

Desensitization of NO/cGMP Signaling in Smooth Muscle: Blood Vessels Versus Airways

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ABSTRACT

The NO/cGMP signaling pathway plays a major role in the cardiovascular system, in which it is involved in the regulation of smooth muscle tone and inhibition of platelet aggregation. Under pathophysiological conditions such as endothelial dysfunction, coronary artery disease, and airway hyperreactivity, smooth muscle containing arteries and bronchi are of great pharmacological interest. In these tissues, NO mediates its effects by stimulating guanylyl cyclase (GC) to form cGMP; the subsequent increase in cGMP is counteracted by the cGMP-specific phosphodiesterase (PDE5), which hydrolyzes cGMP. In platelets, allosteric activation of PDE5 by cGMP paralleled by phosphorylation has been shown to govern the sensitivity of NO/cGMP signaling. Here, we demonstrate that the functional

responsiveness to NO correlates with the relative abundance of GC and PDE5 in aortic and bronchial tissue, respectively. We show a sustained desensitization of the NO-induced relaxation of aortic and bronchial rings caused by a short-term exposure to NO. The NO treatment caused heterologous desensitization of atrial natriuretic peptide-induced relaxation, whereas relaxation by the cGMP analog 8-pCPT-cGMP was unperturbed. Impaired relaxation was shown to be paralleled by PDE5 phosphorylation; this indicates enhanced cGMP degradation as a mechanism of desensitization. In summary, our results demonstrate the physiological impact of PDE5 activation on the control of smooth muscle tone and provide an explanation for the apparent impairment of NO-induced vasorelaxation.

The NO/cGMP signaling pathway is involved in the relaxation of smooth muscle and the inhibition of platelet aggregation. In addition to the well-characterized regulation of vascular tone, NO is also involved in the relaxation of airway smooth muscle, which results in bronchodilation. The main source of NO in the vascular system is the endothelium, in which it is generated by endothelial NO synthase. NO acts as a paracrine messenger and leads to the relaxation of adjacent smooth muscle cells and the inhibition of platelet aggregation. Impaired regulation of the signaling cascade has been associated with endothelial dysfunction, which often precedes severe cardiovascular events. In the lung, NO is believed to be mainly generated in the airway epithelium by endothelial NO synthase and in nonadrenergic noncholinergic neurons by neuronal NO synthase. The inhibition of NO

production can lead to a bronchial hyperresponsiveness to inflammatory mediators in humans (Ricciardolo et al., 1996; Hamad et al., 2003) and to enhanced contraction of murine bronchial rings in response to electrical field stimulation (Kakuyama et al., 1999), demonstrating a role of NO in the regulation of bronchial tone, at least under pathophysiological conditions.

The most important receptor molecule for NO is NO-sensitive guanylyl cyclase (GC), which catalyzes the formation of cGMP. An increase in cGMP leads to the activation of cGMP effector proteins [e.g., cGMP-dependent protein kinases (PKG), cGMP-regulated phosphodiesterases (PDEs), and cGMP-activated ion channels (Biel et al., 1999; Pfeifer et al., 1999; Feil et al., 2003)]. Activation of PKG is mandatory for cGMP-mediated relaxation of vascular smooth muscle and inhibition of platelet aggregation (Pfeifer et al., 1998; Massberg et al., 1999). In smooth muscle, cGMP synthesis is also enhanced by the binding of natriuretic peptides to the extracellular domain of particulate receptor-coupled GCs (Kuhn, 2003). In addition to GC, PDEs, which catalyze the hydrolysis of cyclic nucleotides, determine the amplitude and dura-

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ABBREVIATIONS: GC, guanylyl cyclase; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase; GSNO, S-nitroso glutathione; DEA-NO, 2-(N,N-diethylamino)-diazolot-2-oxide, diethylammonium salt; ProliNONOate, 1-(hydroxyl-NN-azoxy)-L-proline; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; HEK, human embryonic kidney; DTT, dithiothreitol; 5-HT, 5-hydroxytryptamine; PE, phosphatidylethanolamine; ANP, atrial natriuretic peptide.

tion of a cGMP signal (Juilfs et al., 1999). The major cGMP-degrading PDE in platelets and vascular smooth muscle is the cGMP-binding PDE5 (Maurice et al., 2003).

A negative feedback regulation in NO/cGMP signaling has been described in platelets. NO-induced increases in cGMP not only caused the activation of PKG but also enhanced PDE5 activity by cGMP binding to the regulatory GAF-A domain of PDE5 (Mullershausen et al., 2001, 2003; Rybalkin et al., 2003). Maximal stimulation of NO-sensitive GC only elicits a spike-like elevation of cGMP in platelets, because the cGMP increase is rapidly neutralized by this activation of PDE5. The allosteric activation of PDE5 is further stabilized by PKG-mediated phosphorylation (Mullershausen et al., 2004), which is very likely to account for the long-lasting desensitization of NO/cGMP signaling observed in platelets. The signaling cascade with its characteristic features (i.e., the spike-like cGMP response and the desensitization) has been reconstituted in HEK293 cells by coexpression of NO-sensitive GC and PDE5 (Mullershausen et al., 2004). The data corroborate the idea that the desensitization of NO/cGMP signaling by activation of PDE5 is inherent to any GC- and PDE5-expressing cell.

Due to the established relevance of NO/cGMP signaling in the cardiovascular system and its emerging importance in regulating bronchial tone, the mechanisms responsible for the regulation and transduction of NO/cGMP signaling are of major physiological and pharmacological interest. We therefore tested our model of desensitization of NO/cGMP signaling in rat thoracic aorta and second-generation branches of bronchus by assessing the NO-induced desensitization of cGMP-mediated relaxation responses. Herein, we provide evidence for a physiological implication of PDE5 activation in vascular smooth muscle and a as-yet-unidentified dominant role of PDE5 within NO/cGMP signaling in airway smooth muscle.

Materials and Methods

Preparation of Tissue Extracts for Western Blot. Rat thoracic aorta or second-generation bronchial rings were homogenized in buffer containing 50 mM NaCl, 1 mM EDTA, 50 mM triethanolamine/HCl, pH 7.4, 2 mM DTT, and protease inhibitor cocktail (Sigma, St. Louis, MO). After homogenization, 1% SDS was added, and extracts were incubated at 37°C for 60 min and centrifuged (15 min, 20,000g). SDS-polyacrylamide gel electrophoresis, Western blot analysis, and quantification of the signals were carried out as described previously (Mullershausen et al., 2003). Antibodies against α_1 and β_1 subunits of NO-sensitive GC and phospho-PDE5 were raised as described previously (Mullershausen et al., 2004); monoclonal PDE5 antibody was from BD Biosciences (San Jose, CA).

Isometric Tension Recordings. Thoracic aortae from Wistar rats were cut into rings and mounted into organ baths (Myograph 610; Danish Myo Technology, Copenhagen, Denmark) containing Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 7.5 mM glucose), pH 7.4, and gassed with 95% O₂/5% CO₂ in the presence 200 μ M *N*-nitro-L-arginine methyl ester. Resting tension was set to 10 mN (Newton $\times 10^{-3}$), and rings were allowed to equilibrate for at least 60 min at 37°C before the start of experiments.

Second-generation branches of the tracheobronchial tree were prepared from rat lung, cut into rings, and mounted into the organ baths containing Krebs-Henseleit solution and *N*-nitro-L-arginine methyl ester as above. The resting tension was set to 2.5 mN, and the rings were allowed to equilibrate for at least 60 min before the start of the experiments.

Determination of cGMP in Aortic Strips. Determination of cGMP in aortic strips and bronchial rings from Wistar rats was carried out as described previously (Mullershausen et al., 2001). In brief, tissue was incubated with GSNO as indicated, snap-frozen in liquid nitrogen, and homogenized in 70% ice-cold ethanol. The supernatants were dried under nitrogen, and cGMP was determined in a radioimmunoassay (Brooker et al., 1979). The protein content of the pellets was determined for normalization using the BCA method (Pierce, Rockford, IL).

Determination of NO-Stimulated GC Activity and PDE Activity in Aortic and Bronchial Homogenates. Rat thoracic aorta or second-generation bronchial rings were isolated and homogenized immediately in buffer (50 mM triethanolamine/HCl, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μ M pepstatin A, pH 7.4, at 4°C). Homogenates were obtained after centrifugation (800g, 5 min, 4°C). The protein concentration was determined in triplicate and repeated three times (Bradford assay; Bio-Rad, Hercules, CA).

NO-stimulated GC activity was measured for 10 min (at 37°C) using 10 μ l of homogenates (~ 10 – 15 μ g of protein) and 100 μ M 2-(*N,N*-diethylamino)-diazene-2-oxide (DEA-NO; Alexis, Laüfelfingen, Switzerland) as described previously (Russwurm and Koesling, 2005).

PDE activity was measured in homogenates by the conversion of [³²P]cGMP (synthesized from [α -³²P]GTP using purified NO-sensitive GC) to guanosine and [³²P]phosphate in the presence of alkaline phosphatase (Sigma) at 37°C for 7 min. Reaction mixtures (0.1 ml) contained 2 to 5 μ l of the homogenates (~ 7 μ g of protein of aorta, ~ 2 μ g of protein of bronchus), [³²P]cGMP (~ 2 kBq), 1 μ M cGMP, 12 mM MgCl₂, 3 mM DTT, 0.5 mg/ml bovine serum albumin, 2 U of alkaline phosphatase, and 50 mM triethanolamine/HCl, pH 7.4. Reactions were stopped by adding 900 μ l of ice-cold charcoal suspension (30% activated charcoal in 50 mM KH₂PO₄, pH 2.3). After pelleting the charcoal by centrifugation, [³²P]phosphate was measured in supernatant. A 100 nM concentration of sildenafil (a generous gift from Pfizer Inc., New York, NY) was used to inhibit PDE5. GC and PDE assays were carried out in triplicate and repeated three or two times for each homogenate (three to four animals).

Results

To compare the NO/cGMP signaling pathway in vascular smooth muscle with airway smooth muscle, we first examined the expression levels of NO-sensitive GC (α_1 and β_1 subunits) and PDE5 using quantitative Western blot analysis. Figure 1, A and B, shows the expression of PDE5, GC- α_1 , and GC- β_1 in bronchus relative to the expression detected in aorta. Whereas PDE5 expression was 2.5-fold higher in bronchi, the level of either subunit of GC was only approximately 50% of that detected in aorta. As a functional approach, NO-stimulated cGMP formation and cGMP hydrolysis were assayed in the homogenates of aorta and bronchi. To selectively determine PDE5 activity in the samples, the hydrolysis of cGMP was measured in the presence or absence of sildenafil. The amount of PDE activity inhibited by sildenafil (100 nM, with 1 μ M cGMP as substrate) was approximately 65% in aorta and bronchus, indicating that PDE5 is the major cGMP degrading PDE in both tissues. The catalytic activities of NO-sensitive GC and PDE5 shown in Fig. 1, A and B, are in good accordance with the expression levels. PDE5 activity was approximately 2.2-fold higher in the bronchus, whereas cGMP formation was only 53% of the activity found in aorta. The considerably higher ratio of PDE5 to GC expression and activity in the bronchus indicates a relative dominance of cGMP degradation over cGMP synthesis in airway smooth muscle.

To assess the physiological relevance of the expression data, NO-induced relaxations of aortic rings versus bronchial rings were determined in organ bath experiments. Figure 1C shows the concentration-response relationships for the GSNO-induced relaxation of 5-HT-contracted aortic and bronchial rings. The EC_{50} values were approximately 0.05 and 5 μ M GSNO for the relaxation of aorta and bronchus, respectively. The lower potency of GSNO to relax bronchial rings is consistent with the higher ratio of PDE5 to GC expression. To assess the possible contribution of the cGMP-inhibited PDE3 on NO/cGMP-induced relaxations of aortic and bronchial smooth muscle, concentration-responses for NO were recorded in the presence of the specific inhibitor milrinone (1 μ M). The inhibition of PDE3 alone led to a partial relaxation of aortic and bronchial rings (~30%), but the EC_{50} value for NO-induced relaxation was unaltered (data not shown). Therefore, PDE3 does not seem to contribute to NO-induced relaxations of aorta under the conditions used in this study.

Because NO-induced relaxation of smooth muscle is mediated by cGMP, we next analyzed the GSNO-induced cGMP responses in aortic and bronchial rings. Figure 1D shows the time course of the intracellular cGMP accumulation after the addition of GSNO (100 μ M). In both tissues, GSNO elicited a transient elevation of cGMP. The maximal cGMP levels reached after 30 to 60 s were 5- to 10-fold higher in aortic rings than in bronchial rings. The lower cGMP levels measured in bronchial rings are consistent with a higher PDE5-to-GC ratio determined in bronchus compared with aorta.

In this experiment, a rather high concentration of GSNO (100 μ M) was used to induce the maximal cGMP response (Fig. 1D). The GSNO concentration-response curve for cGMP accumulation in aortic rings (Fig. 2A) demonstrates that this concentration is indeed required to achieve a maximal cGMP increase in aortic tissue. In the same graph, the GSNO-concentration-response for relaxation of PE-contracted aortic rings is given for comparison, showing that the EC_{50} values for cGMP accumulation and relaxation (3 and 0.1 μ M, respectively) differ considerably. The higher potency of NO to relax vascular smooth muscle indicates that local increases in

cGMP are sufficient to cause a cellular response even though they are barely detectable when determining the mean cGMP level in intact tissue.

The transient elevation of cGMP induced by NO in aortic and bronchial rings seemed similar to the one observed in platelets (Mullershausen et al., 2001). In these cells, the decrease in cGMP was shown to be caused by cGMP-induced activation of PDE5, which also led to desensitization of the cGMP response. To detect a similar mechanism in vascular smooth muscle, aortic strips were preincubated with a sub-maximally effective concentration of GSNO (10 μ M for 10 min) to induce desensitization. After buffer exchange to remove NO, the tissue was kept under NO-free conditions for 30 or 60 min. Then a maximally effective stimulus was applied (100 μ M GSNO), and cGMP accumulation was measured. As shown in Fig. 2B, preincubation of the tissue led to a reduced cGMP response even 60 min after the removal of NO. As shown previously in platelets (Mullershausen et al., 2001), the short incubation with NO led to a sustained desensitization of the signaling pathway in aorta. Because in bronchial rings the NO-induced cGMP increases were much lower than in aorta (5–10 fold), desensitization of the cGMP response could not be analyzed.

To assess the physiological implication of the NO-induced desensitization, relaxation responses were measured in vascular and airway smooth muscle. Aortic rings were preincubated without or with the rapidly decaying ($t_{1/2}$ = 1.8 s at 37°C) NO donor ProliNNOate (15 μ M, 10 min). After buffer exchange, the rings were contracted with phenylephrine; subsequent to reaching a plateau (30–40 min), relaxation was induced with GSNO (100 nM). As shown in Fig. 3A, relaxation of the preincubated rings was reduced compared with the control. The relaxation induced by 100 nM GSNO was reduced by 40% in the preincubated rings (48 versus 29% relaxation in control and preincubated samples, respectively; Fig. 3A, bar graph). A similar experiment was carried out with bronchial rings using DEA-NO during preincubation (30 μ M, 10 min). After buffer exchange, the rings were contracted with 5-HT (30 μ M), and relaxation was induced with DEA-NO (1 μ M). As shown in Fig. 3B, the relaxation of the

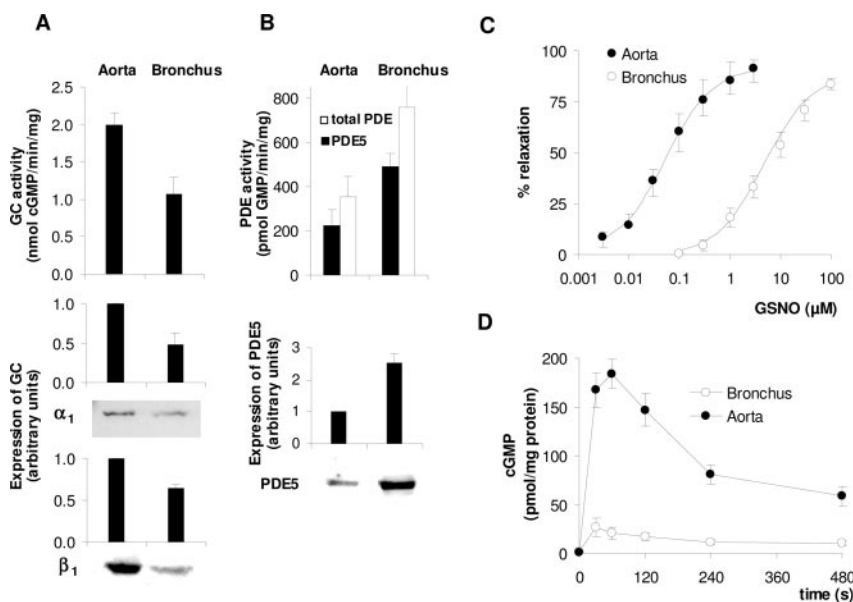


Fig. 1. Comparison of the NO/cGMP signaling pathway in aorta and bronchus. A, NO-stimulated GC activity measured in homogenates of aorta and bronchus (top) and Western blot detection of α_1 and β_1 subunits of NO-sensitive GC in 30 μ g of protein (bottom). B, total PDE and PDE5 activity detected in homogenates of aorta and bronchus (1 μ M cGMP as substrate, top). PDE5 activity represents the sildenafil-inhibited PDE activity (100 nM sildenafil). Bottom, the Western blot detection of PDE5 in 30 μ g of protein. The signal intensities measured in the Western blots were normalized to the value obtained in aorta. Data represent mean \pm S.E.M. of at least three (activities) or 10 (Western blots) independent experiments. C, concentration-response relationships for GSNO-induced relaxation of 5-HT-contracted aorta or bronchus. Data represent mean \pm S.E.M. of four determinations. D, time course of cGMP accumulation in aorta and bronchus after the addition of GSNO (100 μ M). Data are expressed as mean \pm S.E.M. of at least eight independent experiments.

preincubated rings was reduced by 60% (28 versus 11% in control and preincubated samples, respectively; Fig. 3B, bar graph).

We propose that the observed NO-induced desensitization is due to cGMP-induced PDE5 activation, which has been shown to be stabilized by phosphorylation. With antibodies that specifically detect only the phosphorylated form of the enzyme, we studied PDE5 phosphorylation in aortic tissue treated as in the relaxation experiment. As shown in Fig. 3C, phosphorylation of PDE5 was detected directly after preincubation (15 μ M ProliNONOate, 10 min) and 45 min after the removal of NO. The results show that the NO concentration used caused a cGMP increase sufficient to induce phosphorylation. In addition, phosphorylation is shown to be sustained, because it was still detectable 45 min after NO removal. In summary, activation of PDE5, as indicated by the phosphorylation, occurs under these experimental conditions, and is likely to account for the reduced sensitivity toward NO.

In addition to PDE5 activation as the basis for desensitization of NO/cGMP signaling, other mechanisms have been proposed (e.g., reduced NO availability and desensitization of NO-sensitive GC) (Hussain et al., 2001; Munzel et al., 2003). To functionally test these alternative mechanisms, aortic rings were preincubated with ProliNONOate as above (15 μ M, 10 min), and relaxation was induced by ANP to activate particulate GCs. As shown in Fig. 4A, relaxation responses to ANP (3 nM) were reduced by approximately 30% after preincubation with ProliNONOate (30 versus 22% relaxation in control and preincubated samples, respectively). The reduced ANP/cGMP-mediated relaxation clearly demonstrates that the NO-induced desensitization does not occur on the level of NO-sensitive GC or reduced availability of NO. Yet an NO/

cGMP-induced increase in cGMP degradation is sufficient to explain the impaired ANP/cGMP-mediated relaxation.

To test whether mechanisms downstream of cGMP turnover were involved in the NO-induced desensitization, relaxation of aortic rings by a direct activator of PKG was measured. Preincubation with ProliNONOate was carried out as described above, and relaxations were induced by 8-pCPT-cGMP (50 μ M), a nonhydrolyzable cGMP analog. As shown in

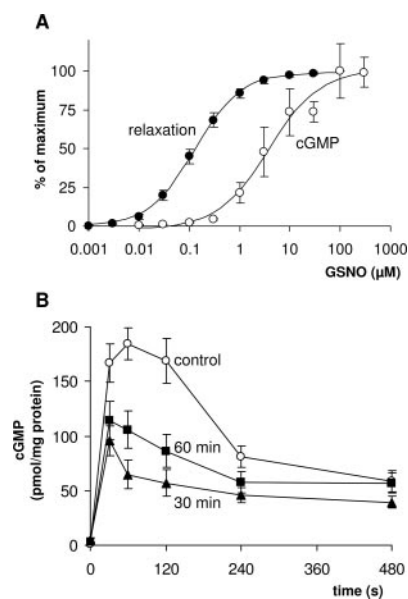


Fig. 2. NO-induced desensitization of cGMP accumulation in aorta. A, concentration-response relationships for GSNO-induced cGMP accumulation (1-min incubation, \circ) versus relaxation of PE-contracted aortic rings (\bullet). Data represent mean \pm S.E.M. of at least five independent experiments. B, aortic strips were preincubated without (control, \circ) or with 10 μ M GSNO for 10 min, washed twice to remove GSNO, and further incubated for 30 (\blacktriangle) or 60 min (\blacksquare). Strips were then stimulated with 100 μ M GSNO, and cGMP was determined at the indicated time points. Data represent mean \pm S.E.M. of 11 independent experiments.

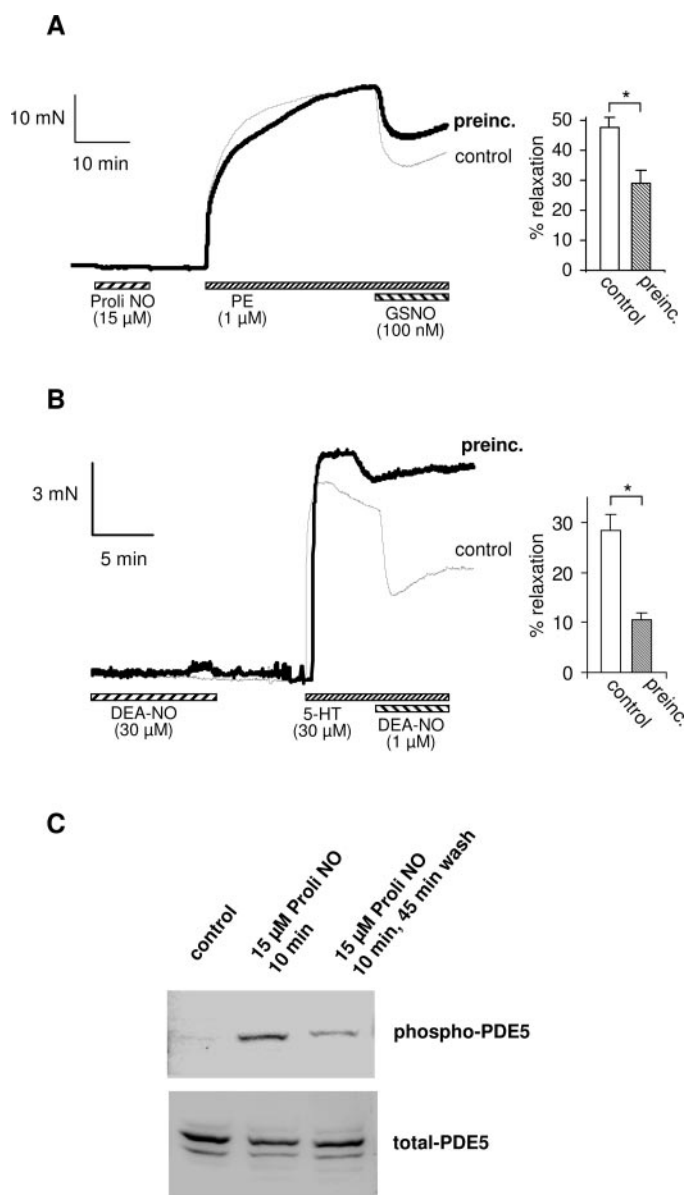


Fig. 3. NO-induced desensitization of smooth muscle relaxation and phosphorylation of PDE5. Aortic and bronchial rings were preincubated in the absence (thin traces, control) or presence (boldface traces, preincubation) of ProliNONOate (15 μ M, 10 min). Then, buffer was changed twice before the contraction of rings. A, aortic rings were contracted with PE (1 μ M); after 35 to 40 min, GSNO (100 nM) was added to induce relaxation. B, bronchial rings were contracted with 5-HT (30 μ M) and relaxed with DEA-NO (1 μ M). Statistical evaluation of the data are shown in the bar graphs; data represent mean \pm S.E.M. of 12 (A) or 6 (B) independent experiments (*, $P < 0.02$, Student's t test). C, aortic segments were treated as in A and were snap frozen in liquid nitrogen at the indicated time point. Homogenization and SDS-polyacrylamide gel electrophoresis were carried out as described under *Materials and Methods*; PDE5 phosphorylation was assessed with Western blot using a phospho-PDE5 antibody.

Fig. 4B, the relaxations of control and preincubated samples were 49 and 50%, respectively. This unaltered relaxation demonstrates that modulation of the effector systems downstream of cGMP is not involved in desensitization. In summary, NO induces desensitization of aortic smooth muscle relaxation, which occurs neither upstream of cGMP formation nor downstream of PKG. Together with the observed PDE5 phosphorylation, the reduced NO responsiveness is likely to result from PDE5 activation, limiting NO-induced cGMP increases.

Discussion

The comparative expression and activity analysis of GC and PDE5 in vascular and bronchial smooth muscle indicates a dominant role of PDE5 in the airway, which is consistent with the relatively low potency of NO donors to relax bronchial rings. Moreover, the high expression of PDE5 was clearly reflected by the modest elevations of cGMP in response to NO. However, the small increases in cGMP were sufficient to cause a full relaxation of 5-HT-contracted bronchial rings (Fig. 1, C and D). In aortic rings, expression of PDE5 was lower, whereas GC expression was higher than in bronchus; consistently, lower concentrations of NO were required for complete relaxation. The EC_{90} value for GSNO-induced relaxation of aortic rings was approximately $2 \mu\text{M}$, a concentration that elicited only 30% of the maximal cGMP response (Fig. 1C). In both tissues, comparable levels of cGMP were required to induce complete relaxation [approximately 45 pmol/mg protein (at $2 \mu\text{M}$ GSNO) in aorta versus 25 pmol/mg protein (at $100 \mu\text{M}$ GSNO) in bronchus]. The data suggest that the potency of NO in aortic and airway smooth muscle is primarily controlled by the ratio of GC and PDE activity rather than by downstream events.

The NO-induced desensitization of relaxation is another

common feature of vascular and bronchial smooth muscle. In both tissues, the short preincubation with NO led to a desensitization of NO-induced relaxation. In aortic rings, the desensitization was also reflected by a reduction of the NO-induced cGMP response. The NO-induced heterologous desensitization of ANP-mediated relaxation indicates a common underlying mechanism of negative feedback regulation in NO/cGMP signal transduction. Because both pathways converge on the level of cGMP degradation, activation of PDE5 would be sufficient to explain the observed effects. This idea is supported by the fact that mechanisms on the level of PKG and further downstream could be ruled out and that desensitization was paralleled by phosphorylation of PDE5. Whereas PDE5 phosphorylation was undetectable under basal conditions, it occurred after NO exposure of aortic rings and was still clearly detectable 45 min after NO removal. This is the first time that NO-induced PDE5 phosphorylation is demonstrated in aortic rings. Because cGMP binding to the regulatory GAF domains and the resulting activation of PDE5 is prerequisite for phosphorylation, the phosphorylation of PDE5 can be considered a reliable marker for PDE5 activation (Turko et al., 1998; Rybalkin et al., 2002; Mullershausen et al., 2003).

The characteristics of the NO-induced cGMP response in aortic and bronchial smooth muscle described here are analogous to the ones observed in platelets and GC/PDE5-expressing HEK cells (Mullershausen et al., 2001, 2003). As in these cells, activation of PDE5 has been shown to be responsible for general features of the cGMP response, it is conceivable that also in smooth muscle cells, PDE5 activation mediates the negative feedback regulation. The resulting NO-induced desensitization in platelets and GC/PDE5-expressing HEK cells has only been demonstrated on the level of cGMP. Yet with the reduced NO responsiveness of smooth muscle relaxation, our results demonstrate for the first time the functional relevance of the negative feedback. Our results suggest that under conditions of high NO production and increased PDE5 activity, the impaired NO-induced relaxation may contribute to pathophysiological phenomena such as endothelial dysfunction and airway hyperreactivity.

Acknowledgments

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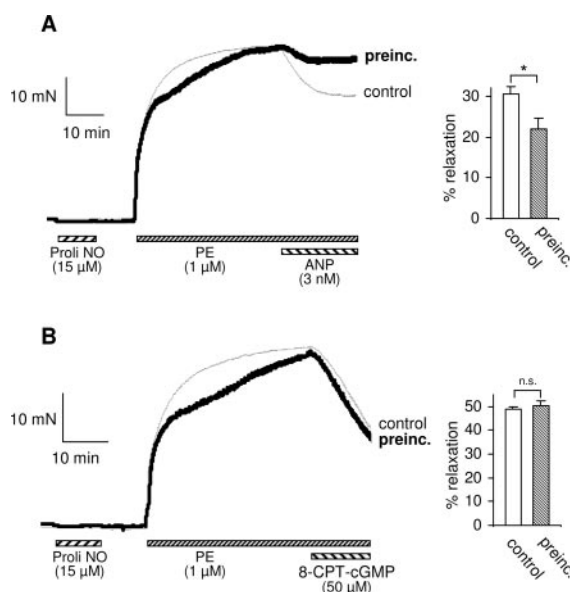


Fig. 4. Heterologous desensitization of smooth muscle relaxation. Aortic rings were preincubated in the absence (thin traces, control) or presence (boldface traces, preinc.) of ProliNONOate ($15 \mu\text{M}$, 10 min). Then, buffer was changed twice before contraction of rings with PE ($1 \mu\text{M}$). Relaxation was induced by 3 nM ANP (A) and $50 \mu\text{M}$ 8-pCPT-cGMP (B). Statistical evaluation is shown in the bar graphs; data represent mean \pm S.E.M. of 12 (A) or 22 (B) independent measurements (*, $P < 0.02$; n.s.: not significant, Student's t test).

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