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Mechanisms Underlying Activation of Soluble Guanylate Cyclase by the Nitroxyl Donor Angeli's Salt[S]

Andreas Zeller, M. Verena Wenzl, Matteo Beretta, Heike Stessel, Michael Russwurm, Doris Koesling, Kurt Schmidt, and Bernd Mayer

Department of Pharmacology and Toxicology, Karl-Franzens-Universität Graz, Graz, Austria (A.Z., M.V.W., M.B., H.S., K.S., B.M.); and Department of Pharmacology and Toxicology, Ruhr-Universität Bochum, Bochum, Germany (M.R., D.K.)

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ABSTRACT

Nitroxyl (HNO) may be formed endogenously by uncoupled nitric-oxide (NO) synthases, enzymatic reduction of NO or as product of vascular nitroglycerin bioactivation. The established HNO donor Angeli's salt (trioxodinitrate, AS) causes cGMP-dependent vasodilation through activation of soluble guanylate cyclase (sGC). We investigated the mechanisms underlying this effect using purified sGC and cultured endothelial cells. AS (up to 0.1 mM) had no significant effect on sGC activity in the absence of superoxide dismutase (SOD) or dithiothreitol (DTT). In the presence of SOD, AS caused biphasic sGC activation (apparent EC_{50} ~10 nM, maximum at 1 μM) that was accompanied by the formation of NO. DTT (2 mM) inhibited the effects of <10 μM AS but led to sGC activation and NO release at 0.1 mM AS even without SOD. AS had no effect on ferric sGC, excluding activation of the oxidized enzyme by HNO. The NO scavenger carboxy-PTIO inhibited endothelial cGMP accumulation induced by AS in the presence but not in the absence of SOD (EC_{50} ~50 nM and ~16 μM, respectively). Carboxy-PTIO (0.1 mM) inhibited the effect of ≤10 μM AS in the presence of SOD but caused NO release from 0.1 mM AS in the absence of SOD. These data indicate that AS activates sGC exclusively via NO, formed either via SOD-catalyzed oxidation of HNO or through a minor AS decomposition pathway that is unmasked in the presence of HNO scavenging thiols.

Activation of soluble guanylate cyclase (sGC) represents an established signaling mechanism downstream of NO synthase activation in a variety of tissues, including endothelial cells, platelets, and neurons (Friebe and Koesling, 2003). NO binds with high affinity to ferrous heme bound to the β-subunit of sGC, resulting in pronounced stimulation of cGMP formation (Griffiths et al., 2003). This effect is blocked in an NO-competitive manner by the sGC inhibitor ODQ, which oxidizes sGC-bound heme to the ferric form (Schrammel et al., 1996). Drugs have been described that stimulate sGC only in the presence of ODQ, suggesting activation of the ferric enzyme (Evgenov et al., 2006). However, a recent study showed that it is the heme-free form of the enzyme that is activated by these drugs (Roy et al., 2008). Because these compounds cause cGMP-dependent vasodilation, heme-free sGC may be present in blood vessels (Evgenov et al., 2006).

The biology of NO is rendered complex by redox reactions yielding NO-related species with distinct biological properties. A prominent example is the NO congener HNO (nitroxyl), which may be formed endogenously by uncoupled NO synthase or reduction of NO by cytochrome *c* oxidase, xanthine oxidase, or hemoglobin (Paolocci et al., 2007; Irvine et al., 2008). The biologically most relevant chemical reaction of HNO is oxidation of low and high molecular mass thiols to the corresponding disulfides with concomitant formation of hydroxylamine (Paolocci et al., 2007). Thus, thiols are useful tools to discriminate between NO and HNO in bioassays (Pino and Feelisch, 1994), and HNO modifies the function of proteins by reaction with essential cysteine residues (Shen and English, 2005). The positive inotropic effect of HNO, for instance, is mediated by sulfhydryl modification of various proteins regulating Ca^{2+} homoeostasis in cardiomyocytes (Paolocci et al., 2007). Other examples are glyceraldehyde-3-phosphate dehydrogenase (Lo-

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ABBREVIATIONS: sGC, soluble guanylate cyclase; AS, Angeli's salt ($Na_2N_2O_3$; sodium trioxodinitrate); carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NONOate); DTPA, diethylene triamine pentaacetic acid; DTT, dithiothreitol; ODQ, 1*H*-(1,2,4)oxadiazolo(4,3-α) quinoxaline-1-one; SIN-1, 3-morpholino sydnonimine; SOD, superoxide dismutase; TEA, triethanolamine; HNO, nitroxyl.

pez et al., 2007) and mitochondrial aldehyde dehydrogenase (DeMaster et al., 1998), which are irreversibly inhibited by HNO-triggered cysteine modification.

Although these effects of HNO are cGMP-independent, a large body of evidence indicates that HNO released from AS causes vasodilation through stimulation of sGC (Irvine et al., 2008). In line with a report showing that NO is the sole nitrogen monoxide redox form that activates sGC (Dierks and Burstyn, 1996), vasodilation by AS has been attributed to oxidation of HNO to NO by SOD (Murphy and Sies, 1991; Liochev and Fridovich, 2002) or other cellular pathways (Paolocci et al., 2007; Irvine et al., 2008). The effect of SOD has raised considerable confusion because of the difficulties in deciding whether SOD-induced increases of NO bioavailability are due to scavenging of superoxide or oxidation of HNO to NO. Thus, although we interpreted the SOD dependence of NO formation by neuronal NO synthase as evidence for enzymatic cogeneration of NO and superoxide (Mayer et al., 1995), others claimed that the initial enzymatic product is HNO that is oxidized to NO by SOD (Schmidt et al., 1996). Another example is bioconversion of nitroglycerin by mitochondrial aldehyde dehydrogenase that results in substantial formation of NO in the presence but not in the absence of SOD (Beretta et al., 2008). Again, it remained unclear whether the effect of SOD reflected cogeneration of superoxide or oxidation of HNO, a putative product of vascular nitroglycerin metabolism (Booth et al., 2000). Although the case of NO synthase was later decided in favor of the superoxide hypothesis (Riethmüller et al., 1999), this issue is still unresolved in the case of nitroglycerin bioactivation.

Contrasting the general view that AS-induced activation of sGC results from oxidation of HNO to NO (Zamora et al., 1995), several studies reported that vasodilation in response to AS was blocked by ODQ but not by the NO scavenger carboxy-PTIO, although the effects of NO donor compounds or endothelial NO synthase activation were inhibited as expected (Li et al., 1999; Costa et al., 2001; Wanstall et al., 2001; Irvine et al., 2003, 2007). This discrepancy is still unresolved and is taken as evidence for NO-independent sGC activation by HNO (Irvine et al., 2008). Likewise, the lack of effect of carboxy-PTIO on AS-induced neuronal cell death led to the exclusion of NO as a mediator of AS toxicity (Hewett et al., 2005). The present study was designed to clarify the mechanisms underlying sGC activation by AS. In particular, we investigated the role of SOD and thiols, the possible activation of ferric sGC by AS-derived HNO, and the puzzling lack of effect of carboxy-PTIO on AS-induced vascular cGMP accumulation that has been reported previously.

Materials and Methods

Materials. Bovine lung sGC was purified as described previously (Russwurm and Koesling, 2005). [α-^{32}P]GTP (400 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Vienna, Austria). AS was from Cayman Europe (Tallin, Estonia). DEA/NO, SIN-1, and ODQ were from Alexis Corporation (Lausen, Switzerland) and purchased via Eubio (Vienna, Austria). DEA/NO and AS were dissolved and diluted in 10 mM NaOH. All other chemicals were from Sigma (Vienna, Austria).

Determination of cGMP in Cultured Porcine Aortic Endothelial Cells. Porcine aortic endothelial cells were isolated as described previously (Schmidt et al., 1989) and cultured at 37°C/5% CO_2 for up to three passages in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 μg/ml amphotericin B. For the determination of intracellular cGMP accumulation, endothelial cells were grown in 24-well plates, washed, and preincubated for 15 min at 37°C in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 1 μM indomethacin, and where indicated 1000 U/ml SOD, 0.1 mM carboxy-PTIO, or 0.1 mM ODQ. Reactions were started by the addition of donor compounds and terminated after 2 min by removal of the incubation medium and addition of 0.01 N HCl. Within 1 h, intracellular cGMP was completely released into the supernatant and measured by radioimmunoassay.

Determination of sGC Activity. The sGC preparation used for most experiments in the present study exhibited a specific activity of ~20 μmol of cGMP $\cdot$ min^{-1} $\cdot$ mg^{-1} and was stored at a protein concentration of 0.1 mg/ml. Dilution in the assay resulted in a final DTT concentration of 10 μM. The purified enzyme (50 ng) was incubated at 37°C for 10 min in a final volume of 0.1 ml in the presence of 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-^{32}P]GTP, ~250,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, and 0.1 mM DTPA. SOD, DTT, donor compounds, and scavengers were present as indicated in the text and figures. To minimize HNO scavenging by endogenously present DTT, some experiments were performed with 740 ng of a more concentrated sGC preparation (7.4 mg/ml; specific activity, ~10 μmol of cGMP $\cdot$ min^{-1} $\cdot$ mg^{-1}) in a total volume of 0.2 ml under otherwise identical conditions, resulting in a final DTT concentration of 1 μM. Reactions were terminated by the addition of 450 μl of zinc acetate (120 mM) and 450 μl of sodium bicarbonate (120 mM), followed by isolation of [^{32}P]cGMP by chromatography over Al_2O_3 columns. Blank values were determined in the absence of sGC.

Determination of NO Release. NO release was measured with a Clark-type electrode (World Precision Instruments, Berlin, Germany), calibrated daily with acidified nitrite as described previously (Mayer et al., 1995). AS or SIN-1 was incubated in 1 ml of 50 mM TEA, pH 7.4, containing 0.1 mM DTPA in the absence or presence of SOD and DTT as indicated in the text.

Determination of Hydroxylamine Formation. AS and DEA/NO (0.1 mM each) were incubated at 37°C for 5 min in 0.2 ml of 50 mM TEA buffer, pH 7.4, containing 0.1 mM DTPA in the absence and presence of 2 mM DTT. Hydroxylamine was measured by light absorbance spectroscopy at 700 nm after condensation with 8-hydroxyquinoline and oxidation to indooxine as described previously (Arnelle and Stamler, 1995). Calibration curves were established with authentic hydroxylamine in the absence and presence of 2 mM DTT.

Results

Fig. 1A shows activation of purified sGC by increasing concentrations of AS. AS had a very minor effect even at the highest concentration tested (0.1 mM) in the absence of SOD and DTT but caused pronounced biphasic sGC activation in the presence of 1000 U/ml SOD. Note that cGMP formation was slightly stimulated by SOD alone. This effect was sensitive to ODQ and NO scavengers (Fig. 4), suggesting that it reflects the stimulation of sGC by airborne NO (Friebe et al., 1996) that is stabilized by SOD. The apparent EC_{50} value for the stimulating effect of AS was <10 nM under these conditions, and maximal rates of cGMP formation (12.7 $\pm$ 0.30 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) were observed at 1 μM concentration of the donor compound. Activation of sGC caused by up to 1 μM AS in the presence of SOD was significantly inhibited by DTT, but the effects of high AS concentrations ($\geq$ 10 μM) were more pronounced in the presence of DTT, presumably because of triggering SOD-independent sGC activation by AS (Fig. 1, ●). As shown in Supplemental Fig. S1, cGMP forma-

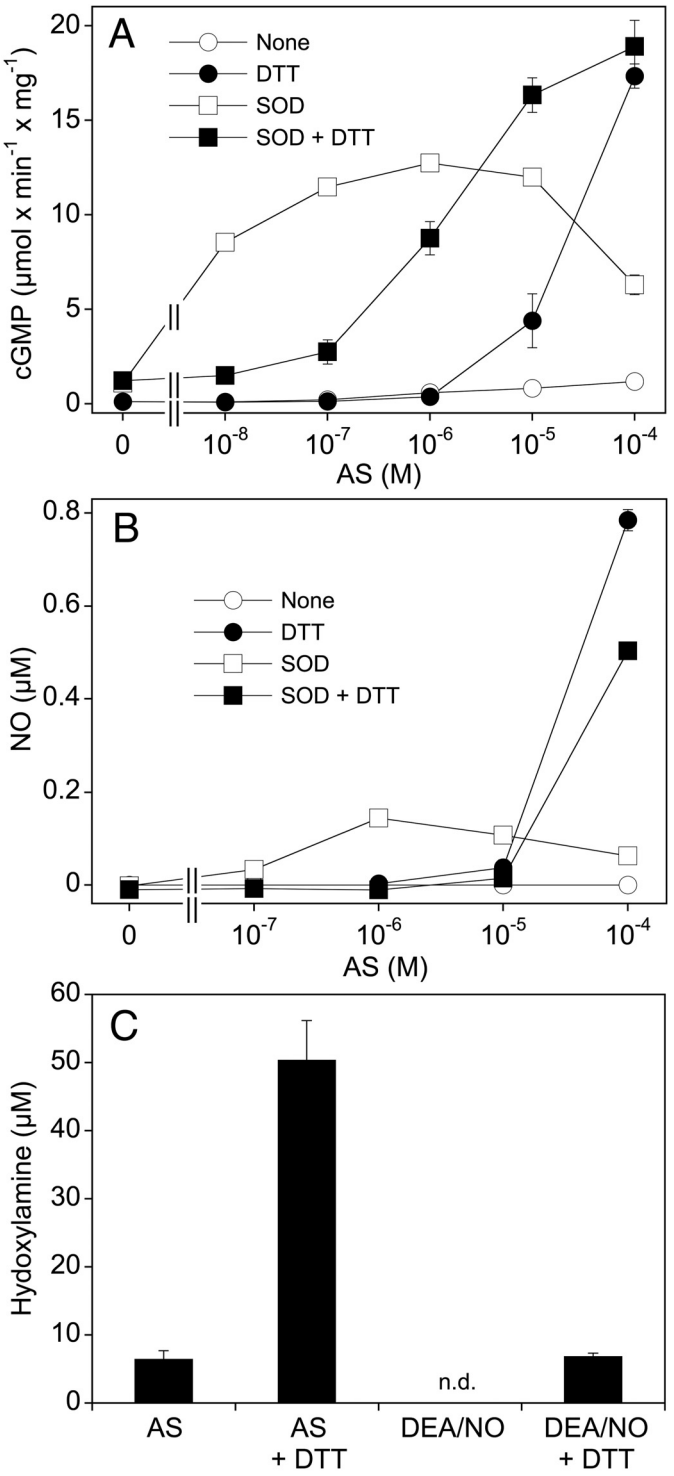

Fig. 1. Effects of AS on activation of purified sGC (A), release of NO (B), and hydroxylamine formation (C). A, purified bovine lung sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.1 ml with increasing concentrations of AS in the presence of 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-^{32}P]GTP (~250,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, and 0.1 mM DTPA. SOD (1000 U/ml) and DTT (2 mM) were present as indicated. Formation of [^{32}P]cGMP was determined as described under *Materials and Methods*. Data are mean values ± S.E. of three experiments. B, AS was incubated at the indicated concentrations in 1 ml of TEA buffer, pH 7.4, containing 0.1 mM DTPA, in the absence and presence of SOD and DTT as indicated, and NO formation was measured with a Clark-type electrode. Data are expressed as mean NO peak concentration ± S.E. of three experiments. C, AS and DEA/NO (0.1 mM each) were incubated at 37°C for 5 min in 0.2 ml of 50 mM TEA buffer, pH 7.4, containing 0.1 mM DTPA in the absence and presence of 2 mM DTT. Hydroxylamine was measured by light absorbance spectroscopy at 700 nm after condensation with 8-hydroxyquinoline and oxidation to indooxine. Data are mean values ± S.E. of three experiments. n.d., not detectable.

tion induced by 1 μM AS in the absence or presence of DTT increased in a roughly linear manner for 8 to 10 min in the presence of SOD.

Determination of NO release yielded similar results. As shown in Fig. 1B, AS (0.1 μM to 0.1 mM) did not release detectable NO (limit, ~5 nM) upon incubation in buffer alone, but a biphasic response was observed in the presence of SOD with maximal NO release, corresponding to a peak concentration of 0.14 ± 0.008 μM, occurring at 1 μM AS. Under these conditions, the peak concentration of NO released from 1 μM DEA/NO was 0.85 ± 0.01 μM, indicating that competing reactions preclude quantitative oxidation of AS-derived HNO to NO by SOD. DTT completely prevented the SOD-induced NO release from up to 10 μM AS but led to pronounced release of NO from 0.1 mM AS in the absence and presence of SOD (peak concentrations of 0.79 ± 0.023 and 0.50 ± 0.009 μM, respectively). Formation of NO was not detectable upon incubation of 0.1 mM nitrite with 2 mM DTT under identical conditions (data not shown). To illustrate the pattern of NO release, representative traces of NO signals obtained from 1 μM and 0.1 mM AS in the absence and presence of SOD and DTT are shown in Supplemental Fig. S2.

The effect of SOD was most likely due to oxidation of AS-derived HNO to NO, suggesting that inhibition of SOD-induced NO release by DTT reflects scavenging of HNO yielding hydroxylamine. To test this hypothesis, we measured hydroxylamine formation from AS in the absence and presence of DTT in comparison with the NO donor DEA/NO, a structurally related compound with similar half-life. As shown in Fig. 1C, incubation of 0.1 mM AS in the absence and presence of 2 mM DTT led to the formation of 6.42 ± 1.28 and 50.3 ± 5.86 μM hydroxylamine, showing that HNO released from AS is efficiently scavenged by DTT. Hydroxylamine formation from 0.1 mM DEA/NO was 6.85 ± 0.50 μM in the presence of 2 mM DTT but was not detectable (limit, ~0.5 μM) in the absence of the thiol, confirming that HNO release is a minor pathway of DEA/NO decomposition at physiological pH.

It was of interest to see whether differences in the SOD concentration-response would allow us to distinguish the two major effects of SOD on NO bioavailability (i.e., prevention of peroxynitrite formation under conditions of NO/superoxide cogeneration and oxidation of HNO to NO). For this purpose, we studied the effects of increasing SOD concentrations on purified sGC stimulated with fixed concentrations of either the HNO donor AS (1 μM) or the NO/superoxide donor SIN-1 (0.1 mM). As shown in Fig. 2A, the rates of cGMP formation induced by AS and SIN-1 were similarly increased by 1 to 1000 U/ml SOD, indicating that the sGC bioassay does not allow any judgment on the mechanism underlying the effect of SOD. Determination of NO release from AS and SIN-1 (Fig. 2B) yielded seemingly discrepant results, because up to 10 U/ml SOD, which led to more than half-maximal sGC stimulation, caused hardly detectable NO release from both donors. However, despite being very close to the detection limit of the electrode, the NO signals obtained from AS and SIN-1 in the presence of 10 U/ml SOD allowed a rough estimate of NO peak concentrations of approximately 3 nM, which would be sufficient for significant sGC activation based on a NO binding affinity of approximately 1 nM (Griffiths et al., 2003). In any case, these results highlight the

potential pitfalls of comparing the biological activity of NO with quantitative analysis of NO formation.

Because HNO triggers reductive nitrosylation of ferric heme, resulting in the formation of ferrous nitrosyl-heme complexes, we tested whether HNO activates oxidized sGC. For this purpose, we incubated the purified enzyme with 0.1 μM AS, a concentration that corresponds to an almost maximally active concentration of the related compound DEA/NO in the presence of ODQ (0.1 mM) to oxidize sGC-bound heme. As shown in Fig. 3A, AS had no effect on the rates of cGMP formation measured in the absence of SOD with and without ODQ, and the effect of SOD was completely inhibited by ODQ, as expected for NO-mediated sGC activation. To test for a low-affinity effect of AS and to minimize HNO scavenging by DTT present endogenously in the sGC preparations, these experiments were repeated with a more concentrated enzyme preparation (final DTT concentration, ~1 μM) and a 1000-fold higher AS concentration (0.1 mM). However, ODQ did not cause significant sGC activation by AS in the absence of SOD under these conditions (data not shown). In the presence of SOD, the degree of inhibition by ODQ decreased with increasing AS concentrations (Fig. 3, B and C), indicat-

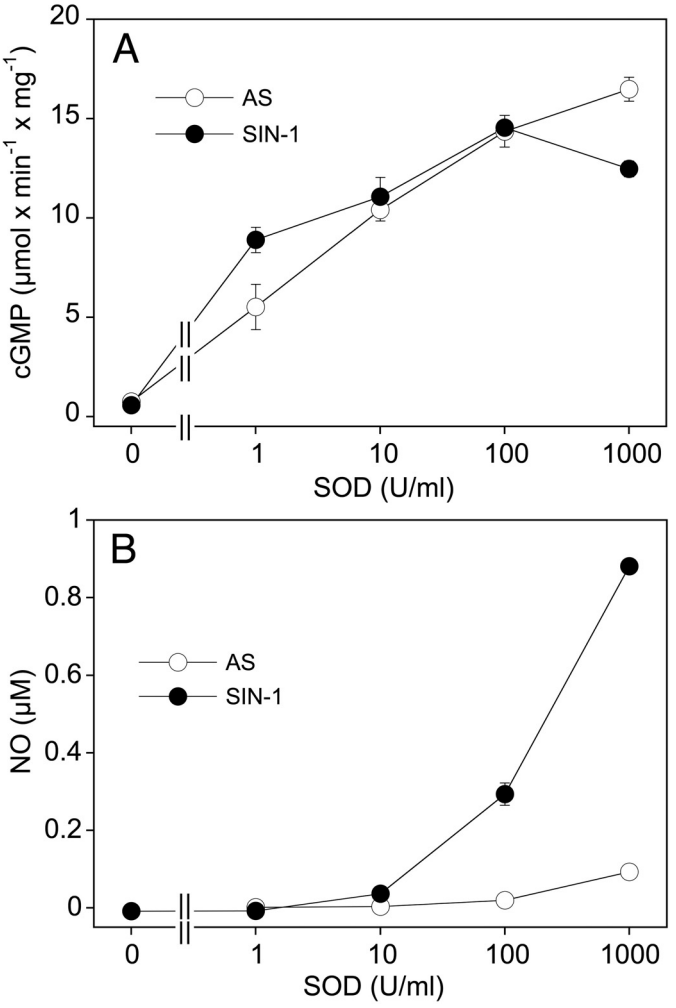

Fig. 2. Effects of SOD concentration on activation of purified sGC (A) and release of NO (B). Activation of sGC (A) and formation of NO (B) were determined in the presence of 1 μM AS or 100 μM SIN-1 and the indicated concentrations of SOD as described in the legend to Fig. 1. Data are mean values ± S.E. of three experiments.

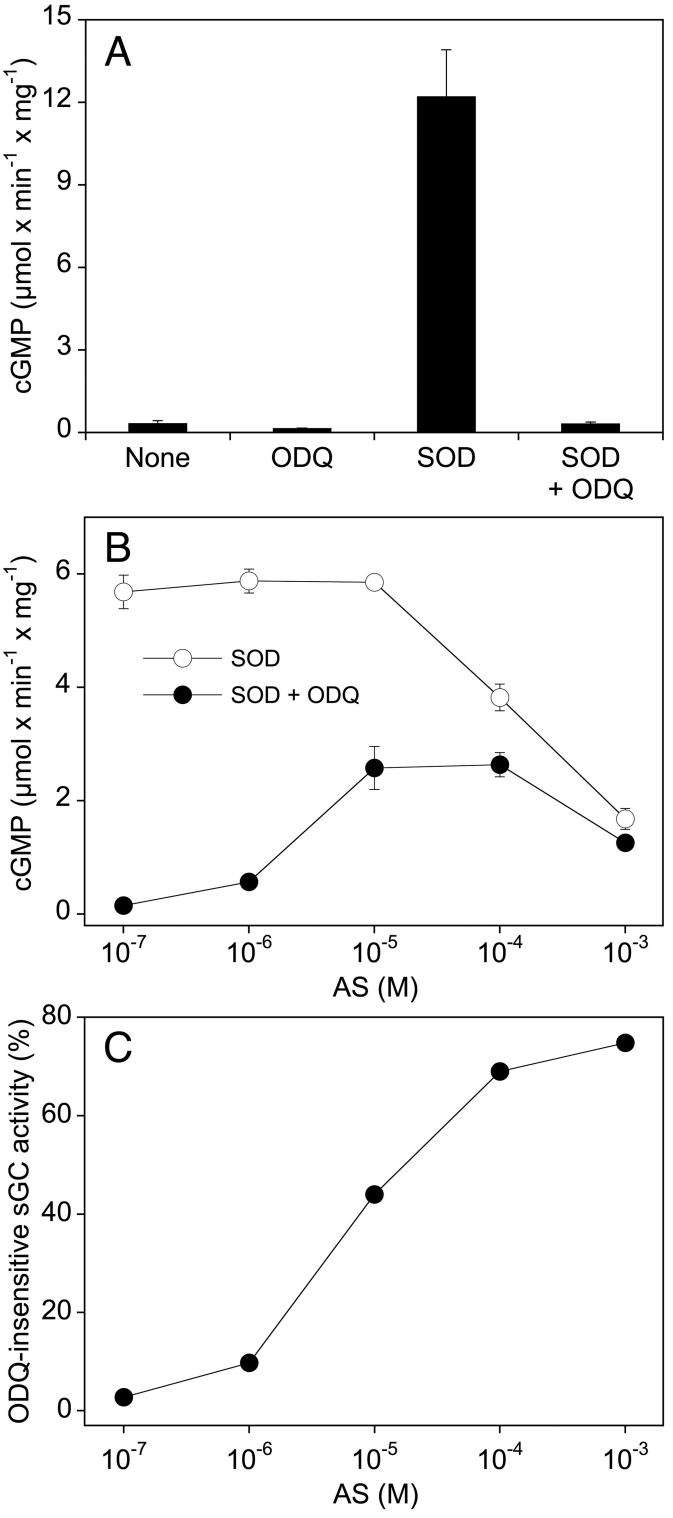

Fig. 3. Effect of ODQ on the activation of purified sGC by AS. A, purified sGC (50 ng) was incubated as described in the legend to Fig. 1 with 0.1 μM AS in the absence and presence of ODQ (0.1 mM) and SOD (1000 U/ml). B, purified sGC (740 ng) was incubated in 0.2 ml with increasing concentrations of AS in the presence of SOD (1000 U/ml) with and without 0.1 mM ODQ. Other experimental conditions were as described in the legend to Fig. 1. Data are mean values ± S.E. of three experiments. C, mean values of sGC activity measured in the absence and presence of ODQ (shown in B) were taken to plot the fraction of residual sGC activity in the presence of ODQ as a function of AS concentration.

ing that the effect of ODQ is attenuated under these conditions by AS-derived NO. This observation agrees well with a previous report on NO-competitive sGC inhibition by ODQ (Schrammel et al., 1996) that was confirmed by repeating the experiment shown in Fig. 3B with DEA/NO instead of AS. A plot of ODQ-resistant sGC activity as a function of DEA/NO concentration is shown in Supplemental Fig. 3S.

Figure 4A shows the effects of the NO scavengers carboxy-PTIO and hydroxy-cobalamine (0.1 mM each) on sGC in the absence and presence of DEA/NO (1 μM), AS (1 μM), and SIN-1 (100 μM) in the presence of 1000 U/ml SOD. As expected, sGC activation by the three donor compounds was largely prevented by both scavengers, even though the effect of SIN-1 was significantly less sensitive than that of AS ($p = 0.01$). The reason for this difference is unknown. To further elucidate the effects of carboxy-PTIO, we tested the effects of the scavenger on sGC stimulation by increasing concentrations of AS in the absence and presence of SOD. As shown in Fig. 4B, carboxy-PTIO completely inhibited the effect of low AS in the presence of SOD, but the degree of inhibition decreased with increasing AS concentration, hinting at an additional effect of carboxy-PTIO counteracting NO scavenging. This effect became clearly apparent in the absence of SOD. Under these conditions, carboxy-PTIO caused pronounced activation of sGC that was maximal with equimolar AS (0.1 mM), followed by a sharp decrease at 1 mM AS. The carboxy-PTIO concentration-response curve shown in Fig. 4C further illustrates the unexpected sGC activation in the presence of equimolar concentrations of AS and carboxy-PTIO. As shown in the inset to Fig. 4C, incubation of AS and carboxy-PTIO (0.1 mM each) gave rise to an electrochemical NO signal corresponding to a peak concentration of 45.9 ± 2.77 nM NO (mean ± S.E., $n = 3$), indicating that a minor reaction of carboxy-PTIO with either AS or AS-derived HNO results in the formation of NO and consequent activation of sGC. Formation of NO was not affected by 1 mM urate (data not shown), presumably excluding the NO_2 radical as reactive intermediate.

The biological relevance of our findings was assessed by the determination of endothelial cGMP accumulation in response to AS and DEA/NO. Figure 5A shows that DEA/NO caused approximately 20-fold increases in cGMP levels (from 3.24 ± 0.62 to 70.6 ± 3.7 pmol/10^6 cells at 1 μM DEA/NO) with an EC_{50} of 0.13 ± 0.013 μM. SOD caused an approximately 3-fold leftward shift of the DEA/NO concentration response (EC_{50} = 0.041 ± 0.015 μM). Carboxy-PTIO completely inhibited the effects of up to 0.1 μM DEA/NO and increased the EC_{50} of the donor 6-fold to 0.81 ± 0.19 μM. At 1 μM DEA/NO, carboxy-PTIO inhibited cGMP accumulation by approximately 30%, whereas inhibition of purified sGC stimulated with the same concentration of the donor was significantly more pronounced (Fig. 4A). This could be a consequence of different exposure periods (2 min in the cell experiments versus 10 min in the sGC assays) relative to the half-life of DEA/NO. The effects of DEA/NO were completely blocked by 0.1 mM ODQ (data not shown). As shown in Fig. 5B, AS was significantly less potent than DEA/NO in the absence of SOD (EC_{50} = 16 ± 2.3 μM), but exhibited similarly high potency in the presence of SOD with an EC_{50} of 0.048 ± 0.008 μM, corresponding to a 330-fold increase in AS potency by SOD. Carboxy-PTIO (0.1 mM) caused a pronounced 200-fold rightward shift of the AS concentration-response curve in the presence of SOD (EC_{50} = 9.3 ± 2.2 μM). In contrast, the effect of high AS observed without added SOD was not inhibited by carboxy-PTIO (EC_{50} = 6.9 ± 2.1 μM). ODQ completely inhibited the cGMP response to AS both in both the absence and presence of SOD. Considering the NO-competitive action of ODQ (Schrammel et al., 1996), the more pronounced effect of ODQ on endothelial cGMP accumulation compared with inhibition of purified sGC (Fig. 3B) indicates that sGC inside the cells is exposed to much lower NO concentrations, possibly as a result of efficient consumption of NO as described previously (Schmidt and Mayer, 2004).

Discussion

Theoretical chemistry predicts that AS decomposes in aqueous solution via reactions [1] and [2] governed by proton concentration, with HNO formation dominating at physiological pH (Dutton et al., 2004):

[1] $N_2O_3^{2-} + H^+ \rightarrow HNO + NO_2^-$
[2] $N_2O_3^{2-} + 2H^+ \rightarrow 2NO + H_2O$
[3] $HNO + NO \rightarrow N_2O_2^- + H^+$

The fractional contribution of reaction [2] to AS decomposition is difficult to determine experimentally because HNO reacts rapidly ($k = 2 \times 10^9\ M^{-1} \cdot s^{-1}$) with NO according to reaction [3] (Liochev and Fridovich, 2002). However, the reaction of HNO with thiols according to reaction [4] should unmask NO release and allow a rough estimate of the partitioning between reactions [1] and [2]: [4] $HNO + 2RSH \rightarrow NH_2OH + RSSR$.

Accordingly, the significant release of NO from ≥10 μM AS that we observed in the presence of DTT (Fig. 1B) may reflect thiol-mediated scavenging of HNO and, thus, prevention of NO consumption via reaction [3]. Determination of hydroxylamine formation from AS in the presence of DTT suggests 50% efficiency of reaction [4], but the actual scavenging efficiency of DTT may have been higher because of alternative reactions between HNO and thiols that do not yield hydroxylamine (Paolocci et al., 2007). The amount of NO released from 0.1 mM AS in the presence of DTT was similar to that detected upon decomposition of 1 μM concentration of the related compound DEA/NO (data not shown). Thus, under the assumption that DTT scavenged 50 to 100% of AS-derived HNO, reaction [2] accounts for 1 to 2% of AS decomposition at pH 7.4. The prevention of reaction [3] through HNO scavenging certainly provides a reliable explanation for SOD-independent activation of purified sGC by AS in the presence of DTT. Considering the high intracellular concentrations of GSH, this pathway may contribute significantly to endothelial cGMP accumulation in response to ≥10 μM AS (Fig. 5B).

However, the amount of NO released from AS in the presence of thiols is too small to explain cGMP-mediated vasodilation induced by nanomolar concentrations of AS in some vascular beds (Zamora et al., 1995; Irvine et al., 2003; Favaloro and Kemp-Harper, 2007), hinting at additional mechanisms of sGC activation in tissues. The most relevant mechanism is oxidation of HNO to NO by Cu(II), Zn(II) SOD (Murphy and Sies, 1991; Liochev and Fridovich, 2002). The reaction results in the formation of Cu(I), Zn(II) SOD, which may be reoxidized slowly to the Cu(II) form by NO, giving rise to formation of $^3NO^-$, which reacts with O_2 to yield peroxyni-

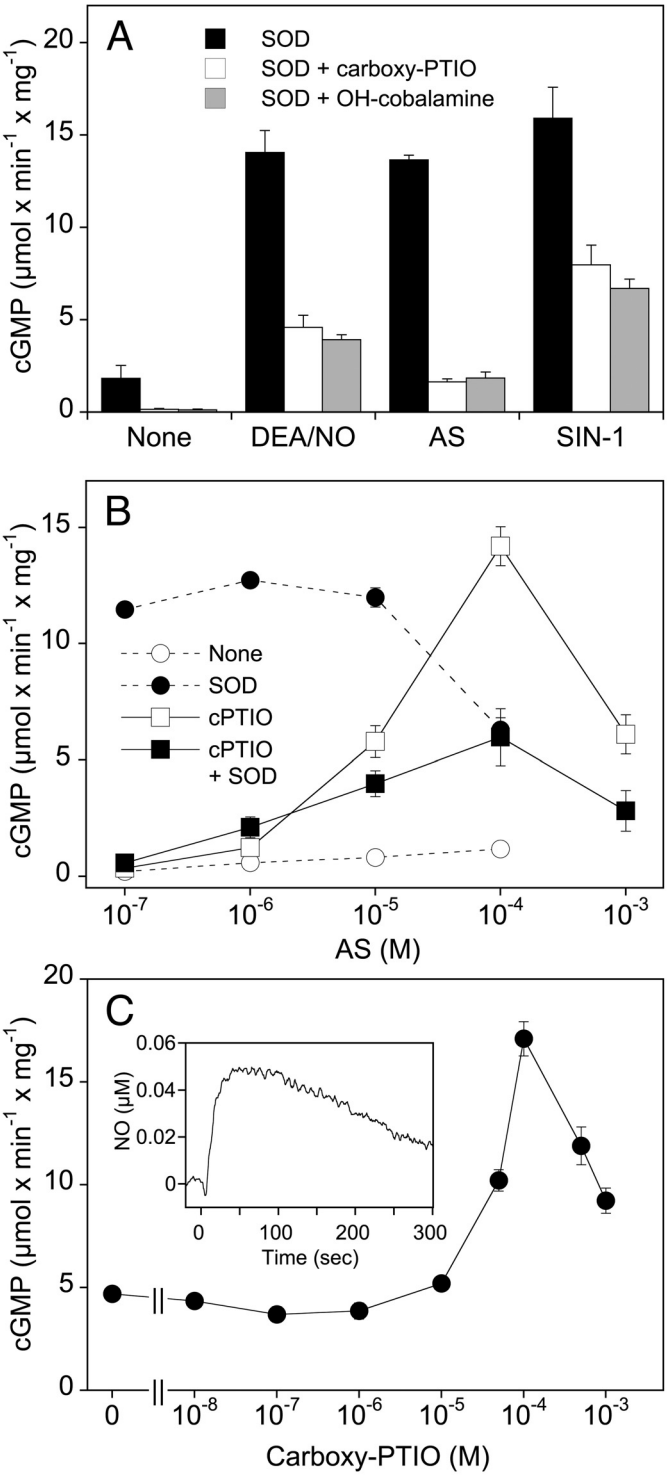

Fig. 4. Effects of carboxy-PTIO and hydroxy-cobalamine on AS-induced activation of purified sGC. Purified sGC (50 ng) was incubated as described in the legend to Fig. 1. A, effects of carboxy-PTIO and hydroxy-cobalamine (0.1 mM each) on sGC activity measured in the presence of SOD (1000 U/ml) and DEA/NO (1 μM), AS (1 μM), or SIN-1 (100 μM). B, effects of AS concentration on sGC activity in the presence of carboxy-PTIO with and without SOD (1000 U/ml). The data on enzyme activity measured in the absence of carboxy-PTIO taken from Fig. 1A are included for better comparison. C, effects of increasing carboxy-PTIO concentrations on sGC activity measured in the presence of 0.1 mM AS in the absence of SOD. Data are mean values ± S.E. of three experiments. C, inset, representative trace of NO formation in the presence of AS and carboxy-PTIO (0.1 mM each; n = 3); OH-cobalamine, hydroxy-cobalamine; cPTIO, carboxy-PTIO.

trite and eventually nitrate as a stable end product (Miranda et al., 2005). In the presence of 1000 U/ml SOD, corresponding to a SOD concentration of 16 μM, sGC activation and NO release were both biphasic with maximal efficacy at 1 μM AS. Thus, depletion of Cu(II), Zn SOD may explain decreased efficacy of ≥10 μM AS, although we cannot exclude that high concentrations of AS caused sGC inhibition through modification of essential cysteine residues, as described recently for dinitrosyl-iron complexes (Mayer et al., 2009). Oxidation of HNO most likely explains the more than 300-fold potentiation of AS-induced endothelial cGMP accumulation by SOD. In comparison, SOD only moderately increased the potency of DEA/NO, presumably through scavenging of superoxide generated via undefined reactions in the culture medium (Fig. 5). The present data do not allow judging the relative contribution of HNO oxidation by intracellular SOD versus thiol-induced NO release to AS-induced cGMP accumulation, but the potency of AS to activate sGC in a given type of tissue is

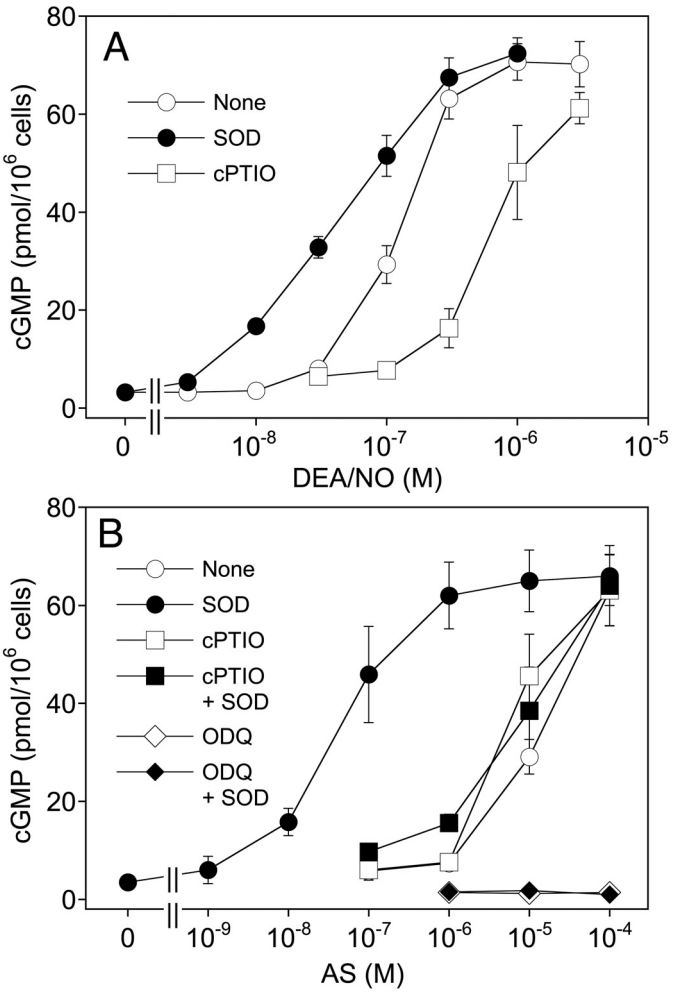

Fig. 5. Effects of DEA/NO (A) and AS (B) on cGMP accumulation in cultured porcine aortic endothelial cells. Porcine aortic endothelial cells were grown in 24-well plates, washed, and preincubated for 15 min at 37°C in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 1 μM indomethacin, and where indicated 1000 U/ml SOD, 0.1 mM carboxy-PTIO, or 0.1 mM ODQ. The cells were then incubated for 2 min with the indicated concentrations of DEA/NO (A) or AS (B), followed by the determination of intracellular cGMP as described under *Materials and Methods*. Data are mean values ± S.E. of three independent experiments. cPTIO, carboxy-PTIO.

expected to be determined by both SOD and thiol levels. The two pathways mediating sGC activation by AS are schematically illustrated in Fig. 6.

While a previous version of this article was under review, Miller et al., (2009) reported that AS caused partial activation of purified sGC that was heme-dependent and occurred in the absence of deliberately added agents known to oxidize HNO to NO. These results led the authors to propose that HNO interacts with sGC-bound heme to activate the enzyme. The discrepancy between their observation and our data showing hardly any increase in cGMP formation with up to 0.1 mM AS in the absence of SOD (Fig. 1A, ○) could be due to different experimental conditions (different enzyme preparations, aerobic versus anaerobic incubation, etc.). More importantly, however, without measuring NO, it cannot be excluded that sGC was activated by minor amounts of HNO-derived NO formed through some unknown reaction in their assay mixtures. Generation of picomolar concentrations of NO would have been sufficient to explain sGC activation without invoking a direct interaction of HNO with ferrous heme iron.

It was one of the goals of the present study to clarify whether AS causes cGMP accumulation through activation of ferric sGC by HNO according to reaction [5]: sGC-Fe(III) + HNO → sGC-Fe(II)-NO + H^+. If this reaction were significant, AS would be expected to stimulate sGC in the presence of the heme oxidant ODQ (Schrammel et al., 1996), as reported for a certain class of sGC-activating drugs (Evgenov et al., 2006). However, in the absence of SOD, neither low (0.1 μM) nor high (0.1 mM) AS caused significant activation of ODQ-treated sGC, and ODQ completely blocked AS-induced accumulation of cGMP in endothelial cells in the presence and absence of added SOD (Fig. 5B). At first glance, the data shown in Fig. 3B might suggest that 10 to 100 μM AS activates ODQ-treated sGC in the presence of SOD. However, replot of the data as ODQ-insensitive sGC activity as a function of AS concentration (Fig. 3C) revealed that ODQ inhibited cGMP formation in an AS-competitive manner. These results were expected considering the fact that AS acts via NO in the presence of SOD together with NO-competitive sGC inhibition by ODQ (Fig. S3). A recent study also reported on the lack of activation of ferric sGC by HNO (Miller et al., 2009). This may be explained by a slow reaction of HNO with Fe(III)-sGC compared with the nearly diffusion-controlled reaction of NO with Fe(II)-sGC (Griffiths et al., 2003). The rate constant of reaction [5] is not known, but the rates for reductive nitrosylation of other ferric heme proteins are in the range of 10^4 to 10^5 $M^{-1} \cdot s^{-1}$ (Miranda et al., 2003). Our data indicate that the affinity of Fe(III)-sGC for HNO is similarly low, precluding activation of the oxidized enzyme as a relevant mechanism of HNO-induced cGMP accumulation in vivo.

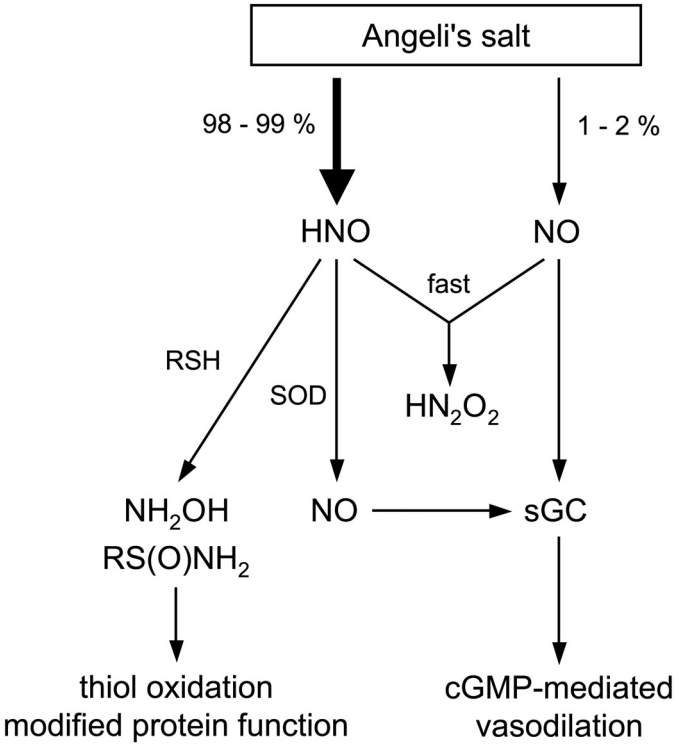

Fig. 6. Schematic illustration of AS decomposition pathways leading to sGC activation. At physiological pH, the predominant pathway of AS decomposition yields HNO, which is oxidized to NO by Cu(II), Zn SOD and possibly other physiological oxidants. NO formed via an alternative decomposition pathway, which predominates at acidic pH (Dutton et al., 2004), is efficiently scavenged by the excess of AS-derived HNO, preventing NO-mediated activation of sGC in the absence of SOD and thiols (RSH). HNO reacts rapidly with low molecular mass thiols (DTT in the present study), yielding hydroxylamine (NH_2OH) and the corresponding disulfides. At millimolar RSH concentrations, this reaction outcompetes oxidation of HNO to NO by SOD as well as HNO-mediated NO scavenging (thereby unmasking NO release from AS). Reaction of HNO with protein sulfhydryls may result in the formation of the corresponding *N*-hydroxysulfinamide derivatives [$RS(O)NH_2$] and consequent irreversible changes in the function of specific HNO target proteins (Paolocci et al., 2007).

Since the first description of NO scavenging by carboxy-PTIO (Akaike et al., 1993), this drug has been used frequently to discriminate between NO-dependent and NO-independent biological processes. However, effects of carboxy-PTIO unrelated to NO scavenging have questioned the usefulness of this drug as a selective pharmacological tool (Pfeiffer et al., 1997). Nevertheless, the failure of carboxy-PTIO to inhibit cGMP-mediated vasodilation to AS is still taken as evidence for NO-independent sGC activation by HNO (Irvine et al., 2008). Our results with cultured endothelial cells clearly show that carboxy-PTIO inhibits cGMP accumulation induced by AS in the presence of extracellular SOD, demonstrating that the drug does scavenge AS-derived NO as expected. However, in line with several published studies (Li et al., 1999; Costa et al., 2001; Wanstall et al., 2001; Irvine et al., 2003, 2007; Hewett et al., 2005), carboxy-PTIO did not block the effect of AS in the absence of added SOD (Fig. 5B). The different sensitivities of low and high AS to carboxy-PTIO could be explained by a predominant reaction of the drug with NO formed extracellularly by SOD at low concentrations of AS, whereas the effect of high AS (in the absence of SOD) might occur intracellularly and hence be resistant to carboxy-PTIO.

Alternatively, the formation of NO in the combined presence of carboxy-PTIO and AS (Fig. 4) may counteract the NO-scavenging effect of the drug. Our observation confirms a previous report from Ellis et al., (2001), who suggested that carboxy-PTIO oxidizes HNO to NO. The biphasic effect of carboxy-PTIO is hard to explain without knowing the rate constants of the relevant reactions that may take place with AS, HNO, and putative reaction intermediates. It would seem that scavenging and/or consumption of NO prevails

over NO formation at excess carboxy-PTIO. Considering the manifold unspecific effects of carboxy-PTIO that limit the usefulness of this drug as a pharmacological tool, we refrained from further investigating the mechanism underlying carboxy-PTIO-triggered NO release from AS and/or HNO.

Taken together, our data suggest that AS activates sGC exclusively through NO formation. Besides the established oxidation of HNO to NO by Cu(II), Zn SOD, we found that scavenging of HNO by thiols unmasks NO release via a minor pathway of AS decomposition that may partially account for AS-induced cGMP accumulation in tissues. Activation of ferric sGC by HNO could be excluded as a relevant mechanism of action of AS.

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Address correspondence to: Dr. Bernd Mayer, Department of Pharmacology and Toxicology, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria. E-mail: mayer@uni-graz.at