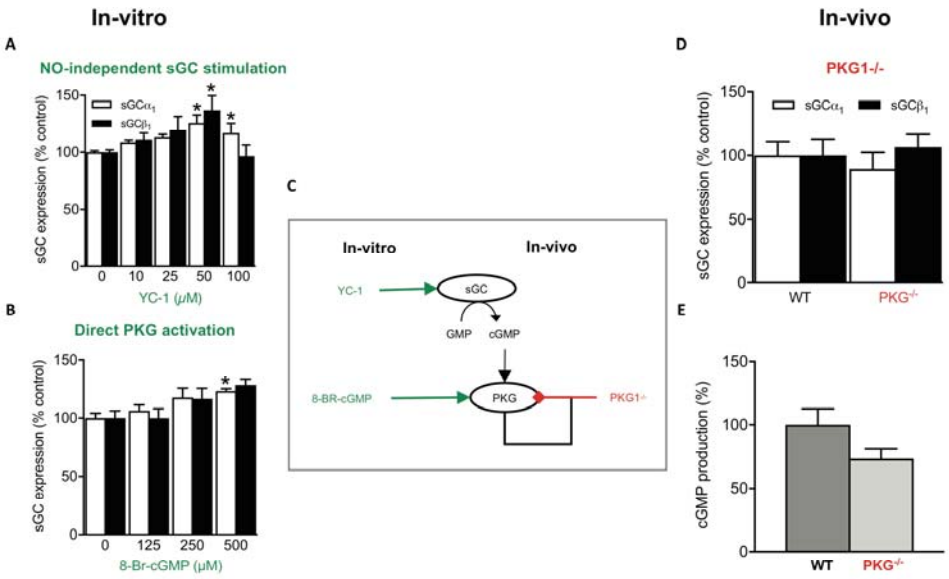

Fig. 2. PKG does not mediate the down-regulation of sGC protein and activity by chronic NO. When PPAEC were incubated for 72 h in the absence or presence of increasing concentrations of **(A)** the NO-independent sGC stimulator, YC-1 (N=6), this did not cause a down-regulation of $sGC\alpha_1$ and $sGC\beta_1$ 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 down-regulation 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.

Figure 3

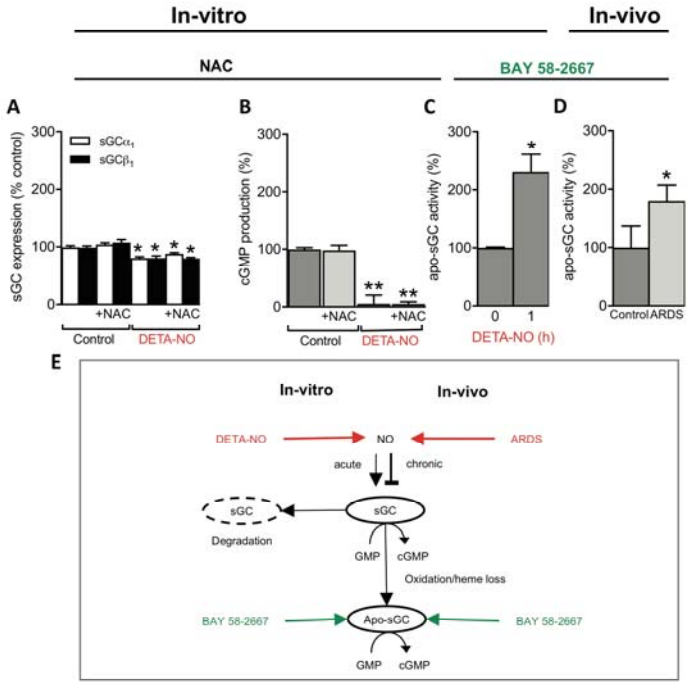

Fig. 3. NO-induced sGC down-regulation is thiol-independent but involves sGC loss and a shift towards apo-sGC. When PPAECs were exposed for 72 h to DETA-NO (100µM) in the absence and presence of N-acetyl-L-cysteine (NAC; 1mM), NAC neither affected sGC protein levels (N=5) (**A**) nor activity (N=4) (**B**).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