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Scavenging of nitric oxide by hemoglobin in the tunica media of porcine coronary arteries

Alexander Kollau[a], **Michael Russwurm**[b], **Andrea Neubauer**[a], **Gerald Rechberger**[c,d], **Kurt Schmidt**[a], **Doris Koesling**[b], **John Fassett**[a], **Astrid Schrammel**[a], and **Bernd Mayer**[a,*]

[a]Institute of Pharmaceutical Sciences, Department of Pharmacology and Toxicology, University of Graz, Austria

[b]Department of Pharmacology and Toxicology, Ruhr University Bochum, Germany

[c]Institute of Molecular Biosciences, NAWI Graz, University of Graz, Austria

[d]Omics-Center, BioTechMed-Graz, Austria

Abstract

Scavenging of nitric oxide (NO) often interferes with studies on NO signaling in cell-free preparations. We observed that formation of cGMP by NO-stimulated purified soluble guanylate cyclase (sGC) was virtually abolished in the presence of cytosolic preparations of porcine coronary arteries, with the scavenging activity localized in the *tunica media* (smooth muscle layer). Electrochemical measurement of NO release from a donor compound and light absorbance spectroscopy showed that cytosolic preparations contained a reduced heme protein that scavenged NO. This protein, which reacted with anti-human hemoglobin antibodies, was efficiently removed from the preparations by haptoglobin affinity chromatography. The cleared cytosols showed only minor scavenging of NO according to electrochemical measurements and did not decrease cGMP formation by NO-stimulated sGC. In contrast, the column flow-through caused a nearly 2-fold increase of maximal sGC activity (from 33.1 ± 1.6 to 54.9 ± 2.2 μmol × min^{-1} × mg^{-1}). The proteins retained on the affinity column were identified as hemoglobin α and β subunits. The results indicate that hemoglobin, presumably derived from *vasa vasorum* erythrocytes, is present and scavenges NO in preparations of porcine coronary artery smooth muscle. Selective removal of hemoglobin-mediated scavenging unmasked stimulation of maximal NO-stimulated sGC activity by a soluble factor expressed in vascular tissue.

Keywords

cGMP; Nitric oxide scavenging; Heme protein; Selective removal; Affinity chromatography; Haptoglobin

*Corresponding author. University of Graz, Institute of Pharmaceutical Sciences, Department of Pharmacology and Toxicology, Humboldtstrasse 46/I, 8010, Graz, Austria. mayer@uni-graz.at (B. Mayer).

1 Introduction

As a signaling molecule nitric oxide (NO) is involved in a variety of physiological and pathological processes ([1–3]). In the vasculature NO plays a major role in maintaining homeostasis by causing smooth muscle relaxation as well as inhibition of platelet aggregation and cell proliferation [4]. Vascular NO is biosynthesized from the amino acid L-arginine by endothelial NO synthase [5] and acts through activation of soluble guanylate cyclase (sGC) in the adjacent smooth muscle layer, resulting in accumulation of 3′,5′-cyclic GMP (cGMP) and vascular relaxation ([6–8]). Many cardiovascular diseases, in particular coronary artery disease, are associated with endothelial dysfunction evident as impaired NO signaling in blood vessels [9]. The mechanisms underlying the development of endothelial dysfunction are manifold, but scavenging of NO by superoxide anion generated in conditions of oxidative stress is considered as a major culprit [10]. Thus, besides substituting endogenous NO with donor compounds such as nitroglycerin, which release NO in the vasculature, antioxidants and superoxide scavengers are promising tools for the treatment of endothelial dysfunction [11].

In the course of our ongoing work on the molecular mechanisms of vascular GTN bioactivation, we discovered a pathway of GTN-induced relaxation of bovine and porcine blood vessels that does not involve aldehyde dehydrogenase-2, the major enzyme catalyzing GTN biotransformation in human and rodent vessels [12]. Although GTN caused potent cGMP-mediated endothelium-independent relaxation of porcine coronary arteries (PCA), the rates of GTN denitration were about 10-fold lower than ALDH2-catalyzed denitration in vessels from rats, mice and guinea pigs, suggesting the presence of a highly efficient mechanism of GTN biotransformation that yields stoichiometric amounts of 1,2-glycerol dinitrate and NO. Since relaxation was not significantly affected by any drug reported previously to interfere with GTN bioactivity, we wished to mimic GTN-induced cGMP formation in cell-free preparations in order to enable isolation and biochemical characterization of the active principle. However, both total homogenates as well as cytosolic PCA preparations exhibited extensive NO scavenging that was not prevented by up to 1000 U/ml of superoxide dismutase (SOD), indicating a superoxide-independent pathway. It was the aim of the present study to characterize the underlying mechanism and, if possible, to remove the scavenging principle from the cytosolic coronary artery preparations.

2 Materials and methods

2.1 Materials

Bovine lung sGC was purified as described [13]. Polyclonal antibodies against Hb subunit α were obtained from Santa Cruz (Heidelberg, Germany). 2,2-Diethyl-1-nitroso-oxyhydrazine (DEA/NO) was from Enzo Life Sciences (Lausen, Switzerland) and obtained *via* Eubio (Vienna, Austria). [α-^{32}P]GTP (800 Ci/mmol; NEN Radiochemicals) was obtained from PerkinElmer Life and Analytical Sciences (Vienna, Austria). All other chemicals, including horseradish peroxidase-conjugated anti-rabbit IgG, Hb (bovine, lyophilized), and Sepharose® 4B were obtained from Sigma–Aldrich (Vienna, Austria).

2.2 Preparation of cytosols

Porcine hearts and aortas were obtained from a local slaughterhouse and immediately transported to the laboratory. Coronary arteries were carefully explanted and surrounding fat and connective tissue were dissected. After multiple washes in ice-cold PBS, tissue was cut in small pieces with scissors and incubated in collagenase type II solution (1.5 mg/ml in 10 mM Tris–HCl buffer, containing 125 mM KCl, 3 mM $CaCl_2$, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 μg/ml amphotericin B, pH 7.4) for 3–4 h at ambient temperature. After removing collagenase and washing vigorously with PBS, tissue was homogenized in 10 mM Tris–HCl buffer (pH 7.4), containing 125 mM KCl, 5 mM EGTA, and 2 mM $MgCl_2$ using a glass-teflon Potter-Elvehjem homogenizer. Homogenates were centrifuged at 100,000 × *g* for 1.5 h. Supernatants were collected, concentrated by ultrafiltration (Merck ultra filtration chamber, 10 kDa molecular weight cut off), and regarded as cytosols. They were stored at −80 °C until use.

To obtain cytosols from different aortic layers, surrounding tissue was dissected from porcine aortas and the vessels were cut open longitudinally. After rinsing with ice-cold PBS *tunica intima* (endothelium and basal lamina), *tunica media* (smooth muscle) and *tunica adventitia* (outermost connective tissue) were separated using forceps and scalpel. The individual layers were processed as described for coronary arteries.

Cytosolic preparations were reduced or oxidized by addition of sodium dithionite (~1 mg/ml) or potassium ferricyanide (~1 mg/ml), respectively. Both compounds were subsequently removed from cytosols using PD-10 desalting columns (GE Healthcare, Vienna, Austria).

Protein concentration was determined with the Pierce BCA™ Protein Assay Kit (Fisher Scientific Austria GmbH, Vienna, Austria).

2.3 Determination of sGC activity

Purified bovine lung sGC (50 ng) was incubated at 37 °C for 10 min in a final volume of 0.1 ml in the presence of the different vascular preparations (as indicated in the figure legends). Assay mixtures contained 50 mM TEA (pH 7.4), 0.5 mM [α-^{32}P]GTP (~200,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, 1 mM IBMX, 1 mM EGTA, 2 mM DTT, 0.1 mM DTPA, 5 mM CP, 152 U/l CK, and 1000 U/ml SOD. Reactions were started by addition of DEA/NO (final concentration as indicated in the text and figure legends) and terminated by the addition of 0.45 ml zinc acetate (120 mM) and 0.45 ml sodium bicarbonate (120 mM). After centrifugation (20,000 × *g* at 4 °C for 10 min) supernatants were applied onto Al_2O_3 columns (acidified with 0.1 M perchloric acid). After washing the columns with distilled water, ^{32}P-cGMP was eluted with sodium acetate (50 mM) and quantified by liquid scintillation counting. Blank values were measured in the absence of sGC.

2.4 Determination of nitric oxide

NO concentration was monitored electrochemically with a Clark-type electrode (World Precision Instruments, Berlin, Germany) as described previously [14]. The electrode was

calibrated daily with acidified nitrite after equilibration in a solution of KJ (100 mM) in H_2SO_4 (100 mM).

For the determination of cytosolic NO consumption, the electrode was equilibrated in 50 mM TEA buffer (pH 7.4) containing the indicated amounts of cytosolic protein. Unless stated otherwise, the final volume was 500 µl. Measurements were performed in an open vessel under constant stirring at 37 °C after injection of DEA/NO (final concentration as indicated in the figure legends).

2.5 Light absorbance spectroscopy

Light absorbance spectra were recorded with a Hewlett–Packward 8452A Diode Array spectrophotometer in 1 cm quartz cuvettes that allow measurements in volumes of 300 µl. Average absorbances between 700 and 800 nm were subtracted from each spectrum. Absorbance at specific wavelengths was corrected for background by three-point drop line. The heme content of the preparations was calculated with an extinction coefficient of 15.3 $mM^{-1} \times cm^{-1}$ at 576 nm [15].

2.6 Preparation of Haptoglobin-Sepharose® column

Haptoglobin (Hp) was isolated from porcine plasma by affinity chromatography as described [16]. For this purpose, 50 mg of bovine Hb was coupled to cyanogen bromide-activated Sepharose® 4B (1 g of powder) according to the manufacturers instructions. The Hb-Sepharose® was then packed into a Poly-Prep® chromatography column (Bio-Rad, Vienna, Austria).

In order to isolate Hp, fresh porcine blood (immediately mixed with EDTA, final concentration 0.1%) was obtained from a local slaughterhouse. After centrifugation (3000 × *g* at 4 °C for 20 min) non-hemolytic plasma was mixed with solid ammonium sulfate to yield 30% saturation. After equilibration for 20 min at 4 °C under constant stirring, the precipitate was separated by centrifugation at 10,000 × *g* for 10 min and discarded. The ammonium sulfate saturation in the supernatant was raised to 60% by further addition of solid ammonium sulfate. After equilibration for 30 min at 4 °C under constant stirring, the precipitate was collected by centrifugation at 20,000 × *g* for 20 min, redissolved in binding buffer (0.01 M Tris–HCl, 0.5 M NaCl, pH 7.0) and dialyzed against the same buffer over night with several changes of the solution. The dialysate was passed through the Hb-Sepharose® column using a peristaltic pump with a flow rate of 1 ml/min. The column was washed with 5 bed volumes (~20 ml) of binding buffer, followed by 10 bed volumes of 1.6 M guanidine in binding buffer (1 ml/min). Hp was eluted with 3.5 M guanidine in binding buffer (pH 5.0), collected in 2-ml fractions and analyzed by SDS-PAGE. The binding buffer was removed from pooled Hp containing fractions by ultrafiltration (Amicon® ultra centrifugal filters, 50 kDa molecular weight cutoff, Merck-Millipore, Vienna, Austria) and replaced by 0.1 M $NaHCO_3$ (pH 8.4), containing 0.5 M NaCl. Finally, Hp was coupled to cyanogen bromide-activated Sepharose® 4B according to the manufacturer's instructions and placed into a Poly-Prep® column.

2.7 Electrophoresis and western blotting

PCA cytosol (1 ml) was loaded onto the Hp-affinity column and allowed to pass through by gravity. The flow-through was collected and used in separate experiments. The column was washed with 10 ml of homogenization buffer (10 mM Tris–HCl, containing 125 mM KCl, 5 mM EGTA, and 2 mM $MgCl_2$, pH 7.4) and bound protein was subsequently eluted with 3.5 M guanidine (in 0.01 M Tris–HCl, containing 0.5 M NaCl, pH 5.0). Samples were concentrated by ultrafiltration using Amicon® Ultra centrifugal filters (10 kDa molecular weigh cutoff, Merck-Millipore, Vienna, Austria), and the buffer replaced by an equal volume of distilled water. This desalting step was repeated once, and samples were eventually concentrated by ultrafiltration. Lysates from human and porcine erythrocytes were used as standards and prepared as follows: 100 μl of blood was mixed with 900 μl of PBS and centrifuged at 3000 × *g* for 5 min at 4 °C. The supernatant was discarded and the pellet (i.e. mainly erythrocytes) was resuspended in 1 ml of PBS. The washed erythrocytes were sedimented by centrifugation at 3000 × *g* for 5 min at 4 °C. The supernatant was discarded and the pellet resuspended in 500 μl of 10 mM Tris–HCl (pH 7.4) and mixed vigorously to lyse erythrocytes. After incubation for 10 min at 4 °C cell debris were sedimented by centrifugation at 20,000 × *g* for 10 min at 4 °C, and the clear red supernatant was collected. Eluates and erythrocyte lysates were stored at −20 °C. For SDS-PAGE, they were mixed with an equal volume of sample buffer (50 mM Tris–HCl, containing 1.6% sodium dodecyl sulfate (w/v), 7% glycerol (v/v), 8 M urea, 5% 2-mercaptoethanol (v/v), and 0.016% bromophenol blue (w/v), pH 6.8) and denatured for 5 min at 95 °C. Denatured eluates and lysates (10 and 0.5 μg of protein, respectively) were separated by SDS-PAGE on 15% gels and transferred onto nitrocellulose membranes (0.45 μm). After blocking with 5% nonfat dry milk ((w/v) in Tris-buffered saline, containing 0.05% Tween-20 (v/v)) for 1 h, membranes were incubated overnight at 4 °C with polyclonal primary antibodies against human hemoglobin subunit α (1:1000). After incubation of membranes with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000), immunoreactive bands were visualized by chemiluminescence using ECL Prime Western Blot Detection Reagent (GE Healthcare, purchased *via* VWR, Vienna, Austria).

2.8 LC-MS/MS analysis of proteins retained on the Hp affinity column

The concentrated guanidine eluate (see above) was separated by SDS-PAGE and stained with Coomassie-brilliant blue. Bands were excised from the gel, tryptically digested and analyzed by nanoLC-ESI-MS/MS as described elsewhere [17]. MS/MS spectra were identified by search of the Swiss-Prot non-redundant public database using SpectrumMill software (Agilent). The following search parameters were used: tryptic digestion with one missing cleavage; carbamidomethylation as fixed modification; oxidized methionine, N-terminal pyro-glutamic acid, acrylamide at cysteine as variable modifications; sequence tag length >3; minimum detected peaks of 4; minimum matched peak intensity of 50%; precursor ion mass tolerance of ±2.5 Da, and product mass tolerance of ±0.7 Da. Acceptance parameters were three or more identified distinct peptides with minimum sequence coverage of 20% and probability score of >95%.

3 Results

3.1 NO scavenging of PCA cytosols

As shown in Fig. 1A, purified sGC exhibited a specific activity of 37.9 ± 3.2 μmol cGMP × min^{-1} × mg^{-1} in the presence of 0.1 μM DEA/NO. Formation of cGMP was almost completely abolished by PCA cytosols (0.1 mg of protein), resulting in an apparent sGC activity of 2.0 ± 0.6 μmol cGMP × min^{-1} × mg^{-1}, which was virtually identical to the basal activity of the enzyme in the absence of NO stimulation (not shown). Similar results were obtained with cytosolic preparations of the smooth muscle layer (*tunica media*) of porcine aortas (6.3 ± 3.5 μmol × min^{-1} × mg^{-1}), while the innermost part of the aortas (*tunica intima*, mainly consisting of the endothelium) had no effect.

The data clearly indicate that interference with NO-stimulated sGC activity is located in the smooth muscle layer and not a specific feature of coronary arteries. The effect was observed in the presence of the non-selective phosphodiesterase inhibitor IBMX and a GTP regenerating system and, moreover, not prevented by a phosphatase inhibitor cocktail (PhosSTOP®, Roche) (not shown), excluding the involvement of enzymatic cGMP hydrolysis or limited substrate availability through GTPase activity. Therefore, we considered that the cytosolic preparations efficiently scavenged NO released from DEA/NO.

Fig. 1B shows representative traces of NO release from 1 μM DEA/NO measured with a Clark-type electrode. Addition of PCA cytosols decreased the NO signal and delayed its appearance in a protein-dependent manner. In the presence of 0.1 mg of cytosolic protein the NO signal was reduced to 50% as compared to buffer controls, and 0.8 mg of protein completely abolished the NO signal. These results strongly suggest that cytosolic preparations of blood vessels interfere with sGC-catalyzed cGMP formation through consumption of DEA/NO-derived NO.

Although scavenging was observed in the presence of a high concentration of SOD (1000 U/ml), we considered that the reaction of NO with NADPH oxidase-derived superoxide [18], resulting in formation of peroxynitrite [19], could be involved. However, several NADPH oxidase inhibitors, including the well established inhibitor diphenyleneiodonium [20], had no effect (data not shown).

3.2 UV/Vis spectroscopy of PCA cytosols

Based on these results we speculated that NO could be scavenged by a reduced heme protein present in PCA cytosols. Cytoglobin was identified as a soluble scavenging heme protein in vascular smooth muscle [21], and hemoglobin α was shown to limit NO diffusion through myoendothelial junctions if reduced by cytochrome *b5* reductase 3 [22]. To test whether high affinity binding of NO to reduced (ferrous) heme iron contributes to NO scavenging, we recorded UV/Vis spectra of PCA cytosols under control conditions and after reduction and oxidation by dithionite and ferricyanide, respectively. Fig. 1C shows that the untreated preparations exhibited a Soret band at 413 nm and α/β bands at 540 and 576 nm. This spectrum is virtually identical to that of O_2-bound hemoglobin ([23,24]). The presence of dithionite had no effect on absorbance, while ferricyanide caused a blue-shift of the Soret

band to 405 nm and loss of the α/β bands, indicating that PCA cytosols contain a reduced heme protein that is oxidized by ferricyanide.

According to the absorbance at 576 nm, the heme content of the various preparations varied between 140 and 470 pmol/mg of total protein. This allowed us to correlate the scavenging efficacy of the preparations with their heme content. As shown in Fig. 1D, there was a positive correlation (R = 0.942) between NO consumption (released from 0.5 μM DEA/NO) and heme content. The slope of the linear regression shown in Fig. 1D was 0.73, indicating that about 0.7 mol of NO were consumed per mol of heme. These results strongly suggest that NO is scavenged by a reduced heme protein present in the cytosolic preparations of porcine blood vessels.

3.3 Effects of heme iron oxidation on NO scavenging by PCA cytosol

As shown in Fig. 2A, the effect of cytosolic PCA protein (0.1 mg) on sGC activation by DEA/NO (0.1 μM) was prevented by pretreatment of the preparations with the heme oxidant ferricyanide (1.6 ± 0.5 and 32.5 ± 5.2 μmol × min^{-1} × mg^{-1} in the presence of untreated and oxidized PCA cytosols, respectively). Likewise scavenging of NO released from 1 μM DEA/NO was almost completely prevented by ferricyanide treatment of the cytosolic preparations (Fig. 2B).

3.4 Selective removal of the NO scavenging heme protein from PCA cytosols

Since the observed NO scavenging precludes studies on NO-triggered effects in cell-free preparations of blood vessels, we developed a method for selective removal of heme proteins from PCA cytosols. In a first attempt, we tried to achieve this by immunoprecipitation using antibodies against human Hb subunit α. However, NO scavenging was not affected by precipitation, presumably due to relatively low cross-reactivity of the antibody with porcine Hb. Indeed, dot blots with erythrocyte lysates revealed that reactivity of the anti-Hb antibody with porcine Hb was about 20-fold lower than its reactivity with the human protein (data not shown).

To avoid problems associated with species-specific binding, affinity chromatography with haptoglobin (Hp), which selectively binds Hb, was applied ([16,25]). For this purpose we isolated Hp from porcine plasma and prepared an affinity column by coupling the protein to cyanogen bromide-activated Sepharose® 4B. As shown in Fig. 3A, the electrochemical signal obtained with 1 μM DEA/NO was about 75% of buffer controls in the presence of cytosolic preparations (0.4 mg of protein) that had been passed through the Hp affinity column, while the NO signal was almost completely abolished by the cytosol prior to chromatography.

The effect of Hp affinity chromatography of PCA cytosols on DEA/NO-stimulated sGC activity is shown in Fig. 3B. Formation of cGMP was abolished in the presence of ≥0.1 mg of protein prior to chromatography (pre-column), while no considerable reduction was observed with up to 0.2 mg of PCA cytosol that had been passed through the affinity column. Surprisingly, sGC activity was increased almost 2-fold by the post-column fractions (from 33.1 ± 1.6 to 54.9 ± 2.2 μmol × min^{-1} × mg^{-1}). Since endogenous sGC activity was hardly detectable in PCA cytosols (data not shown), this observation suggests that the

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preparations contain a factor that activates maximal sGC activity. In untreated cytosolic preparations, this effect is masked by high affinity binding of NO to a hemoglobin-like protein.

The NO scavenging heme protein was tentatively identified by immunoblotting using a specific antibody against Hb subunit α. Fig. 3C shows that the antibody reacted with the eluate from the Hp affinity column (10 μg of protein). The visualized band at 16 kDa agrees well with the molecular mass of monomeric Hb [26]. Note the low reactivity of the anti-Hb antibody with porcine as compared to human erythrocyte lysates. An antibody raised against cytoglobin detected small amounts of the protein in PCA cytosols but not in the eluates, suggesting that cytoglobin was not retained on the Hp column (data not shown).

The proteins retained on the Hp affinity column were eluted with guanidine, concentrated and identified by LC-MS/MS as Hb subunit α (5 peptides identified, amino acid coverage: 44%, SpectrumMill Score: 91) and Hb subunit β (7 peptides identified, amino acid coverage: 52%, SpectrumMill Score: 137).

4 Discussion

Numerous pathways have been described that consume or scavenge NO, thereby interfering with downstream NO signaling. Molecular oxygen is certainly the reactant that is most widely abundant in cells and tissues. Although NO autoxidation yielding inorganic nitrite is second order with respect to NO in aqueous solution [27] and, therefore, slow at nanomolar NO concentrations, we recently found that NO/O_2-triggered thiol nitrosation can occur at physiologically low NO concentrations in a first order reaction that is stimulated by Mg^{2+} ions ([28,29]). Thus, tissue pO_2 may regulate NO availability through nitrosation of cellular thiols. Superoxide anion from NADPH oxidases and other sources is another well established NO scavenger. To avoid the fast, almost diffusion-controlled reaction of NO with superoxide, yielding peroxynitrite ([30,31]), we routinely perform sGC assays with cell-free preparations and NO measurements in the presence of high concentrations of SOD (1000 U/ml).

As a free radical, NO reacts rapidly with unpaired electrons in metal centers of proteins [32]. The most prominent biologically relevant example is the central heme iron of hemoglobin, which binds NO with high affinity, a reaction that is considered to limit the availability of endothelium-derived NO and regulate vascular tone [33]. Reduced hemoglobins not only bind NO but exhibit dioxygenase activity, resulting in formation of met-Hb and nitrate, with efficient turnover requiring associated reductases [34]. The reaction catalyzed by microbial flavohemoglobins is considered as a phylogenetically early mechanism of NO detoxification [35]. However, identification of a wide variety of NO dioxygenation pathways in mammalian tissues indicates that the defense against NO poisoning is essential for all types of organisms. Moreover, besides NO detoxification, the hemoglobin/NO reaction can be beneficial by regulating NO-mediated biological processes [36].

In vascular tissue, two hemoglobins were identified that appear to be essentially involved in the physiological regulation of NO signaling, cytoglobin and Hb α subunit. Cytoglobin, an

ubiquitously expressed 21 kDa protein with multiple biological functions [37], is another heme protein with NO dioxygenase function that was shown to limit NO availability in vascular smooth muscle [21]. According to Western blot analysis cytoglobin was present in PCA cytosols but not detected in the Hp affinity column eluates, indicating that it had not been retained on the resin. Thus, cytoglobin could contribute to the residual NO scavenging observed after Hp affinity chromatography (cf. Fig. 3A).

The reduced Hb α subunit, expressed in myoendothelial junctions, was shown to bind NO, thereby limiting NO transfer from the endothelium to the underlying vascular smooth muscle cells [22]. NO consumption by Hb α requires continuous recycling of the reduced (ferrous) protein by cytochrome *b5* reductase, suggesting that a redox switch regulates vascular relaxation by endothelium-derived NO [38]. Myoendothelial junctions containing Hb α are expected to be present in the endothelium, but dissection of PCA indicated that NO scavenging occurred exclusively in the smooth muscle layer (*tunica media*; *cf.* Fig. 1A). Since the scavenging protein was unequivocally identified as Hb by amino acid sequencing, it is conceivable that myoendothelial junctions were removed together with the smooth muscle layer. However, our preparations contained both α and β subunits, while myoendothelial junctions were reported to exclusively express Hb α. Contamination of the preparations with blood-derived Hb appears unlikely, because the vessels were extensively cleaned prior to homogenization. Moreover, no scavenging occurred in the endothelium, which is expected to be more prone to blood-derived contamination than the underlying smooth muscle layer.

Expression of both subunits of Hb was shown for several cell types beyond erythrocytes, including mesanglial cells, dopaminergic neurons, and alveolar epithelial cells (see Ref. [39] and references therein). However, we did not detect Hb in smooth muscle cells cultured from PCA (data not shown), arguing against unexpected Hb biosynthesis in vascular smooth muscle. Specialized microvessels that migrate from the adventitia into the media, termed *vasa vasorum*, are known to supply large blood vessels with oxygen and nutrients. Neovascularization with *vasa vasorum* is considered as an early process that occurs in hypercholesteremia and promotes coronary atherosclerosis ([40,41]), but the microvessels also play a major role in normal vessel wall biology [42]. Therefore, erythrocytes of *vasa vasorum* present in the media of PCA are the most likely source for Hb α and β identified as NO scavenging proteins in our preparations.

Interestingly, PCA cytosols significantly increased the activity of maximally NO-stimulated purified sGC after removal of the scavenging heme protein. To our knowledge the resulting specific activity of almost 60 μmol cGMP × min^{-1} × mg^{-1} is the highest activity ever reported for this enzyme. We are not aware of a similar effect caused by any agent described in the literature. Work on the characterization of the active principle is underway in our laboratory. This unexpected finding highlights the importance of identifying and eliminating pathways of NO scavenging that may hamper biochemical studies on NO signaling in cell-free preparations of blood vessels and other tissues.

Acknowledgments

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Abbreviations

cGMP	3′,5′-cyclic GMP
CP	creatine phosphate
CK	creatine kinase
DEA/NO	2,2-diethyl-1-nitroso-oxyhydrazine
DTPA	diethylene triamine pentaacetic acid
DTT	dithiothreitol
EGTA	ethylene glycol tetraacetic acid
Hb	hemoglobin
Hp	haptoglobin
IBMX	3-isobutyl 1-methylxanthine
GTN	nitroglycerin
NO	nitric oxide
PCA	porcine coronary arteries
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl polyacrylamide gel electrophoresis
SOD	superoxide dismutase
sGC	soluble guanylate cyclase
TEA	triethanolamine

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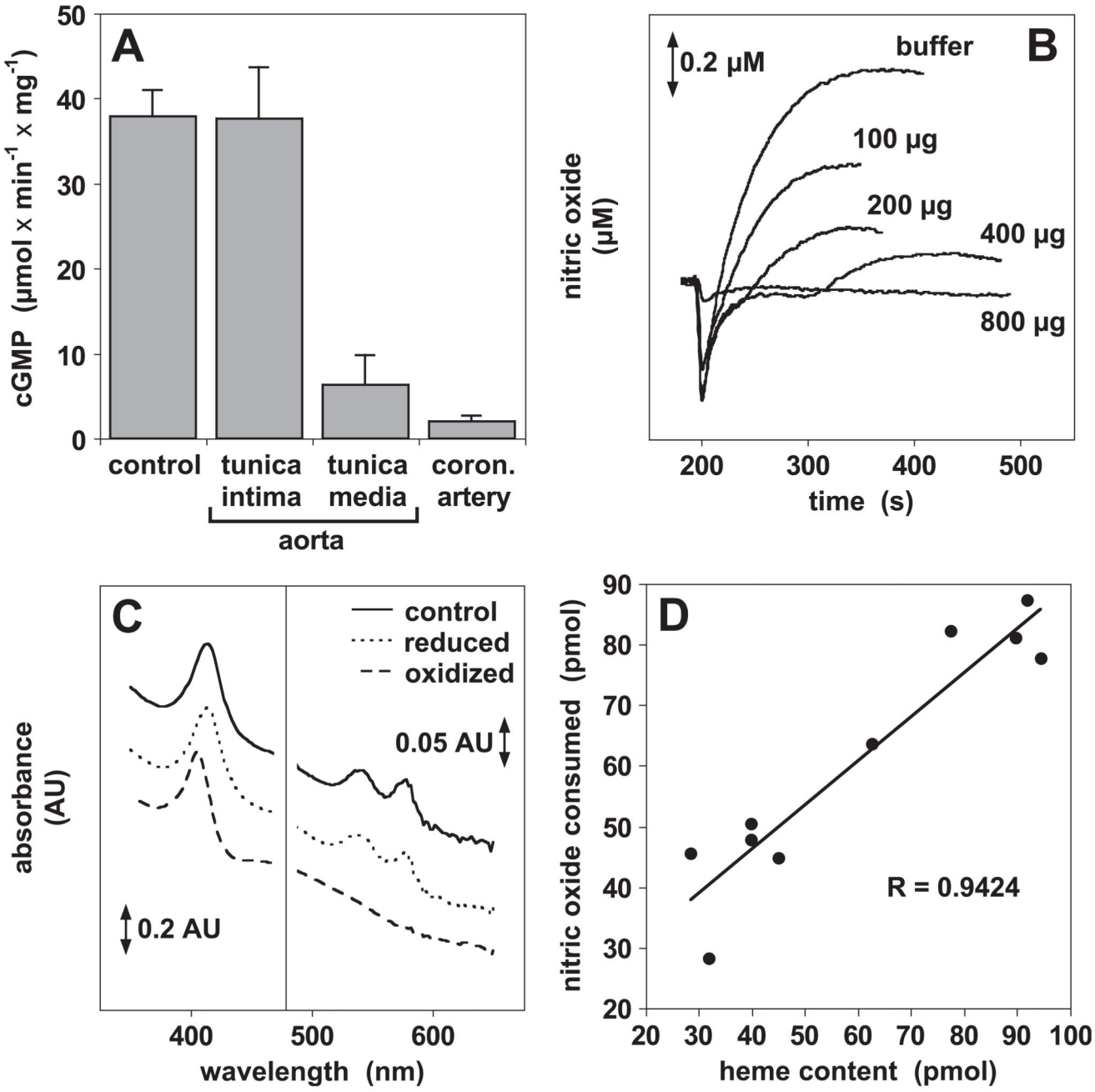

Fig. 1.

A: Formation of cGMP by purified sGC (50 ng) in the presence of 0.1 µM DEA/NO with and without cytosolic preparations of *tunica intima* and *tunica media* from porcine aortas or PCA (0.1 mg of protein each). Data are mean values ± S.E.M. of three independent experiments. B: NO release from 1 µM DEA/NO measured with a Clark-type electrode in the presence of increasing amounts of PCA cytosol (0.1–0.8 mg). The traces are representative for three individual experiments. C: Representative UV/Vis spectra of PCA cytosols reduced or oxidized with sodium dithionite (~1 mg/ml) and ferricyanide (~1 mg/

ml), respectively. D: Correlation of NO scavenging with heme content of the preparations. The amount of NO (released from 0.5 μM DEA/NO) that was consumed by 200 μg of total cytosolic protein (200 μl final volume) was plotted against the corresponding heme content. Each data point represents an individual preparation.

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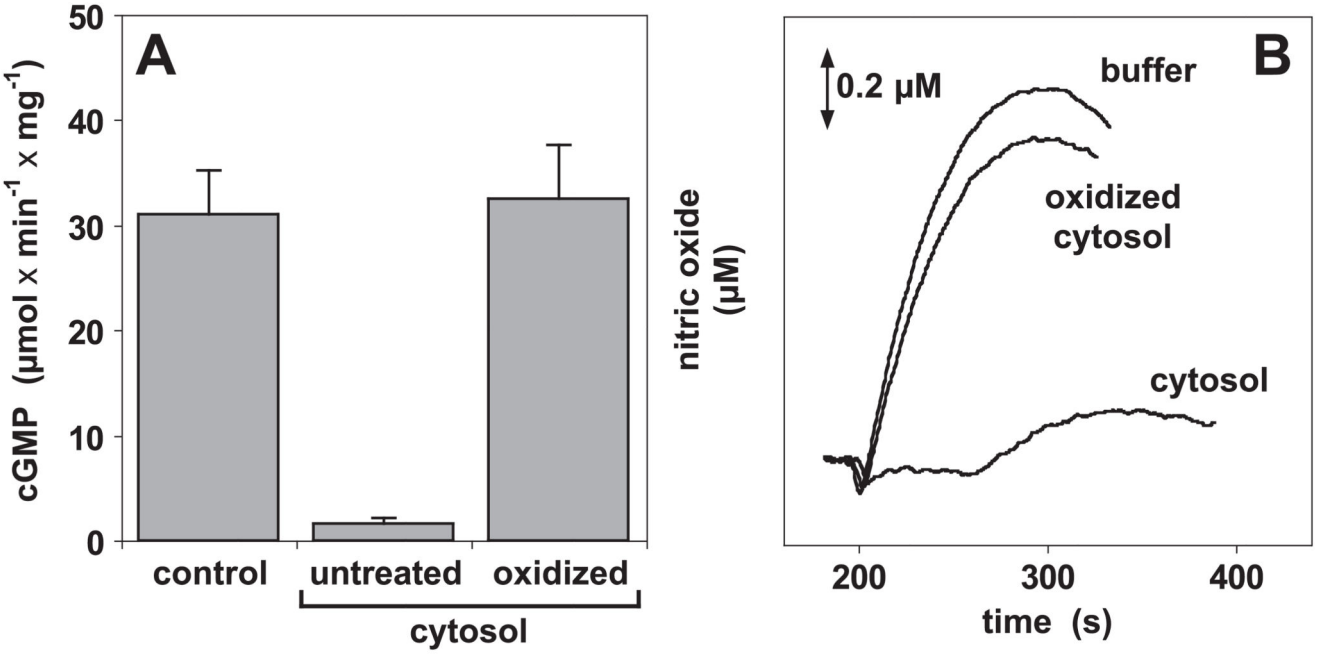

Fig. 2.

A: Formation of cGMP by purified sGC (50 ng) in the presence of 0.1 µM DEA/NO in the absence or presence of PCA cytosols (0.1 mg of protein) that were either untreated or oxidized with ferricyanide (1 mg/ml), followed by desalting to remove the oxidant. Data are mean values ± S.E.M. of three independent experiments. B: NO release from 1 µM DEA/NO measured with a Clark-type electrode in the absence or presence of PCA cytosols (0.1 mg of protein) that were either untreated or oxidized with 1 mg/ml ferricyanide. The traces are representative for three individual experiments.

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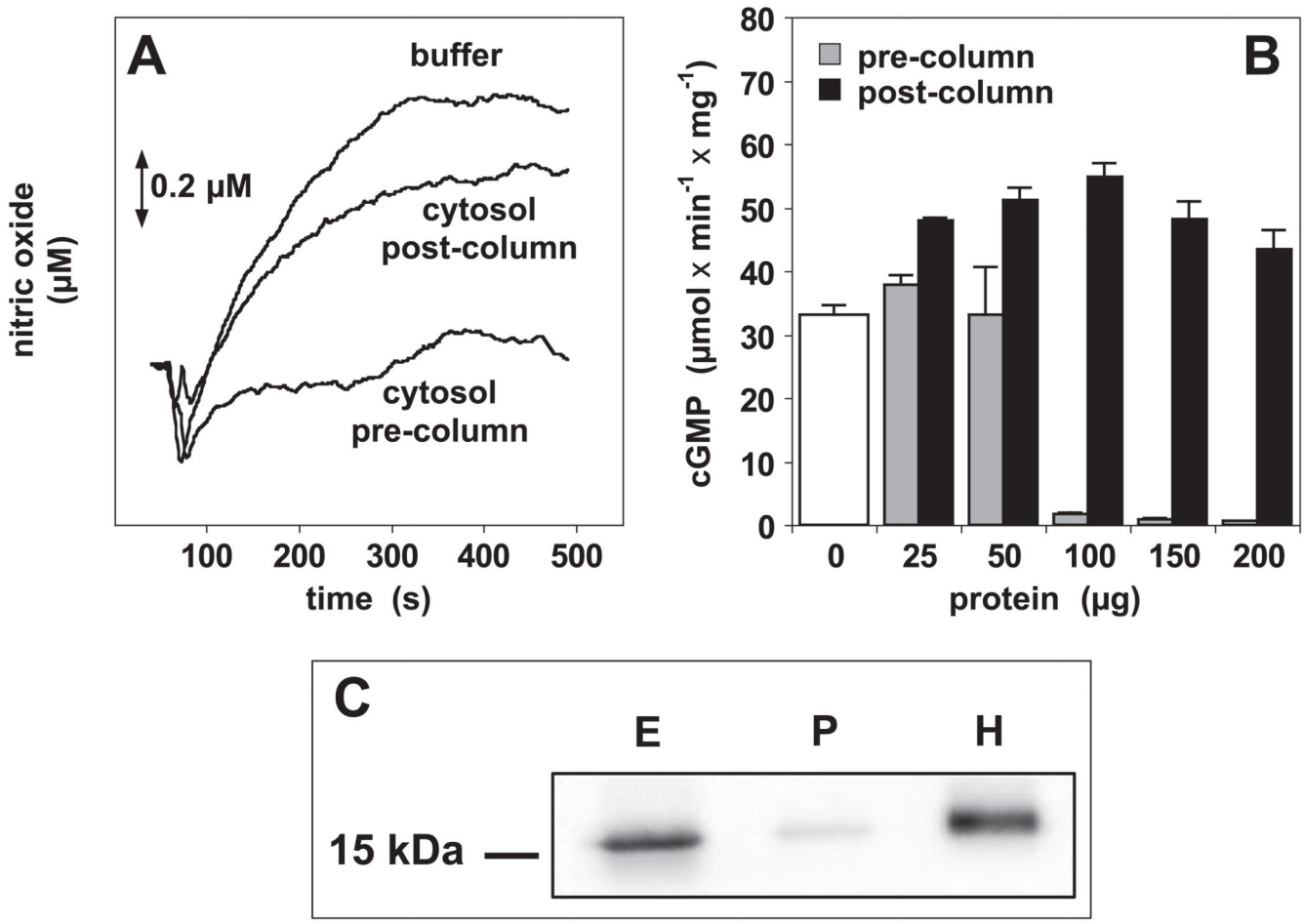

Fig. 3.

A: NO release from 1 µM DEA/NO measured with a Clark-type electrode in the absence or presence of PCA cytosols (0.4 mg of protein) before and after Hp affinity chromatography (pre- and post-column, respectively). The traces are representative for three individual experiments. B: Formation of cGMP by purified sGC (50 ng) in the presence of 0.1 µM DEA/NO and increasing amounts of PCA cytosols before and after Hp affinity chromatography (pre- and post-column, respectively). Data are mean values ± S.E.M. of three independent experiments. C: Representative immunoblot (n = 3) of the protein (10 µg) that had been eluted from the Hp affinity column with 3.5 M guanidine in 0.01 M Tris–HCl, containing 0.5 M NaCl (pH 5.0) as well as porcine and human erythrocyte lysates (0.5 µg of protein, each). E, eluate from Hp-affinity resin; P, porcine erythrocyte lysate; H, human erythrocyte lysate.