Reduction in soluble guanylyl cyclase-specific activity following prolonged treatment of porcine pulmonary artery with nitric oxide

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Perkins WJ, Taniguchi M, Warner DO, Chini EN, Jones KA. Reduction in soluble guanylyl cyclase-specific activity following prolonged treatment of porcine pulmonary artery (PA) with nitric oxide. Am J Physiol Lung Cell Mol Physiol 293: L84–L95, 2007. First published March 23, 2007; doi:10.1152/ajplung.00368.2006.— In a newly characterized cultured porcine pulmonary artery (PA) preparation, 24-h treatment with the nitric oxide (NO) donor (Z)-[N-(2-aminoethyl)-N-(2-ammonioethyl)aminodiacetate-1-u-m,1,2-diolate (DETA-NO) decreased the response to acutely applied DETA-NO compared with 24-h control (−log EC50 6.55 ± 0.12 and 5.02 ± 0.21, respectively). Treatment of PA with the cell-permeable superoxide dismutase mimetic, Mn(III) tetra(4-benzoic acid) porphyrin chloride, did not change NO responsiveness in either freshly prepared or 24-h DETA-NO-treated PA. cGMP and cAMP phosphodiesterase activities were approximately equal in PA. Twenty-four-hour DETA-NO treatment did not change either cGMP or cAMP phosphodiesterase activities. Twenty-four hours in culture had no significant effect on soluble guanylyl cyclase (sGC) subunit mRNA expression, but 24-h DETA-NO treatment significantly decreased the expression of both sGCα1 and sGCβ1, sGCβ2 protein expression was 42 ± 4 ng/mg soluble protein. Twenty-four-hour cultures in culture without and with DETA-NO reduced sGCβ1 protein expression (36 ± 3 and 31 ± 3 ng/mg soluble protein, respectively, P < 0.025). Basal tissue cGMP ([cGMP]i) was significantly increased, and NO-induced (cGMP), was significantly decreased by 24-h DETA-NO treatment. (cGMP), normalized to the amount of sGC protein expressed in PA was significantly lower in PA treated for 24 h with DETA-NO compared with both freshly isolated and 24-h cultured PA. We conclude that prolonged NO treatment induces decreased NO responsiveness in part by decreasing both sGC expression and sGC-specific activity.

The nitric oxide (NO) signaling system plays a significant role in the regulation of arterial smooth muscle tone and thus of systemic hemodynamics. Multiple factors regulate this system, including NO production and bioavailability, cGMP production by soluble guanylyl cyclase (sGC), cGMP breakdown by phosphodiesterases (PDE), and transduction of the cGMP signal by cGMP-dependent protein kinases (cGK) and their associated protein targets involved in decreasing myoplasmic calcium (15) and myofilament calcium sensitivity (56). Variability in the NO responsiveness of tissues occurs over both very short and longer time intervals and appears to play a role both in normal physiological conditions and in pathophysiological states such as endotoxic shock (62) and pulmonary hypertension (31). In vivo changes in NO responsiveness fall into two broad classes of mechanisms: increased neuroendocrine counterregulatory responses and decreased NO responsiveness intrinsic to the NO-cGMP signaling system (43). Evidence for variability in the NO responsiveness attributable to the latter includes a reduction in the hemodynamic response to NO in mice overexpressing endothelial NO synthase (eNOS) (61), acute NO supersensitivity in endothelium-denuded arterial preparations (37, 52) and in eNOS−/− mice (6), acute sGC desensitization with a stable NO signal (4, 5), and an acute reduction in NO response with agents that lead to oxidation of the Fe2+ in sGC heme (18). In aggregate, these findings indicate that NO, in addition to acutely activating sGC, also decreases physiological and biochemical responsiveness to NO. NO-induced NO hyporesponsiveness occurs, in large part, by mechanisms distinct from that involved in acute tolerance to organic nitrates such as nitroglycerin (10, 57), since it occurs with NO produced by NO synthase and NO donors that do not require mitochondrial aldehyde dehydrogenase activity to release NO.

Reduced NO responsiveness involving the NO-cGMP signaling system in vascular smooth muscle has been successfully related to decreased NO bioavailability due to increased NADPH oxidase expression/activity and superoxide production (44, 45), a reduction in cGMP production due to a parallel reduction in sGC expression (14, 58), increased cGMP PDE activity (41), and decreased response to a given cGMP level (54). It remains unclear, however, whether and to what extent such NO desensitizing processes occur following prolonged treatment with NO in a form that does not require bioconversion. In addition, a mechanism for NO-induced NO hypersensitivity that has been implicit in previous results, NO-induced reduction in sGC specific activity, has yet to be semiquantitatively addressed.

The present studies, a cultured pulmonary artery (PA) preparation was characterized and used to test the hypothesis that prolonged NO treatment induces NO hyporesponsiveness in PA, in part by reducing both sGC expression and reduced sGC-specific activity.

MATERIALS AND METHODS

Tissue Preparation

Following Institutional Animal Care and Use Committee approval, pigs (domestic crossbreds, weight 35–77 kg) were anesthetized with intravenous pentobarbital (100 mg/kg) and exsanguinated by bilateral transection of the carotid arteries. The lungs were excised and immersed in chilled physiological salt solution (PSS) with a composition (in mM) of 110.5 NaCl, 25.7 NaHCO3, 5.6 dextrose, 3.4 KCl, 2.4

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CaCl₂, 1.2 KH₂PO₄, and 0.8 MgSO₄. Third-generation PA was dissected from the lung parenchyma, cut into rings, and cleaned of adventitia under microscopic observation, and the endothelium was removed by gentle rubbing of the luminal surface with a moist cotton swab. For isometric force measurements, 0.1- to 0.2-mm width, 1-cm length, and 0.2- to 0.3-mg wet wt PA strips were prepared. Third-generation PA was used in these studies due to ease of preparation, better definition of myocyte orientation, and the amenability of the tissue size to biochemical studies.

**Organ Culture**

In organ culture experiments, endothelium-denuded PA rings were placed in 10-ml minimum essential medium with Earle’s salts and l-glutamine (Invitrogen, Carlsbad, CA) with 100 units penicillin and 100 µg streptomycin/ml at 37°C in a humidified 5% –95% air incubator (Forma Scientific), as previously described for basilar artery preparations (8). Rings were incubated with 0, 1, 10, 100, and 1,000 µM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) for 24 or 48 h. DETA-NO was selected since it slowly releases authentic NO (22), giving rise to stable relaxations and NO concentrations at a given DETA-NO concentration (13) and does not require biotransformation to release NO (32). The highest concentration of DETA-NO used in these studies results in a solution NO ≈ 1 µM that was stable for 24 h at 37°C (see Fig. 1, left). NO was measured using a World Precision Instruments NO electrode with NO gas as the standard, as previously described (13).

**Mechanical Measurements**

Strips were suspended in 5-ml tissue baths filled with PSS (37°C), aerated with 94% O₂–6% CO₂, pH 7.4. One end of the strips was anchored to a metal hook at the bottom of the tissue bath; the other end was attached to a calibrated force transducer (model FT03D, Grass Instruments/Astro-Med, West Warwick, RI). During a 3-h equilibration period, the strips were repeatedly contracted isometrically using 40 mM KCl and then relaxed. The length of the strips was increased after each contraction-relaxation cycle until active force was maximal (optimal length). All strips were subsequently maintained at optimal length. Relaxed PA strips were then contracted with norepinephrine (1 µM), and the absence of endothelium was verified by failure of acetylcholine (1 µM) to cause relaxation. Strips were then relaxed again until commencement of a study. Before concentration-response studies, all strips were incubated with 10 µM indomethacin to prevent the formation of prostanooids. In previous studies, contraction of tracheal smooth muscle (TSM) and pulmonary artery smooth muscle (PASM) during incubation with indomethacin had no effect on tissue cGMP [(cGMP),] (20).

**Concentration-Response Curves**

Concentration-response curves were performed using PA strips that were freshly prepared, 24-h cultured, 24-h cultured + DETA-NO, 48-h cultured, and 48-h cultured + DETA-NO. In one set of PA strips, phenylephrine was accumulatively added in concentrations ranging from 10⁻⁸ to 10⁻³ M in half-log concentration increments. Incremental concentrations were added to the bath 5 min after a stable isometric force was attained. Each n represents PA prepared from a separate animal and n = 6 for each study, unless otherwise specified. In another set of experiments, the concentration response of PA to the phenylephrine and DETA-NO response curves in freshly prepared PA, 24-h cultured, and 24-h cultured + DETA-NO was determined. The length, width, and thickness of strips were measured for calculation of isometric force per unit area and unit volume.

**Measurement of Superoxide Production**

Lucigenin (5 µM) was used to measure superoxide production using a previously described method (53) from freshly prepared PA and PA cultured for 24 h in culture medium without and with 1 mM DETA-NO. Results are reported as counts per minute per milligram tissue dry weight.

**Relative Actin and Myosin Expression**

The amount of actin and myosin present in PA tissue homogenate was obtained by loading 50 µg total protein and running a 7.5% SDS-PAGE. The resulting gels were zinc stained (Bio-Rad, Hercules, CA), scanned using a flat bed scanner, and digitized using Adobe Photoshop. Actin and myosin band intensities were compared with those obtained with tissue homogenates from freshly prepared PA. A preliminary study using purified actin and myosin in the same gel format and the assumption that these are the predominant proteins in smooth muscle homogenates at the molecular weight was used to assign the bands to a specific protein.

**DNA Isolation and Quantitation**

Genomic DNA was prepared from 25- to 50-mg finely minced PA tissue samples using a QIAamp Mini Kit (Qiagen, Valencia, CA). DNA was eluted from the QIAamp silica gel membrane with water and was quantitated using outer diameter (OD) 260-nm measurement and converting this to a concentration of total RNA obtained from a sample using the relation OD 260 nm = 1 at 50 µg/ml double-stranded DNA. Only DNA samples with OD 260 nm/OD 280 nm > 1.8 were used. sGC Total RNA Preparation and Quantitation

Total RNA was prepared from RNAlater-treated (Ambion, Austin, TX) PA tissue samples by extraction using guanidinium isothiocyanate and subsequent sedimentation through CsCl (12). Following precipitation of total RNA with isopropanol, washing with ethanol and suspension of the resultant total RNA in water, RNA quality was assessed by electrophoresis on an agarose gel. Only samples in which the ratio of 28S:18S ribosomal RNA band intensity (in arbitrary units) was at least >1.2, with little evidence of laddering below or between these bands, were studied. OD 260 nm/280 nm was > 1.8 for these samples, and the OD 260 nm was used to quantitate the amount of total RNA obtained from a sample using the relation OD 260 nm = 1 at 40 µg/ml RNA. cDNA from PA was prepared from the total RNA samples using Avian Myeloblastosis Virus reverse transcriptase (Clontech, Mountain View, CA) and was stored at −20°C until used in sGC subunit sequencing and mRNA expression measurements.

**sGC Subunit Sequencing**

cDNA was probed using rapid amplification of cDNA ends (Clontech), using adapter sequences and a sequence for the sGC subunit that was highly conserved across species. For sGCα₁, nucleotides 2198-2200 of the rat sequence (46) (accession no. M36075), and for sGCβ₁, nucleotides 1701-1679 of the human sequence (17) (antisense, access-
sion no. X66533) were used as the initial probes for touchdown PCR. The resulting cDNA band was purified from an agarose gel and subcloned into pCR2.1 vector using TOPO TA cloning kit (Invitrogen). Positive *E. coli* colonies were tested for the insert using colony PCR using M13 forward and reverse primers. Plasmid containing the insert was purified from a broth culture of a positive colony using Wizard plasmid miniprep (Promega, Madison, WI). The sequence of the insert was initially characterized with M13 forward and M13 reverse primers using the Sanger dyeoxy method (51) at the Mayo Clinic College of Medicine DNA Sequencing and Synthesis Core Facility, Rochester, MN. These sequences were used to generate new primers to extend the sequence until the complete open frame was obtained. Overlapping sequences were used to design gene-specific sequencing primers for obtaining the full-length open-reading frame sequence of each sGC subunit in triplicate. The resulting porcine sGCo1 and sGCβ1 sequences were submitted to the National Center for Biotechnology Information and have accession numbers DQ008577 and DQ008578, respectively. Oligonucleotide primers and probes were synthesized at the Mayo Rochester Core Facility. Oligonucleotide primers designed on the basis of published sequences were used to probe for sGCo1, sGCo2, and sGCβ3 subunit isoforms (accession nos. X63282, Z50053 and M57507, respectively).

**Measurement of sGC mRNA Expression**

Quantitative RT-PCR was used for this determination (3, 16). The exogenous internal standards (mimes) were derived from pBR322 and composite primers for PCR amplifying; these were produced as previously described, except that porcine sGCo1, (sense 1247-1269, antisense 1705-1685) and sGCβ1, (sense 261-286, antisense 658-632) sequences were used. The pBR322 sequence was selected to have a melting point by nearest neighbor estimation (Oligo Software, Cascade, CO) within 2°C of the sGC subunit sequence being amplified. The respective size of the mime/sGC amplicon sequences were 345/225 and 348/398 bp for sGCo1 and sGCβ1, respectively. The amplified mime sequences were purified and subcloned into pCR2.1 plasmid using TOPO TA cloning (Invitrogen), and colonies containing the mime insert were selected as described above in the DNA sequencing section. Plasmid was purified from the *E. coli* pellet using a QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA). Confirmation of insertion was obtained by sequencing the plasmid and testing for amplification using sGC subunit selective primers. Plasmid and mime concentration were quantitated using OD 260 nm. Meme cDNA concentrations for determination of sGC cDNA concentration in total RNA from TSM and PASM were 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 amol for both sGC subunits, as previously described (28). Samples underwent PCR for 30 cycles with the following cycling conditions: 30-s initial denaturation at 94°C, followed by 25 cycles of 10-s denaturation at 94°C and 2-min 30-s annealing and extension at 68°C, with a final extension at 72°C for 3 min. The PCR products were run on a 2.5% composite agarose gel (1.5% LMP Ultrapure agarose; Invitrogen) containing 500 ng/ml ethidium bromide for staining. Gels were digitized using an Alphalmotech ChemiImager 400 imaging system, and the resulting images were analyzed using Optiquant software (PerkinElmer, Boston, MA). The amount of gene-specific mRNA was determined using linear regression analysis of a plot of log (intensity mime/intensity sample) vs. log (mole mime in PCR reaction). The point at which the regression line crosses zero on the y-axis determines the number of moles of sGC subunit mRNA present in the original total RNA sample. The quantity of gene-specific mRNA is expressed in attomoles per microgram total RNA.

**Measurement of sGC Protein Concentration**

PA strips were flash-frozen by rapid immersion in liquid nitrogen and stored at −70°C until proteins were to be extracted. Then they were placed in a chilled mortar on dry ice and pulverized, and the resulting powder was suspended in extraction buffer composed of (in mM) 10 KCl, 5 NaCl, pH 7.0, 1 dithiothreitol, 1 EDTA, 1 phenylmethylsulfonyl fluoride, 5 NaF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A. The sample was centrifuged at 4,000 g for 10 min to pellet insoluble material. Protein concentration in the extraction buffer supernatant was determined using the Bradford method. Ten micrograms of soluble protein from the tissue extracts were loaded into wells, and known amounts of purified sGC protein (Alexis Biochemicals, Lausen, Switzerland) were loaded in adjacent lanes to generate a standard curve. Proteins were separated by electrophoresis in SDS-7.5% polyacrylamide precast minigels (BioRad) using Tris-glycine-SDS buffer. The proteins were transferred to a polyvinylidene difluoride membrane for 45 min at 100 V, and the membrane was subsequently washed with 10 mM Tris-buffered saline containing 5% (wt/vol) bovine serum albumin for 15 min (25°C). The membrane was then treated overnight with Tris-buffered saline containing 0.2% Tween 20 and 1:10,000 dilution of sGC polyclonal rabbit antibody (Cayman Chemical, Ann Arbor, MI) overnight. After washing, the membrane was treated with 1:10,000 anti-rabbit horseradish peroxidase-conjugated IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min and then washed again. Membranes were then treated with enhanced chemiluminescence Western blotting reagents (ECL, Amersham, Piscataway, NJ) for 1 min, apposed to Kodak BioMax film, and developed for a period of time that avoided saturation of any bands. The film was scanned and digitized using Adobe Photoshop and subsequently analyzed using Optiquant software (Packard Instruments).

**cGMP Measurements**

PA strips for these studies were either freshly prepared, 24-h cultured, or 24-h cultured + 1 mM DETA-NO. They were then washed extensively with PSS and were placed in 37°C baths containing PSS aerated with 94% O2–6% CO2, pH 7.4. In the presence of 500 µM isobutylmethlyxanthine, a membrane-permeant PDE inhibitor, PA strips were incubated with or without 1 mM DETA-NO for 1 min. The PA strips were then frozen with liquid nitrogen and stored in a −70°C freezer. Strips were then homogenized, and the soluble extract was assayed for cGMP using a commercially available RIA kit (Amersham Biosciences, Piscataway, NJ), as previously described (49). The protein concentration in the tissue homogenate was determined by the method described by Lowry et al. (30), using bovine serum albumin dissolved in 1 N NaOH as the standard. (cGMP), was expressed in picomoles per milligram protein.

**cGMP measurements** were also made in the soluble fraction of tissue homogenates from 24-h cultured, with and without 1 mM DETA-NO, according to a previously described method (38). sGCβ1 protein was quantified in the homogenates, and results of the assay are reported as picomoles cGMP per minute per nanogram sGCβ1. In these studies, 100 µM sodium nitroprusside were used to activate sGC in the tissue homogenate, as described previously (38).

**PDE Activity Measurements**

Tissue was homogenized in an ice-cold medium containing (final concentrations) 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 µM leupeptin, 0.1 µM pepstatin, and 0.1 µM phenylmethylsulphonyl fluoride, 0.1% Triton X-100, and 20 mM Tris·HCl, adjusted to pH 7.4. The homogenate was centrifuged for 60 min at 100,000 g. The supernatant, denoted “extract”, was used for PDE assay. The samples were assayed immediately or stored after addition of ethylene glycol (final concentration 30%) at −20°C until used (maximum 6 days). The protein concentration was determined by the Bradford method.

Tissue extracts were incubated in a final volume of 110 µl of the following medium (final concentrations): 10 mM MgSO4, 2 mM EGTA, 0.1% bovine serum albumin, 15 mM Tris·HCl (pH 7.4), and either 0.5 mM [3H]cAMP or [3H]cGMP as substrate. In experiments
examining the activity of Ca\(^{2+}\)-calmodulin (CaM)-dependent PDEs, CaCl\(_2\) was added to the reaction mixture from 2 mM stock to obtain 10 \(\mu\)M Ca\(^{2+}\) and 10 \(\mu\)g/ml of CaM. The PDE activity in aliquots incubated without Ca\(^{2+}\) and CaM (but with 2 mM EGTA) was determined as a basal activity. The reaction was initiated by addition of the PA homogenate and was incubated at 30°C for 5–20 min, depending on the amount of protein added. The reaction was stopped in dry ice, and samples were then boiled for 90 s. After conversion of S'-AMP/GMP products into adenosine or guanosine by treatment with S' nucleotidase for 15 min at 37°C, samples were applied to DEAE-sephadex A-25. \[^{3}H\]adenosine or \[^{3}H\]guanosine was eluted by three washes with 20 mM ammonium formate, pH 7.4, and radioactivity was measured with scintillation counting. Hydrolysis of cAMP or cGMP was <20% of the substrate and was linearly proportional to incubation time and enzyme protein. Ca\(^{2+}\)/CaM activation of PDE was calculated by subtracting the basal PDE activity from the Ca\(^{2+}\)/CaM PDE activity (11).

**Materials**

The NO donor, DETA-NO, was purchased from Alexis Biochemical (Ann Arbor, MI). [2,8-\(^{3}H\)]cAMP/cGMP were from DuPont NEN. The QAE-Sephadex A-25 resin was purchased from Pharmacia (Piscataway, NJ). Unless otherwise specified in the text, all other drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO). All drugs and chemicals were dissolved in distilled water.

**Statistical Analysis**

Data are expressed as means ± SE; \(n\) represents the number of pigs. The effects of atrial natriuretic peptide on isometric force and (cGMP) was assessed by repeated-measures ANOVA with post hoc analysis using Student-Newman-Keuls method. cGKI mRNA expression, cGKI protein expression, and phosphotransferase activities between PASM and TSM were compared by unpaired Student’s \(t\)-test. \(P\) value < 0.05 was considered statistically significant. Concentration-response curves were compared by nonlinear regression analysis, as described by Meddings et al. (34). In this method, force (\(F\)) at any concentration of drug (C) is given by the equation \(F = F_m C / (EC_{50} + C)\), where \(F_m\) represents the maximal isometric force. \(EC_{50}\) represents the concentration that produces half-maximal isometric force for that drug. Nonlinear regression analysis was used to fit values of \(F_m\) and \(EC_{50}\) to data for \(F\) and \(C\) for each condition studied. This method allows comparison of curves to determine whether they are significantly different and whether this overall difference can be attributed to differences in \(F_m\), \(EC_{50}\), or both parameters. A \(P\) value <0.05 was considered statistically significant.

**RESULTS**

**Physiological Studies**

Up to 48 h in culture did not significantly change DNA content of porcine PA compared with freshly prepared tissue (Fig. 1, middle). Similarly, although there appeared to be a trend for a reduction in total RNA, there was no significant reduction in total RNA for up to 48 h in culture, with or without 1 mM DETA-NO (Fig. 1, right). The quality of the RNA in porcine PA samples, as assessed by the 18S to 28S band intensities and the absence of laddering below the 18S band, was also not affected by up to 48 h in culture, with or without 1 mM DETA-NO. There was no eNOS or inducible NOS detected by immunoblotting in freshly prepared and 24- and 48-h cultured, endothelium-denuded PA used in these studies (data not shown).

The concentration-response curve of porcine PA to the \(\alpha\)-adrenergic receptor agonist phenylephrine was not significantly changed by up to 48 h in culture (Fig. 2, top). There was a small but significant difference in \(pEC_{50}\) (negative logarithm of \(EC_{50}\)) to phenylephrine between PA cultured for 24 h with and without 1 mM DETA-NO, 6.62 ± 0.15 and 6.40 ± 0.09, respectively, \(P < 0.05\) (Fig. 2, top). There was no further change at 48 h (data not shown). An example of a zinc-stained SDS-PAGE with 24-h cultured PA with (NO) and without (Ctl) 1 mM DETA-NO with the actin and myosin bands indicated is shown in Fig. 2, top, inset. The amount of actin and myosin present in 24-h cultured PA with and without DETA-NO relative to that in freshly prepared PA was 95 ± 4 and 97 ± 7% (actin) and 92 ± 13 and 94 ± 11% (myosin), respectively. The phenylephrine-induced maximal isometric force development per unit area or per unit volume of the PA strips was not changed by 24 h in tissue culture with and without 1 mM DETA-NO (Fig. 2, bottom) compared with freshly prepared PA.

The effect of culture and of prolonged exposure to DETA-NO on the isometric force response of PA to acutely applied DETA-NO following a phenylephrine-induced contraction ~50% of maximal isometric force is shown in Fig. 3. The \(pEC_{50}\) for freshly prepared, 24-h, and 48-h cultured PA (6.47 ± 0.09, 6.55 ± 0.12, and 6.52 ± 0.13, respectively) did not differ significantly (Fig. 3A). Treatment of PA for 24 and 48 h with 1 mM DETA-NO resulted in a significant right shift in the response to acutely applied DETA-NO, with \(pEC_{50}\) of

![Graph](http://example.com/graph.png)
compared with 24-h cultured PA, (pEC50 6.64).

PA was cultured for 24 h in the presence of 1 mM DETA-NO. Treatment was concentration dependent. No effect was observed when these values at 24 and 48 h were not significantly different. The concentration response to acutely applied DETA-NO was significantly right shifted, and the maximal extent of relaxation reduced in PA cultured with DETA-NO for 24 h and 48 h.

phenylephrine concentration-response curve in freshly prepared PA and 24-h cultured PA without (24) and with (24 NO) DETA-NO. Data are presented as means ± SE; n = 6.

5.02 ± 0.21 and 5.05 ± 29, respectively, P < 0.01 (Fig. 3A); these values at 24 and 48 h were not significantly different. The effect of prolonged DETA-NO treatment on NO-induced relaxation was concentration dependent. No effect was observed when PA was cultured for 24 h in the presence of 1 mM DETA-NO compared with 24-h cultured PA (pEC50 6.64 ± 0.18 and 6.79 ± 0.19, respectively; Fig. 3B). The pEC50 to DETA-NO was significantly increased by 24-h treatment with 10 and 100 μM DETA-NO, to 6.27 ± 0.04 and 5.6 ± 0.24, respectively, P < 0.05. The pEC50 to DETA-NO in 24-h cultured PA following a 60% increase in the phenylephrine concentration above EC50 was 6.48 ± 0.10, which was not significantly different from that obtained at the phenylephrine EC50.

The SOD mimic MnTBAP (10 μM) had no effect on phenylephrine-induced contraction in PA cultured 24 h with 1 mM DETA-NO (Fig. 4, top). MnTBAP and an equal volume of 50% DMSO without MnTBAP also had no effect on the phenylephrine concentration-response curve in freshly prepared PA (data not shown). MnTBAP (10 μM) had no effect on the DETA-NO pEC50 in PA cultured for 24 h, with and without 1 mM DETA-NO (Fig. 4, bottom). In three separate parallel experiments, 100 μM MnTBAP had no effect on either the phenylephrine or the DETA-NO concentration-response curves in PA cultured for 24 h with and without 1 mM DETA-NO (data not shown). Superoxide levels, as reflected by lucigenin enhanced chemiluminescence (n = 4), were not significantly changed from that of freshly prepared PA in PA cultured for 24 h without and with 1 mM DETA-NO (Fig. 4, top, inset).

sGC Expression Studies

Of the sGC subunits probed for, only sGCα1 and sGCβ1 were found in appreciable quantities in porcine PA. sGCα3 mRNA was present in PA, but accounted for <5% of the sGC subunits. In the open reading frame, the mRNA sequence for porcine PA sGCα1 was 86 and 87% homologous to canine (28) and human sGCα, respectively (accession nos. DQ008575 and Y17523, respectively). The inferred amino acid sequence homology was 92 and 91%, respectively. The calculated molecular weight was 77,246 Da. sGCβ1 mRNA open reading frame sequence homology was 91 and 92% to the canine and human sequences (accession nos. DQ008576 and X66533, respectively). The inferred amino acid sequence homology for sGCβ1 was 99 and 98%, and the calculated molecular weight was 70,541 Da.

As previously reported with canine PA (28), quantitative competitive RT-PCR amplified both the constructed mimeres.
The amino acid sequence this antibody is directed against is identical in the bovine and porcine specimen (data not shown). The amino acid sequence this antibody is directed against is identical in the bovine and porcine specimen (data not shown). The amino acid sequence this antibody is directed against is identical in the bovine and porcine specimen (data not shown). The amino acid sequence this antibody is directed against is identical in the bovine and porcine specimen (data not shown). The amino acid sequence this antibody is directed against is identical in the bovine and porcine specimen (data not shown).

cDNA and the sample cDNA (data not shown). Serial dilutions of mime produced linear changes in band intensity that encompassed the intensity of sGC subunit selective bands (data not shown). sGCα1, and sGCβ1 mRNA expression in freshly prepared porcine PA did not significantly differ, 0.29 ± 0.08 and 0.19 ± 0.03 amol/μg total RNA, respectively (Fig. 5, A and B, respectively). Following 24 h in culture, mRNA expression for sGCα1 (Fig. 5A) and sGCβ1 (Fig. 5B) were not significantly changed from that in freshly prepared PA. Twenty-four-hour treatment with 1 mM DETA-NO in organ culture resulted in a significant reduction in the mRNA expression of both sGC subunits to 0.16 ± 0.03 and 0.10 ± 0.02 amol/μg total RNA for sGCα1 and sGCβ1, respectively.

An example of an SDS-PAGE result in which tissue homogenate was loaded into lanes adjacent to a set of six concentrations of purified sGC from bovine lung is shown in Fig. 6, top, inset. We confirmed that the antibody used in this study was selective to the sGCβ1 subunit by examining the labeling of recombinant porcine sGCα1 and sGCβ1 expressed in E. coli (data not shown). The amino acid sequence this antibody is directed against is identical in the bovine and porcine sequence. A plot of arbitrary intensity units vs. the concentration of purified sGC resulted in a nonlinear standard curve best fit with a sigmoid curve fit (Fig. 6, top). Equal loading of protein in the porcine PA homogenate suggests that sGCβ1 protein expression is reduced by 24 h in culture and further reduced by 24-h culture in the presence of DETA-NO (Fig. 6, top). This is demonstrated in Fig. 6, bottom, in which the level of sGC protein expression is significantly reduced from that present in freshly prepared porcine PA, 42 ± 4, to 36 ± 3 and 31 ± 3 ng/mg total homogenate protein following 24-h culture without and with DETA-NO, respectively.

**cGMP Measurements**

**Intact** (cGMP) measurements. (cGMP) levels 10 min after treatment with isobutylmethylxanthine were 9.73 ± 2.91 and 11.72 ± 3.34 pmol/mg protein in freshly prepared and 24-h cultured PA, respectively (Fig. 7, top). Following 24-h treatment with DETA-NO, (cGMP) was 48.3 ± 3.11 pmol/mg protein, significantly higher than in PA not treated with DETA-NO for 24 h, P < 0.05. Acutely applied DETA-NO resulted in a significant increase in (cGMP), for both freshly prepared and PA cultured without and with DETA-NO for 24 h, but the extent of the increase was significantly less in PA cultured for 24 h with DETA-NO, P < 0.05 (Fig. 8, top). (cGMP), normalized to the amount of sGC protein (measured separately from those in the sGC expression measurement in Fig. 6) in freshly prepared and PA cultured without and with DETA-NO was 5.24 ± 0.81, 6.95 ± 0.70, and 3.76 ± 0.45

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**Fig. 4.** Effect of the superoxide dismutase mimetic Mn(III) tetra(4-benzoic acid) porphyrin chloride (MnTBAP) on the phenylephrine and DETA-NO response curves in porcine PA. **Top:** the phenylephrine concentration response curves for PA cultured for 24 h with 1 mM DETA-NO in the absence (○) and presence (○) of 10 μM MnTBAP were not significantly different. **Inset:** superoxide in PA detected by lucigenin chemiluminescence was not increased by 24-h treatment with 1 mM DETA-NO. Twenty-four-hour treatment with 1 mM DETA-NO in organ culture resulted in a significant increase in (cGMP)i for both freshly prepared PA and PA cultured without and with DETA-NO for 24 h, but the extent of the increase was significantly less in PA cultured for 24 h with DETA-NO, P < 0.05. Acutely applied DETA-NO resulted in a significant increase in (cGMP), for both freshly prepared and PA cultured without and with DETA-NO for 24 h, but the extent of the increase was significantly less in PA cultured for 24 h with DETA-NO, P < 0.05 (Fig. 8, top). (cGMP), normalized to the amount of sGC protein (measured separately from those in the sGC expression measurement in Fig. 6) in freshly prepared and PA cultured without and with DETA-NO was 5.24 ± 0.81, 6.95 ± 0.70, and 3.76 ± 0.45

**Fig. 5.** Quantitative competitive RT-PCR measurement of soluble guanylyl cyclase (sGC) subunit mRNA expression in porcine PA. **A:** sGCα1 subunit expression did not differ from that in freshly prepared (0 h) PA following 24 h in culture (24 h), but was significantly reduced following 24 h in culture with DETA-NO (24 h NO). **B:** the amount of sGCβ1 subunit mRNA expression in PA did not differ significantly from sGCα1, and was not affected by 24 h in culture. sGCβ1 expression was significantly reduced following 24-h culture with DETA-NO. Data are presented as means ± SE; n = 6. *Different from 0 h; #different from 24 h: P < 0.05.
pmol cGMP·min⁻¹·ng sGCβ₁⁻¹, respectively. Compared with that in freshly prepared tissue, the normalized (cGMP) was significantly higher in PA cultured 24 h without DETA-NO and significantly lower in PA cultured 24 h with DETA-NO, $P < 0.05$ (Fig. 7, bottom). Normalized (cGMP), was also significantly different between PA cultured 24 h without and with DETA-NO, $P < 0.05$.

**Tissue homogenate cGMP measurements.** Basal cGMP production in the soluble fraction of tissue extracts from PA cultured for 24 h without and with 1 mM DETA-NO was 2.65 ± 0.37 and 3.53 ± 0.41 pmol cGMP·min⁻¹·ng sGCβ₁⁻¹, respectively ($n = 4$, $P < 0.05$). Sodium nitroprusside (100 μM)-activated cGMP production was 6.38 ± 0.81 and 4.72 ± 0.44 pmol cGMP·min⁻¹·ng sGCβ₁⁻¹, respectively ($n = 4$, $P < 0.05$) in PA following 24-h treatment without and with 1 mM DETA-NO.

**PDE Activity Measurements**

There was no difference in basal cGMP PDE activity in homogenates from freshly prepared and 24-h cultured PA with and without 1 mM DETA-NO (Fig. 8, top). There was a significant increase in cGMP PDE activity in the presence of Ca²⁺ and CaM, but there was again no difference in this activity across the same tissue homogenate preparations (Fig. 8, top). The difference between the cGMP-PDE activity in the presence and absence of CaM was also not significantly different between freshly prepared and 24-h cultured PA with and without DETA-NO treatment. Basal cAMP PDE activity was significantly increased by 24 h in culture, $P < 0.05$ (Fig. 8, bottom), but was not significantly increased following 24 h in culture in the presence of 1 mM DETA-NO. There was no significant increase in cAMP PDE activity in porcine PA homogenates in the presence of CaM and in the difference in PDE activity with and without CaM.

**DISCUSSION**

The significant findings of this study are that prolonged exposure of porcine PA to an NO donor that spontaneously releases NO reduces responsiveness to both acutely applied NO and the α-adrenergic receptor agonist phenylephrine. This was associated with a reduction in sGC subunit mRNA, sGC protein expression, and NO-induced cGMP production. Furthermore, prolonged exposure to NO decreased the amount of cGMP produced per unit of sGC protein in porcine PA. This suggests a form of sGC activity regulation beyond that associated with acute binding of NO to sGCβ₁ subunit heme iron.

Cultured vessels have proven useful to characterize smooth muscle responsiveness (9), including studies of NO-mediated responses (26, 27). They permit assessment of a physiologically relevant endpoint, isometric force, for both relaxation and contraction.
minimizes the role of variable NO release kinetics and bioconversion in these processes.

Within 24 h, exposure to DETA-NO significantly reduced the responsiveness of cultured PA to an acutely applied NO donor. An additional 24-h exposure did not further change responsiveness, suggesting that within 24 h the smooth muscle adapts to higher NO levels with stable changes in the NO-sGC-cGMP signaling system. The NO-induced reduction in NO donor response was present at concentrations that approach the EC50 range, which suggests that this process may be operative at physiologically relevant NO concentrations. This is consistent with prior work finding that the low levels of NO from eNOS under basal conditions are capable of inducing a reduction in NO donor responsiveness (37) without a reduction in the amount of sGC protein expressed (40).

There are several possible mechanisms capable of reducing NO responsiveness in vascular smooth muscle. Focusing on those mechanisms intrinsic to the smooth muscle, these include the following: 1) reduced NO bioavailability due to decreased bioconversion or increased NO scavenging, 2) reduced sGC activity due to decreased sGC protein expression, 3) reduced sGC-specific activity, 4) increased cGMP PDE activity, and 5) decreased cGMP responsiveness, which may result from decreased responses in downstream cGMP sensors or in the localization of sGC. While reduced cGMP responsiveness may contribute to NO-induced NO hyporesponsiveness (54), it was not the subject of these studies and will not be further addressed in this discussion.

**NO Bioavailability**

Prolonged exposure of vascular smooth muscle to organo-nitrates induces a reduction in NO donor responsiveness due to increased superoxide production (44, 45). This mechanism should decrease the vascular smooth muscle response to all NO sources, whether from NOS or any NO donor that releases NO. If this mechanism contributes to the reduction in pulmonary arterial NO responsiveness after 24-h treatment with DETA-NO, two predictions would follow: 1) scavenging of superoxide with a membrane-permeant SOD mimic should increase the NO responsiveness, and 2) increased superoxide production should be detected. Neither prediction was borne out in these studies. It is possible that the concentration of the SOD mimic was insufficient to observe an increase in NO responsiveness, although a 10-fold higher concentration also failed to increase NO responsiveness with or without prolonged treatment with DETA-NO. The results suggest that NO from DETA-NO does not give rise to increased superoxide production in endothelium-denuded PA under the conditions used in these studies and suggest that nitrile-induced nitrate hyporesponsiveness is, in part, mechanistically distinct from NO-induced NO hyporesponsiveness, as recently reported (10).

**sGC Expression**

Another potential mechanism producing NO-induced NO hyporesponsiveness is decreased sGC subunit protein expression. The primary sGC subunit mRNAs found in porcine PA were sGCα1 and sGCβ1. We also found a small amount of sGCα2 mRNA, consistent with recent findings in mouse aorta (35). In agreement with previous results in canine PA (28), sGCα1 and sGCβ1 mRNA expression were nearly equal and in

contraction in conjunction with biochemical assessment of factors regulating the response to NO. Smooth muscle cells in the cultured vessels maintain a contractile phenotype for 24 h or more, in contrast to most cultured vascular smooth muscle cell preparations. The cultured artery preparation also permits assessment of prolonged NO donor treatment effects in the absence of relevant, but confounding, counterregulatory neurohumoral mechanisms present when studies are performed in vivo. We showed that the cultured PA model maintains normal DNA, total RNA, actin and myosin protein levels, specific force, sensitivity to an α-adrenergic receptor agonist, and sensitivity to NO donor-induced relaxation following agonist-induced submaximal contraction after 48 h in culture. The cultured PA preparation thus permits assessment of the effects of NO donor treatment over this period in a preparation that remains similar to that of freshly isolated PA.

DETA-NO, an NO donor that spontaneously releases NO in buffered solutions with a half time ≈24 h (22), was used in these studies for two reasons. First, stable levels of NO are attained in culture medium for up to 24 h (13). Second, DETA-NO releases authentic NO and does not require other factors to induce NO release. This is in contrast to almost every other class of NO donor, which either requires a protein or low molecular weight thiol or a redox active metal ion to facilitate production of either NO or a bioactive NO precursor, such as an S-nitrosothiol species. Thus use of DETA-NO to both induce and then examine NO-induced NO hyporesponsiveness...
Reduced expression of sGC protein should decrease sGC activity proportional to the reduction in protein expression, unless sGC-specific activity changes. An additional and largely unexplored mechanism by which NO may induce NO hyporesponsiveness is by reducing sGC-specific activity. Assessment of this possibility requires assaying sGC enzyme activity and normalizing this activity to the amount of sGC protein present in the sample. sGC activity was estimated by measurement of intracellular cGMP concentration in intact PA strips. Our results for the cGMP measurements agree well with those obtained by others in intact smooth muscle tissue samples (28, 38, 49). Following 24-h treatment with DETA-NO, (cGMP) levels were almost five times greater than in PA not treated with DETA-NO. This is unlikely to be due to residual DETA-NO in the tissue due to extensive washing and the freely diffusible nature of both NO and DETA-NO. Rather, it may reflect the development of intracellular NO “stores” that have slower NO release kinetics than the original NO donor, as recently suggested (29). This persistent elevation in basal (cGMP), in tissue treated with DETA-NO for 24 h may play a role in the α-adrenergic receptor hyporesponsiveness described later.

In intact tissue, acutely applied NO resulted in a ∼20-fold increase in (cGMP), in fresh and 24-h cultured PA, but resulted in only an approximately twofold increase in PA treated with DETA-NO for 24 h. The amount of cGMP produced in PA treated with DETA-NO for 24 h following acute application of NO was less than one-half that observed in freshly prepared and 24-h cultured PA. This reduction in (cGMP), in PA treated with DETA-NO for 24 h was greater than the reduction in sGCβ1 protein expressed. It is possible, however, that the reduction in sGC activity observed following prolonged treatment with NO was due to confounding factors related to the intact preparation, such as localized or general reduction in sGC substrate (GTP) or cofactors. The results suggested that sGC-specific activity was significantly reduced by prolonged treatment with NO. If this is the case, it would be predicted that a significant reduction in sGC activity would also be observed in PA-soluble homogenates prepared following prolonged treatment with DETA-NO. The results from the homogenate studies confirmed this prediction. The reported activity in homogenates from PA not treated with DETA-NO for 24 h compared well with those in a previous report (38), although the activity in that study was not normalized to the amount of sGC present in aortic tissue. Taken together, the intact tissue results and those obtained in PA homogenates strongly suggest that sGC “basal”-specific activity is increased and NO-activated sGC-specific activity is significantly decreased by prolonged treatment of PA with DETA-NO. The relative contribution of the reduction in sGC-specific activity vs. the reduction in sGC expression is difficult to precisely determine, but both appear to play a significant role in contributing to NO-induced NO hyporesponsiveness in PA. This result contrasts with a previous report, indicating that the salient mechanism for NO-induced NO hyporesponsiveness following 4-h incubation with NO in aortic smooth muscle was reduced sGC protein expression, rather than specific activity (59). Differences in the NO donor used, the NO treatment time, and in the
semiquantitative methods used in the two studies probably account for these differences.

Possible mechanisms by which prolonged NO treatment results in reduced sGC-specific activity in the present study are speculative at this point, but those for which there is a precedent include altered expression of sGC-associated proteins, such as the heat shock proteins and other chaperonins (2, 19, 24), oxidation of the Fe(II) heme in sGCβ1 (1), and direct thiol redox modulation of sGC activity (36, 60). Recent results in support of the heme oxidation mechanism demonstrate the presence of either heme empty or heme oxidized sGC. This finding was associated with both reduced sGC-specific activity and NO responsiveness in several disease states in animals and humans (55).

PDE Activity

NO-induced NO hyporesponsiveness may also reflect increased cGMP breakdown. PA smooth muscle contains the two PDE primarily involved with breaking down cGMP to GMP: PDE 1A and PDE 5 (48, 50). Prolonged exposure of animals and cells to nitrates increased PDE 1A expression while decreasing nitrate responsiveness (23), suggesting a role for increased PDE activity in nitrate-induced nitrate hyporesponsiveness. Increased PDE 5 activity via cGK-mediated PDE 5 phosphorylation (39) has been correlated with desensitization to acutely applied NO following short-term treatment with S-nitrosoglutathione. In the present studies, Ca2+-independent PDE activity slightly exceeded Ca2+-CaM-dependent cGMP PDE, which may indicate PDE 5 > PDE 1 activity in porcine PA, consistent with observations in other vascular smooth muscle preparations (33). There was, however, no significant increase in either the Ca2+-independent or Ca2+-CaM-dependent PDE activity observed following 24-h treatment of cultured PA with DETA-NO. In addition, prolonged treatment with DETA-NO did not result in increased cAMP PDE activity, as reported in cultured rat PA cells following prolonged treatment with S-nitrosoglutathione (7). Possible explanations for the discrepancy between present observations and those previously reported include differences in the NO-donors used, differences in the incubation times, and differences in the tissue preparation used. It is also possible that localized or compartmentalized changes in specific PDE expression/activity occur that would not be detected by the PDE assay methods used in the present studies. However, since intracellular, localized PDE activity measurements are problematic and since pharmacological tools for assigning PDE activity to a specific PDE isofrom in a complex tissue sample containing more than six different PDE isoforms are not yet ideal, the simple approach of measuring overall cGMP and cAMP PDE has merit. The tissue homogenate PDE activity results in the present studies do not indicate a significant role for NO or cGMP-mediated increases in PDE activity in the observed NO-induced NO hyporesponsiveness. The findings of reduced sGC expression and specific activity were determined under conditions in which PDE activity either does not impact the measurement (expression) or is inhibited (sGC-specific activity).

Adrenergic Responsiveness

Prolonged treatment with DETA-NO also decreased responsiveness to an α-adrenergic receptor agonist, phenylephrine. There are several possible explanations for this that can be broadly classified as cGMP dependent and cGMP independent. The possibility that NO-induced hyporesponsiveness was due to the use of a higher phenylephrine concentration to attain 1 = 50% maximal isometric force is unlikely because increasing the phenylephrine concentration in control strips by an amount corresponding to the shift in the EC50 in PA cultured without and with DETA-NO did not decrease the response to acutely applied DETA-NO (data not shown). The finding that cGMP production remains elevated in PA cultured for 24 h with DETA-NO following extensive washing suggests that the α-adrenergic receptor agonist hyporesponsiveness observed in these studies may be due to a cGMP-dependent mechanism, although a cGMP-independent mechanism reported by others (21, 47) may also contribute to NO-induced agonist hyporesponsiveness.

Summary

Prolonged exposure of cultured PA to the NO donor DETA-NO decreased both α-adrenergic receptor agonist and acutely applied NO donor responsiveness, with the latter response decreased by more than an order of magnitude. The observed NO-induced NO hyporesponsiveness was associated with a reduction in sGC subunit mRNA and a more modest reduction in sGC subunit protein expression. Semiquantitative measurement of sGC subunit protein expression in conjunction with measurement of sGC activity reveals for the first time that prolonged NO treatment results in a significant reduction in NO-activated sGC-specific activity and an increase in basal sGC-specific activity.

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