inflammatory drugs—1. *Biochem Pharmacol* **38**: 4389–4395, 1989.
4. Williams K, Day R, Knihinicki R and Duffield A, The stereoselective uptake of ibuprofen enantiomers into adipose tissue. *Biochem Pharmacol.* **35**: 3403–3405, 1986.
5. Kawashima Y, Hirose A and Kozuka H, Modification by clofibric acid of acyl composition of glycerolipids in rat liver. *Biochim. Biophys. Acta* **795**: 543–551, 1984.
6. Mentlein R, Lembke B, Vik H and Berge RK, Different induction of microsomal carboxylesterases, palmitoyl-CoA hydrolase and acyl-L-carnitine hydrolase in rat liver after treatment with clofibrate. *Biochem. Pharmacol.* **35**: 2727–2730, 1986.
7. Kawashima Y, Katoh H and Kozuko H, Differential effects of altered hormonal state on the induction of acyl-CoA hydrolases and peroxisomal β-oxidation by clofibric acid. *Biochim. Biophys. Acta* **750**: 365–372, 1983.
8. Skrede S and Halvorsen O, Increased biosynthesis of CoA in the liver of rats treated with clofibrate. *Eur J Biochem* **98**: 223–229, 1979.
9. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
10. Sallustio BC, Abas A, Hayball PJ, Purdie YJ and Meffin PJ, Enantiospecific high performance liquid chromatographic analysis of 2-phenylpropionic acid, ketoprofen and fenoprofen. *J Chromatogr* **374**: 329–337, 1986.
11. Krisans SK, Mortensen RM and Lazarow PB, Acyl-CoA synthetase in rat liver peroxisomes. *J Biol Chem* **255**: 9599–9607, 1980.
12. Gill JL, *Design and Analysis of Experiments in Animal & Medical Sciences.* Iowa State University Press, Iowa, 1981.
13. Groot PHE, Scholte HR and Hulsmann WC, Fatty acid activation: specificity, localization and function. *Adv Lipid Res* **14**: 75–126, 1976.
14. Caldwell J, Hutt AJ and Fournel-Gigleux S, The metabolic chiral inversion and dispositional enantioselectivity of the 2-arylpropionic acids and their biological consequences. *Biochem Pharmacol* **37**: 105–114, 1988.
15. Sallustio BC, Knights KM and Meffin PJ, The stereospecific inhibition of endogenous triacylglycerol synthesis by fenoprofen in rat isolated adipocytes and hepatocytes. *Biochem Pharmacol* **40**: 1414–1417, 1990.

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Thiol-independent stimulation of soluble guanylate cyclase by NO-containing compounds

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NO-containing compounds are in clinical use for the treatment of coronary heart disease and arterial hypertension. Among these substances are the metal–nitroso complex sodium nitroprusside (SNP*), the acyl-sydnonimine molsidomine and various organic nitrates as glycerol trinitrate. Like the recently identified endothelium-derived relaxing factor [1] they induce vasorelaxation and inhibition of platelet aggregation by activating soluble guanylate cyclase (sGC, EC 4.6.1.2.), which catalyses the formation of cyclic GMP [2]. The active metabolites of the NO-containing compounds however, are still not defined. Thiols could mediate the generation of active metabolites of these compounds. Activation of sGC by organic nitrates requires the addition of specific thiols, preferably cysteine [3]. *S*-Nitrosothiols have been suggested as general active intermediates of NO-containing compounds [4, 5], though findings of others are contrary [6]. NO is another candidate [7, 8]. It is possible that various NO-containing compounds generate various active intermediates [9].

Thiols can influence the responsiveness of sGC for NO-containing compounds, since the enzyme is activated and inactivated upon oxidation with a concomitant loss of responsiveness [10–13]. The activation of sGC by SNP leads to an increase in the amount of reactive thiol groups of the enzyme [14]. Till now, no direct evidence has been given that NO-containing compounds can activate sGC in the absence of thiols. We studied the influence of the NO-containing compounds SNP and 3-(4-thiomorphlinyl)-*S*,*S*-dioxide-sydnonimine (C 78 0698) on a thiol free sGC preparation under anaerobic conditions (pO_2 below 6 Torr). This enabled us to demonstrate a thiol independent activation of sGC by these NO-containing compounds.

Materials and Methods

Materials. Reduced glutathione (GSH) and dithiothreitol (DTT) were purchased from Serva (Heidelberg, F.R.G.), C 78 0698 was from Cassella AG (Frankfurt, F.R.G.), SNP was from Merck (Darmstadt, F.R.G.).

Determination of guanylate cyclase activity. sGC activity was determined in a total volume of 0.1 mL with 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 $MgCl_2$, 0.1 mg/mL γ-globulin and thiols when indicated. The reaction was started by the addition of the enzyme and was performed for 10 min at 16–18° (for technical reasons). Some experiments were performed at 37°. The reaction was stopped by the addition of 0.4 mL 120 mM zinc acetate, followed by 0.5 mL 120 mM sodium carbonate. The isolation of [^{32}P]cGMP and calculation of sGC activity was as described previously [15]. SNP and C 78 0698 were dissolved only prior to use. Incubations under reduced oxygen tension were performed in a glovebox. The glovebox and all solutions were evacuated and then gassed with nitrogen (99.9%) three times. pO_2 inside the glovebox and

* Abbreviations: SNP, sodium nitroprusside; C 78 0698, 3-(4-thiomorpholinyl)-*S*,*S*-dioxide-sydnonimine; sGC, soluble guanylate cyclase; DTT, dithiothreitol; GSH, reduced glutathione.

in solutions was determined with an oxygen electrode. pO_2 inside the glovebox was kept between about 1 to 6 Torr throughout the experiments. [α-^{32}P]GTP was prepared according to Johnson and Walseth [16]. Protein was determined according to Peterson [17]. Experiments were performed in triplicates, representative results are shown. Apparent K_a values are given as mean ± SEM from three separate experiments.

Purification of soluble guanylate cyclase. Bovine lung sGC was purified according to the method described by Gerzer *et al.* [18] with minor modifications. The final purification step, i.e. preparative polyacrylamide gel electrophoresis, was performed under anaerobic conditions (pO_2 below 6 Torr) without addition of a thiol. Purified sGC was stored under nitrogen in 50% glycerol (v/v) at −70°. The enzyme preparation was stable over a period of 6 months.

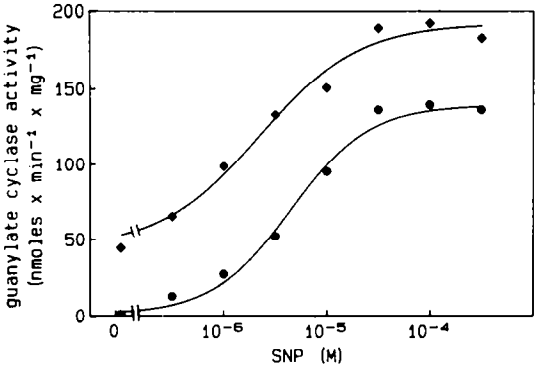

Fig. 1. Stimulation of sGC by SNP and the effect of GSH on this activation. The activity of sGC was determined at various concentrations of SNP in the absence of added thiols (●) or after the addition of 3 mM GSH (◆). Experiments were carried out under anaerobic conditions at 16–18°.

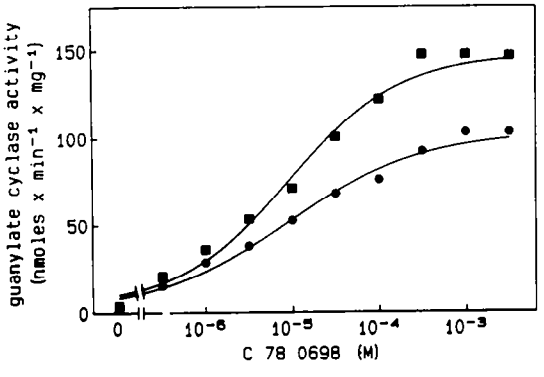

Fig. 2. Stimulation of sGC by C 78 0698 and the effect of DTT on this activation. Guanylate cyclase activity was determined at various concentrations of C 78 0698 in the absence of thiols (●) or after the addition of 3 mM DTT (■). Experiments were carried out under anaerobic conditions at 16–18°.

Results and Discussion

SNP contains a free NO-group and interacts with various redox-reactive substances, including thiols [19]. In order to exclude an involvement of thiols in the generation of the active intermediate, even the presence of small amounts of thiols had to be avoided. Therefore, the last step of enzyme purification (polyacrylamide gel electrophoresis) was carried out without the addition of thiols. The final incubation mixture still contained protein linked thiols. Though, it seems very unlikely that these thiols could significantly influence the stimulation of sGC by NO-containing compounds.

However, sGC had to be protected from oxidative inactivation. In these experiments, purification, storage and assays of sGC activity were conducted under nitrogen at a pO_2 below 6 Torr throughout the experiments and no thiols were added unless indicated.

SNP activated this preparation of sGC at increasing concentrations under anaerobic conditions (Fig. 1). In the absence of added thiols, SNP stimulated sGC at concentrations below 1 μM. The enzyme was activated about 150-fold at 10–30 μM of SNP, the apparent K_a value was 3.4 ± 1.0 μM. The addition of 3 mM GSH increased basal enzyme activity about 50-fold. This effect was additive towards the stimulation of sGC by SNP. GSH did not affect the apparent K_a value for SNP (2.8 ± 1.5 μM) and maximal enzyme activity increased by the amount of GSH dependent enzyme activation (Fig. 2). This effect of GSH is reported in further detail elsewhere [20].

The sydnonimine C 78 0698 activated sGC under anaerobic conditions 100-fold at concentrations between 30 and 100 μM in the absence of added thiols (Fig. 2). An activation of the enzyme was seen at concentrations below 1 μM. The apparent K_a value was at 8.1 ± 3.4 μM. DTT had no effect on this value (8.7 ± 3.7 μM) and increased the maximal activity of sGC after activation by C 78 0698 slightly (Fig. 2). Stimulation of sGC activity by GSH was additive towards the stimulation by C 78 0698 (data not shown). Although DTT is a stronger reducing agent than GSH, it stimulated sGC activity only up to 5-fold.

Both substances, SNP and C 78 0698, were tested with various thiols such as GSH, DTT and cysteine. None of these thiols had a significant influence on the activation of sGC by these compounds. Our results show that substances, which possess a free NO-group (such as SNP), or can generate a metabolite with a free NO-group independent from thiols (such as sydnonimines), stimulate sGC independent from thiols. There is direct evidence against the hypothesis that *S*-nitrosothiols are the general active intermediates for NO-containing substances.

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REFERENCES

1. Palmer RMJ, Ferrige AG and Moncada S, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**: 524–526, 1987.
2. Waldman SA and Murad F, Cyclic GMP synthesis and function. *Pharmacol Rev* **39**: 163–196, 1987.
3. Ignarro LJ and Gruetter CA, Requirement of thiols for activation of coronary arterial guanylate cyclase by

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glyceryl trinitrate and sodium nitrite. *Biochim Biophys Acta* **631**: 221–231, 1980.
4. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ and Gruetter CA, Mechanisms of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of *S*-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* **218**: 739–749, 1981.
5. Ignarro LJ, Kadowitz PJ and Baricos WH, Evidence that regulation of hepatic guanylate cyclase activity involves interactions between catalytic site -SH groups and both substrate and activator. *Arch Biochem Biophys* **208**: 75–86, 1981.
6. Yeates RA, Laufen H and Leitold M, The reaction between organic nitrates and sulfhydryl compounds: a possible model system for the activation of organic nitrates. *Mol Pharmacol* **28**: 555–559, 1985.
7. Katsuki S, Arnold W, Mittal C and Murad F, Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cycl Nucl Res* **3**: 23–35, 1977.
8. Feelisch M and Noack E, Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* **139**: 19–30, 1987.
9. Böhme E, Grossmann G, Herz J, Mülsch A, Spies C and Schultz G, Regulation of cyclic GMP formation by soluble guanylate cyclase. Stimulation by NO-containing compounds. *Adv Cycl Nucl Prot Phos Res* **17**: 259–266, 1984.
10. Böhme E, Graf H and Schultz G, Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic GMP formation in smooth muscle and platelets. *Adv Cycl Nucl Res* **9**: 131–143, 1978.
11. Craven PA and DeRubertis FR, Effects of thiol inhibitors on guanylate cyclase activity. Evidence for the involvement of vicinal dithiols in the expression of basal and agonist-stimulated activity. *Biochim Biophys Acta* **524**: 231–244, 1978.
12. Brandwein HJ, Lewicki JA and Murad F, Reversible inactivation of guanylate cyclase by mixed disulfide formation. *J Biol Chem* **256**: 2958–2962, 1981.
13. Braughler JM, Soluble guanylate cyclase activation by nitric oxide and its reversal. *Biochem Pharmacol* **32**: 811–818, 1983.
14. Kamisaki Y, Waldmann SA and Murad F, The involvement of catalytic site thiol groups in the activation of soluble guanylate cyclase by sodium nitroprusside. *Arch Biochem Biophys* **251**: 709–714, 1986.
15. Schultz G and Böhme E, Guanylate cyclase. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), Vol. 3, pp. 379–389. Verlag Chemie, Weinheim, 1984.
16. Johnson RA, and Walseth TF, The enzymatic preparation of (alpha-^{32}P) ATP, (alpha-^{32}P) GTP, adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterase. *Adv Cycl Nucl Res* **10**: 135–167, 1979.
17. Peterson GL, A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* **83**: 346–356, 1977.
18. Gerzer R, Hofmann F and Schultz G, Purification of soluble, sodium-nitroprusside-stimulated guanylate cyclase from bovine lung. *Eur J Biochem* **116**: 479–486, 1981.
19. Leeuwenkamp OR, Van Bennekom WP, Van Der Mark EJ and Bult A, Nitroprusside, antihypertensive drug and analytical reagent. *Pharmaceut Weekbl-Sci Ed* **6**: 129–140, 1984.
20. Niroomand F, Rössle R, Mülsch A and Böhme E, Under anaerobic conditions, soluble guanylate cyclase is specifically stimulated by glutathione. *Biochem Biophys Res Commun* **161**: 75–80, 1989.