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UNDER ANAEROBIC CONDITIONS, SOLUBLE GUANYLATE CYCLASE IS SPECIFICALLY STIMULATED BY GLUTATHIONE

F. Niroomand[+], R. Rössle, A. Mülsch and E. Böhme[+*]

Pharmakologisches Institut, Universität Heidelberg
Im Neuenheimer Feld 366, D-6900 Heidelberg, F.R.G.

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Various thiols exert non-specific effects on the activity of soluble guanylate cyclase under aerobic conditions. We studied the effects of thiols under anaerobic conditions (pO_2 < 6 Torr) on soluble guanylate cyclase, purified from bovine lung. Reduced glutathione stimulated the enzyme concentration-dependently with half-maximal enzyme stimulation at a concentration of about 0.5 mM. The extend of maximal enzyme stimulation (up to 80-fold) was comparable with the activation by NO-containing substances. The activation by glutathione was additive with the effect of sodium nitroprusside. Cysteine and various other thiols increased the enzyme activity 20-fold and 2- to 5-fold, respectively. The stimulatory effect of these thiols was not related to their reducing potency. Activation of soluble guanylate cyclase by glutathione was dose-dependently reduced in the presence of other thiols (cysteine > oxidized glutathione > S-methyl glutathione). Under aerobic conditions or with Mn-GTP as substrate, the effect of glutathione on soluble guanylate cyclase was suppressed. The results suggest a specific role for glutathione in the regulation of soluble guanylate cyclase activity and a modulation of this effect by redox reactions and other intracellular thiols.

Thiols are known to prevent oxidative activation and inactivation of soluble guanylate cyclase (E.C. 4.6.1.2., GTP:pyrophosphate lyase (cyclizing)) (1-5). It has been proposed that the regulation of the enzyme activity involves redox mechanisms (1,3,5-7). The hypothesis is based on the observations that thiols, thiol blocking agents (e.g. N-ethylmaleimide), thiol oxidizing agents (e.g. diamide) and oxygen affect enzyme activity (2,8,9-11). Other redox reactive substances like reducing dyes, ascorbic acid and fatty acid hydroperoxides affect enzyme actity as well (7,12). It is assumed that critical thiol groups essential for soluble guanylate cyclase activity are affected by

[+]Present address: Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73, D-1000 Berlin 33, F.R.G.

[*]To whom all correspondence should be addressed.

Abbreviations: cGMP, guanosine 3':5'-monophosphate; GTP, guanosine 5'-triphosphate; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; SNP, sodium nitroprusside.

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redox reactive substances, thereby leading to altered enzyme activity (3,5,8-10). The heme moiety of the enzyme is another redox sensible component which is thought to mediate the enzyme activation by NO and NO-containing compounds (13-15). An involvement of sulfhydryl groups in the activation of soluble guanylate cyclase by SNP has been proposed (16). In conclusion, all previous results indicate that reduced thiols function to protect soluble guanylate cyclase from oxidative activation and inactivation.

We studied the effects of different thiols on the activity of soluble guanylate cyclase under anaerobic conditions which were close to pO_2 values in living cells. We show that GSH is a specific activator of soluble guanylate cyclase under these conditions and that this effect is inhibited by structurally related reduced and oxidized thiols.

MATERIALS AND METHODS

Diethylaminoethyl cellulose (DE 32) was obtained from Whatman (Maidstone, England). Blue Sepharose CL-6B was prepared by a modification of the procedure described by Böhme et al. (17) and contained 1 µmol of Cibachron Blue F3G-A per ml of settled Sepharose. Sepharose CL-6B was obtained from Pharmacia (Freiburg, F.R.G.). Cibachron Blue F3G-A was from Ciba-Geigy (Lörrach, F.R.G.), GSH from Serva (Heidelberg, F.R.G.) and Sigma (Deisenhofen, F.R.G.). S-Methyl-GSH was from Sigma. GSSG, reduced coenzyme A, cysteine, cystine and DTT were purchased from Serva. Oxidized DTT was from Calbiochem (Frankfurt, F.R.G.) and 2-mercaptoethanol from Roth (Karlsruhe, F.R.G.) and SNP was from Merck (Darmstadt, F.R.G.).

Purification of soluble guanylate cyclase. Soluble guanylate cyclase was purified from bovine lung according to the method described by Gerzer et al. (4) with minor modifications. The final purification step, i.e. preparative polyacrylamidegel electrophoresis, was performed under anaerobic conditions (pO_2 < 6 Torr) without addition of a thiol. The purified enzyme was stored under nitrogen in 50 % glycerol (v/v) at -70 °C. Some experiments were carried out with a purified enzyme preparation that had been obtained under air with the addition of 50 mM 2-mercaptoethanol during the last step of purification and storage of the enzyme. The purity of the enzyme preparations was assessed by Coomassie blue stained sodium dodecyl sulfate gel electrophoresis. Enzyme activity was stable over a period of 6 months.

Determination of guanylate cyclase activity. Soluble guanylate cyclase activity was determined in a total volume of 0.1 ml of 50 mM triethanolamine/HCl-buffer, pH 7.4, containing 0.1 mM [α-^{32}P]GTP (about 0.5 µCi per tube), 3.0 mM $MgCl_2$ or $MnCl_2$ when indicated, 0.1 mg/ml γ-globuline, thiols and SNP when indicated. The reaction was started by the addition of the enzyme and was conducted for 10 minutes at 16-18 °C (for technical reasons). Some experiments were performed at 37 °C. The reaction was stopped by the addition of 0.4 ml of 120 mM zinc acetate, followed by 0.5 ml of 120 mM sodium carbonate. The isolation of formed ^{32}P-cGMP and calculation of guanylate cyclase activity was as described previously (18). Incubations under reduced oxygen tension were performed in a glovebox. The glovebox and all solutions were evacuated and then gassed with nitrogen (99.99 % N_2) three times. Oxygen tension inside the glovebox and in solutions was determined with an oxygen electrode. pO_2 inside the glovebox was kept below 6 Torr throughout the experiments. [α-^{32}P]GTP was prepared according to Johnson and Walseth (19). Protein was determined according to Peterson (20). Experiments were performed at least twice and in triplicates, representative results are shown.

RESULTS

Under aerobic conditions, the activity of soluble guanylate cyclase was increased by the addition of different thiols to the incubation mixture. Maximal activation of the enzyme was observed at about 3 mM for each thiol tested. GSH lead to a 16-fold increase in enzyme activity. Cysteine and DTT enhanced basal enzyme activity 4- to 5-fold. GSSG lead only to a slight increase in enzyme activity. Under anaerobic conditions in the absence of a thiol, basal enzyme activity was 2- to 3-fold higher than under aerobic conditions. GSH lead to a 40-fold activation of basal enzyme activity. GSSG did not affect the enzyme activity. Cysteine activated the enzyme 7-fold, DTT less than 2-fold (Table 1). In the presence of Mn-GTP instead of Mg-GTP as the substrate, guanylate cyclase activity was increased 4-fold, GSH activated the enzyme only 5- to 6-fold and maximal enzyme activity in the presence of GSH was 2-fold less than with Mg-GTP. Stimulation of soluble guanylate cyclase by GSH appeared to be biphasic with half-maximal and maximal (50-fold) activation at 0.5 and 1 to 3 mM, respectively. In the presence of DTT, the enzyme was stimulated only 2.5-fold (Fig. 1). Enzyme stimulation by the NO-containing compound SNP was additive towards the stimulation with GSH (Table 2). S-Methyl-GSH and cysteine at 10 mM activated soluble guanylate cyclase 3- to 4-fold and 10- to 20-fold, respectively. Reduced coenzyme A and 2-mercaptoethanol activated soluble guanylate cyclase up to 3-fold. Like GSSG, the oxidized thiols, cystine and oxidized DTT had no effect on the enzyme activity at concentrations up to 0.3 mM under anaerobic conditions.
The stimulatory effect of GSH (80-fold) was inhibited by S-methyl-GSH, GSSG and cysteine at increasing concentrations. S-Methyl-GSH and cysteine did apparently not affect the K_a-value for GSH but decreased V_{max}. The effect of GSH was almost completely inhibited in the presence of 3 mM cysteine. S-Methyl-GSH was less effective as an inhibitor of GSH-induced enzyme activa-

Table 1: Effects of various thiols on the activity of soluble guanylate cyclase under aerobic and anaerobic conditions

thiol	aerobic	anaerobic
	guanylate cyclase activity nmoles cGMP x min^{-1} x mg^{-1}	
none	0.5	1.3
GSH	7.9	54.0
GSSG	0.7	1.4
cysteine	2.3	8.8
DTT	2.3	2.3

The activity of soluble guanylate cyclase was determined under aerobic and anaerobic conditions at 16-18 °C without and after addition of various thiols (3 mM) as described under Methods. The enzyme preparation contained 50 mM 2-mercaptoethanol and was diluted 1,000-fold in the incubation mixture.

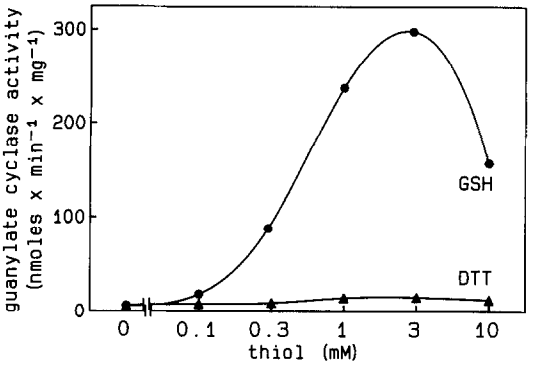

Fig. 1. Effects of GSH and DTT on the activity of soluble guanylate cyclase. The activity of soluble guanylate cyclase was measured in the presence of various concentrations of GSH and DTT under anaerobic conditions. The incubation temperature in this experiment was 37 °C. The enzyme preparation contained no added thiols.

tion. GSSG shifted the dose response curve to the right, and V_{max} was not reached even at 3 mM GSH (Fig. 2).

DISCUSSION

Previous studies have shown that thiols protect soluble guanylate cyclase from oxidative activation and inactivation (1-5). None of these studies has shown any specifity for different thiols. In addition, no attempt has been taken to rule out the possibility of other effects of thiols. Almost all results were obtained under aerobic conditions where the pO_2 value by far exceeds physiological values in living cells. The present study suggests that GSH plays a specific role in the regulation of soluble guanylate cyclase activity in vivo.

Under air, the enzyme activity was enhanced by various thiols with GSH beeing most effective. Basal enzyme activity was higher under anaerobic conditions. GSH stimulated the enzyme dose dependently up to 80-fold. This effect was additive with the stimulatory effect of SNP. The high stimulating effect of

Table 2: Effects of GSH and SNP on the activity of soluble guanylate cyclase under anaerobic conditions

addition	guanylate cyclase activity nmoles cGMP x min^{-1} x mg^{-1}
none	0.9
GSH	44.2
SNP	139.3
GSH and SNP	192.2

The activity of soluble guanylate cyclase was determined under anaerobic conditions and at 16-18 °C with no additions or after the addition of 3 mM GSH or/and 0.1 mM SNP. The enzyme preparation contained no added thiols.

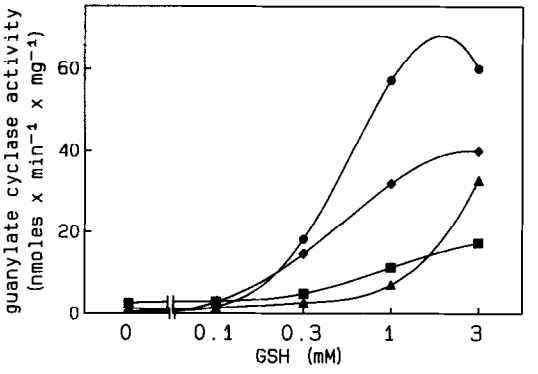

Fig. 2. Inhibition of GSH-activated soluble guanylate cyclase by S-methyl GSH, GSSG and cysteine. The effect of GSH (●) and the inhibitory effect of 3 mM S-methyl-GSH (◆), GSSG (▲) and cysteine (■) on GSH-activated soluble guanylate cyclase was determined under anaerobic conditions at 16-18 °C. The enzyme preparation contained no added thiols.

GSH was not seen under air or in the presence of Mn-GTP as substrate, indicating that the effect of GSH is sensitive towards oxidation. DTT was the strongest reducing thiol we tested. The ability of thiols to activate soluble guanylate cyclase did not correlate with their redox potential. These findings are contrary to the view that GSH does only function to protect soluble guanylate cyclase from oxidative modulation. The different stimulatory effects of thiols on soluble guanylate cyclase are not caused by the oxidation products of these thiols, since GSSG, oxidized DTT and cystine showed no effect on the activity of soluble guanylate cyclase under anaerobic conditions.

The free SH-group appears to be essential for enzyme stimulation by GSH, since S-methyl-GSH showed only a 3- to 4-fold stimulation and was the weakest antagonist of GSH-mediated activation studied. On the other hand, cysteine alone showed a much weaker effect than GSH and even antagonized the effect seen with GSH. Reduced coenzyme A showed only a 2-fold activation of soluble guanylate cyclase activity, indicating that the effect seen with GSH is not solely related to the greater molecular size.

Under our experimental conditions, the stimulatory effect of the various thiols varied by a factor of two. Whether this variation was due to the variation of the pO_2 (between 1 and 6 Torr) could not be evaluated. However, the stimulatory effect of GSH on soluble guanylate cyclase was comparable with two seperate preparations. We cannot exclude the possibility that both preparations contained a minor protein contamination. This contamination could be an enzyme which metabolizes GSH and mediates the activation of soluble guanylate cyclase by GSH. Thus, it is possible that we observed an indirect effect of GSH on soluble guanylate cyclase activity.

If we assume that GSH interacts directly with a domain on soluble guanylate cyclase containing critical thiol groups, S-methyl-GSH and GSSG may competitively occupy this domain. Their surface shape resembles GSH, but they lack a free SH-group. On the other hand, cysteine possesses a free SH-group but may not fulfill the stereochemical requirements necessary for enzyme activation. One may speculate that the interaction of GSH with soluble guanylate cyclase requires reduced SH-groups on either side, enzyme and thiol, since the effect of GSH is strongly attenuated under air and in the presence of manganese. This may explain, why previous investigations failed to show specifity for glutathione in the regulation of soluble guanylate cyclase.

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