Stimulation of soluble guanylate cyclase by superoxide dismutase is mediated by NO

Andreas FRIEBE, Günter SCHULTZ and Doris KOESLING¹

Institut für Pharmakologie, Freie Universität Berlin, D-14195 Berlin, Germany

Soluble guanylate cyclase (sGC), which is found in many cells and tissues, represents the receptor for the intra- and intercellular messenger molecule NO. Superoxide dismutase (SOD), an enzyme involved in the degradation of toxic superoxide radicals, has been proposed as a non-NO activator of sGC. Here we show that SOD stimulated sGC purified from bovine lung up to 10-fold. Activation by SOD was not influenced by the hydroxyl radical scavengers mannitol and DMSO. In contrast, the presence of the NO scavengers oxyhaemoglobin and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, as well as the O₂^{•-}-generating system xanthine oxidase/hypoxanthine, led to inhibition of SOD-stimulated cGMP production. NO-insensitive

sGC mutants were not influenced either by SOD or by xanthine oxidase. We have previously shown that sGC was stimulated by NO present in the normal atmosphere. Here we show that the SOD effect depended on the NO concentration from the atmosphere, as the stimulation of sGC by defined NO gases (0, 120, 330 and 1000 parts per billion NO) was potentiated by SOD. NO stimulation of sGC and its potentiation by SOD were inhibited by oxyhaemoglobin to identical levels. We conclude that the SOD-mediated stimulation of sGC is due to the elimination of superoxide, thereby preventing its reaction with NO to form peroxynitrite.

INTRODUCTION

As mediator in the NO/cGMP signalling pathway, soluble guanylate cyclase [sGC; GTP pyrophosphate-lyase (cyclizing); EC 4.6.1.2] plays a key role in vasorelaxation, inhibition of platelet aggregation and the central nervous system [1–6]. sGC catalyses the conversion of GTP to cGMP, and stimulation of the enzyme by NO leads to a pronounced increase in the catalytic rate. As an intracellular messenger, cGMP acts on several cGMP-regulated receptor proteins, such as cGMP-stimulated protein kinases, cGMP-regulated phosphodiesterases and cGMP-gated ion channels.

sGC consists of two different subunits (α and β) and contains a prosthetic haem group that mediates the stimulation of the enzyme by NO. Spectrophotometric analysis revealed that the non-covalently bound haem is five-co-ordinated with a histidine as the axial ligand [7]. The histidine 105 of the β_1 subunit was identified as the possible haem-co-ordinating residue, as point mutation of this amino acid led to an NO-insensitive haem-deficient enzyme [8,9].

Although the role of NO as the enzyme's physiological activator has been widely accepted, the relevance of regulation of the enzyme by substances other than NO remains unknown. Recently, we and others have identified YC-1 as a novel non-NO activator of sGC [10–12]. YC-1 stimulated cGMP production up to 12-fold in an NO-independent, but haem-dependent, manner. YC-1 sensitized the enzyme towards NO and, in the presence of YC-1, CO was able to activate sGC to almost the same extent as NO [11].

In previous reports, a multitude of different activators and regulators of sGC were described, most of them exhibiting

redox-active properties (for review see [13]). These non-NO activators constitute a rather heterogeneous group of substances; thus, several mechanisms of activation have been postulated and subsumed as 'redox-regulation' of sGC. In a first study, we were able to show that cysteine residues of sGC are unlikely to be the direct targets of this redox regulation [14]. The results with these non-NO activators were mostly obtained using cytosolic or partially purified enzyme preparations [13], making it impossible to differentiate between a direct influence on sGC activity or indirect effects on NO synthases or phosphodiesterases. Therefore, our aim was to reproduce the stimulatory effect of one of these non-NO activators, superoxide dismutase (SOD) [15], on the activity of the purified enzyme and to elucidate the underlying mechanism of activation.

We show here that SOD leads to a concentration-dependent stimulation of purified sGC, which was blocked by the NO scavengers oxyhaemoglobin (oxyHb) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) as well as by superoxide anions. Experiments under defined NO atmospheres indicate that SOD stimulates sGC indirectly by preventing the reaction of dissolved NO with superoxide to peroxynitrite, i.e. by increasing the relative amount of NO in solution.

MATERIALS AND METHODS

Materials

SOD, xanthine oxidase (XO), hypoxanthine (HX) and haemoglobin were obtained from Sigma, DMSO and sodium dithionite were purchased from Merck, carboxy-PTIO was from

Abbreviations used: sGC, soluble guanylate cyclase; SOD, superoxide dismutase; XO, xanthine oxidase; HX, hypoxanthine; oxyHb, oxyhaemoglobin; DEA-NO, 2,2-diethyl-1-nitroso-oxyhydrazine; ppb, parts per billion; ODQ, 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

¹ To whom correspondence should be addressed (e-mail koesling@iname.com).

Calbiochem, and mannitol was obtained from Fluka. 2,2-Diethyl-1-nitroso-oxyhydrazine (DEA-NO) sodium salt was purchased from NCI Chemical Carcinogen Repository. [α - 32 P]GTP (800 Ci/mmol) was from NEN-DuPont. NO gases [0, 120, 330 or 1000 parts per billion (ppb) NO in N₂] were purchased from AGA Gas (Berlin, Germany).

Determination of guanylate cyclase activity

All enzyme activity determinations were performed in room air on the bench top except those under defined NO atmospheres in a glove box. sGC was purified from bovine lung as described previously [16]. The mutant cyclases, β_1 H105F and the N-terminally deleted mutant $\Delta 1-63\beta_1$, were constructed and purified from Sf9 cells as described elsewhere [8,17]. sGC activity (0.1 μ g of enzyme per tube) was measured by the conversion of [α - 32 P]GTP to [32 P]cGMP at 37 °C for 10 min [18]. Reaction mixtures contained 3 mM Mg²⁺ as divalent metal ion, 3 mM dithiothreitol, 0.5 mg/ml BSA, 1 mM cGMP, 300 μ M GTP and 50 mM triethanolamine hydrochloride, pH 7.4, in a total volume of 0.1 ml. Data are expressed either as representative experiments or as means $\pm$ S.E.M. of 3 experiments performed in duplicate.

Guanylate cyclase activities under defined NO atmosphere

Enzyme was preincubated in a glove box (I₂R, Cheltenham, U.S.A.) at 4 °C under the indicated NO atmosphere for 60 min. Before pre-incubation, the glove box and all solutions were evacuated and gassed three times with the respective NO gas. The reaction was started by the addition of substrate and performed under the respective NO atmosphere throughout the entire incubation.

Preparation of oxyHb

OxyHb was prepared in 50 mM triethanolamine hydrochloride, pH 7.0, by reducing bovine methaemoglobin with sodium dithionite. Subsequently, reduced haemoglobin was desalted by passing over a Sephadex G-25 (PD-10) column (Pharmacia). The concentration of oxyHb was determined spectrophotometrically (560, 576 and 630 nm).

RESULTS

To investigate the stimulatory effect of SOD on purified sGC, we co-incubated the enzyme with increasing concentrations of SOD. Stimulation of sGC by SOD was concentration-dependent ($EC_{50} = 0.83 \pm 0.12$ units/ml, $n = 3$; Figure 1), and maximal activation was observed with 10 units/ml SOD, a concentration used in all further experiments. The stimulatory effect of SOD depended on dismutase activity, as heat-denatured SOD did not lead to an enhancement of cGMP production (results not shown). Although maximal stimulation with 10 units/ml SOD was always reproducible qualitatively, the stimulation factor varied between 2- and 10-fold. This variation of the stimulation factor was due to the increase in SOD-stimulated activity, and only high SOD effects were accompanied by minor increases in the non-stimulated activity of the enzyme.

The stimulatory effect of SOD on a partially purified sGC preparation has been attributed to hydroxyl radicals [15]. Therefore, we next investigated the effect of two well-known hydroxyl radical scavengers on the purified enzyme. As shown in Table 1, neither mannitol nor DMSO influenced non-stimulated, SOD- or NO-stimulated sGC activities. In addition, catechol (5 mM) and epinephrine (10 mM) did not inhibit SOD-stimulated sGC but rather had a stimulatory effect on non-stimulated activity

Figure 1 SOD-mediated stimulation of purified sGC

Increasing concentrations of SOD were applied when starting the incubation. Data shown are representative of 4 experiments with similar results. U, units.

Table 1 Effect of hydroxyl radical scavengers mannitol and DMSO on sGC activity

Data are means $\pm$ S.E.M. from 3 separate experiments.

Activator	Addition	Enzyme activity (nmol of cGMP $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹)
None	Control	14 $\pm$ 3
	1 mM Mannitol	17 $\pm$ 6
	1% DMSO	18 $\pm$ 6
SOD (10 units/ml)	Control	58 $\pm$ 26
	1 mM Mannitol	69 $\pm$ 43
	1% DMSO	72 $\pm$ 34
DEA-NO (10 μ M)	Control	3460 $\pm$ 350
	1 mM Mannitol	3490 $\pm$ 260
	1% DMSO	3430 $\pm$ 233

(results not shown). Thus, we conclude that hydroxyl radicals are not likely to mediate the stimulatory effect on sGC.

A possible NO dependence of the SOD effect was investigated with the help of the two different NO scavengers, oxyHb and carboxy-PTIO (Figures 2A and 2B). SOD-mediated sGC stimulation was abolished by increasing concentrations of both scavengers, whereas non-stimulated enzyme activity was only slightly reduced (50% or less). Interestingly, SOD-stimulated as well as non-stimulated sGC activities were inhibited to a similar 'basal level'. The term basal activity will be used for the activity that remains under non-stimulated conditions in the presence of an NO-scavenger. The results with oxyHb and carboxy-PTIO speak in favour of a role for NO in SOD-mediated stimulation.

We have previously shown sGC to be highly sensitive to NO present in the atmosphere, as the tiny amounts of NO in solution resulting from polluted air had a stimulatory effect on the enzyme [19]. Based on the reaction of NO with superoxide to form peroxynitrite [20]:

Figure 2 Inhibition of basal, SOD- and NO-stimulated sGC by oxyHb, carboxy-PTIO and XO

Enzyme activity was determined in the absence of additional activator (○) or in the presence of 10 units/ml SOD (●) or 10 μ M DEA-NO (■). (A) Oxyhaemoglobin was prepared as described in the Materials and methods section and applied to sGC when starting the incubation. (B) The NO scavenger carboxy-PTIO was added when starting the incubation. The higher enzyme activities compared to those in (A) and (C) are explained by the use of a different enzyme preparation. (C) XO and HX (100 μ M) were added to sGC when starting the incubation. Data are representative of 3 similar experiments.

we postulated that elimination of superoxide by SOD increased the NO concentration in solution, thus leading to enzyme activation.

To test this hypothesis, we tried to reverse the SOD-mediated sGC activation by increasing O_2^- . By analogy to the results with oxyHb, Figure 2(B) shows that increasing amounts of the O_2^- -producing enzyme XO (100 μ M HX) resulted in a slight reduction of non-stimulated sGC activity (50% or less) and in a complete abolishment of SOD-mediated sGC stimulation. Although the stimulation factor by SOD varied in different experiments, O_2^- always reduced SOD-stimulated sGC activity to a basal level of enzyme activity similar to the one observed in the presence of the NO scavenger oxyHb (see Figure 2A). In addition, maximal NO-stimulated activity (10 μ M DEA-NO) was inhibited very strongly to almost the same basal activity.

These results suggest that oxyHb and XO/HX only inhibited

the stimulated enzyme whereas the basal activity of sGC remained unaffected. As the findings with O_2^- were in contrast to a recent report [12], we performed additional experiments and confirmed our results using a point-mutated form of sGC. This mutant (β_1 H105F) exhibited intact non-stimulated catalytic activity but was haem-deficient and therefore insensitive to NO [8]. Enzyme activity of the mutant was influenced neither by SOD (10 units/ml) nor by XO/HX (10 milliunits/ml; 100 μ M) (results not shown). Similarly, another haem-deficient NO-insensitive mutant lacking the N-terminal 63 amino acids ($\Delta 1-63\beta_1$) was not affected by these additions (results not shown).

To finally demonstrate that the SOD effect depended on atmospheric NO, we measured the SOD-mediated sGC stimulation under defined atmospheric NO concentrations (0, 120, 330 and 1000 ppb NO in N_2). As shown in Figure 3(A), increasing concentrations of NO in the surrounding atmosphere produced a concentration-dependent increase in cGMP production that was potentiated in the presence of SOD. OxyHb blocked both NO-induced and SOD-mediated activation to similar levels. Under NO-free conditions ('0' ppb NO), we still observed a minute NO-dependent sGC activation (inhibited by oxyHb) and thus also activation by SOD (similarly blocked by oxyHb). This can probably be explained by the fact that, according to the manufacturer, NO-free gas may contain up to 30 ppb NO. As expected, sGC stimulation by a very high atmospheric NO concentration (300 ppm), a stimulation similar to that by maximally effective DEA-NO concentrations (10 μ M), was not further augmented by SOD (Figure 3B). In conclusion, stimulation of sGC by SOD is unlikely to be mediated by hydroxyl radicals but rather by an increase of the NO concentration due to inhibition of peroxynitrite formation.

DISCUSSION

By elimination of the reactive superoxide anion, O_2^- , SOD has an important function in the detoxification of cells and tissues. SOD has been assigned a stimulatory role regarding sGC, as co-incubation with a partially purified sGC preparation resulted in an increase in cGMP production. The SOD-mediated activation of sGC was proposed to be mediated by hydroxyl radicals resulting from the Haber-Weiss reaction [15]. In contrast to this mechanism, we show here that SOD stimulates sGC by an NO-dependent mechanism.

SOD-mediated stimulation of purified sGC varied between 2- and 10-fold, was not altered by scavengers of hydroxyl radicals, but was blocked by two different NO-scavengers, oxyHb and carboxy-PTIO, indicating NO dependence of the activation. We have shown that sGC is stimulated by the minute concentrations of NO present in the atmosphere even without administration of additional NO [19]; this also explains the variability of the SOD-mediated sGC activation (2- to 10-fold), which was elevated on days with high air pollution. Auto-oxidation of thiols, in our case dithiothreitol, at physiological pH [21] could account for the presence of O_2^- in our experiments. Therefore, we assumed that the SOD-catalysed elimination of O_2^- prevented the reaction of O_2^- with NO, and thus NO's inactivation, to peroxynitrite. The fast and spontaneous reaction of O_2^- with NO with the formation of peroxynitrite, which is not an activator of sGC itself, has been demonstrated and is generally accepted as a mechanism of NO inactivation not only *in vitro*, but also *in vivo*.

In accordance with our hypothesis, the SOD-mediated activation of sGC was reversed by increasing the O_2^- concentration, depended on the NO concentration of the surrounding atmosphere and was not additive to the stimulation evoked by

Figure 3 Concentration-dependent activation of sGC by atmospheric NO and potentiation of the NO stimulation by SOD

(A) Measurement of enzyme activity was performed in the absence and presence of SOD (10 units/ml) in different NO atmospheres (white bars). '0' ppb NO ('NO-free' gas) may contain up to 30 ppb NO according to the manufacturer. For comparison, sGC activities in normal laboratory atmosphere (atm; usually not exceeding 50 ppb NO) are also shown (histogram on far-right). Activity in the presence of oxyHb is symbolized by grey bars. (B) Maximal NO stimulation by 300 ppm NO atmosphere was not further augmented by SOD (10 units/ml).

maximally effective NO concentrations. These results support the assumption outlined above that SOD stimulates sGC indirectly by increasing the amount of available NO in solution. Although these concentrations of NO were low, even a small population of sGC molecules in the NO-activated state measurably increased cGMP production due to the extraordinary stimulation factor of NO.

Alternatively, one could argue in favour of a direct effect of superoxide on the catalytic activity of sGC. Yet, similarly to that seen with the two NO scavengers oxyHb and carboxy-PTIO, superoxide did not decrease enzyme activity below a basal level of activity (Figure 2). This, in our opinion, argues against a direct effect of superoxide on the catalytic activity. In addition, a direct NO/haem-independent effect of SOD/O₂⁻ on sGC activity can be ruled out because the enzyme activities of two different NO-insensitive sGC mutants were unaltered in the presence of SOD, oxyHb or the O₂⁻-generating system.

The lack of an effect of O₂⁻ on basal sGC activity was contrasted by a recent report in which O₂⁻ led to a drastic reduction of activity in the absence of an added activator. These incongruous results are likely to be explained by differences in the enzyme preparations, also reflected by the distinct maximal stimulation factor for NO (250-fold for the enzyme used in this study versus 10-fold for the enzyme used by Mülsch et al. [12]), and the different sensitivity towards specific inhibitors of sGC. Whereas ODQ [1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one] only inhibited the NO-stimulated activity of our enzyme, leaving

the basal activity unchanged [22], the ODQ-related substance NS 2028 markedly reduced the non-stimulated catalytic activity of the enzyme used in the study of Mülsch et al. [12]. These differences can be best explained by a varying degree in the prestimulation of the enzyme preparations.

The mechanism of SOD-mediated activation outlined above may be discussed as an *in vitro* artefact because, under physiological conditions, atmospheric NO will not participate in sGC activation (with the possible exception of the respiratory tract) and neither will several of the so-far-described non-NO activators. Nevertheless, the results emphasize the importance of high amounts of SOD as well as the essential influence of intracellular O₂⁻ formation on the concentration of physiologically occurring NO. Especially under conditions of oxidative stress, SOD activity will not only reduce the concentration of toxic oxygen radicals but, by prevention of NO inactivation, will also maintain the NO/cGMP signalling pathway.

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