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Explanation of the discrepancy between the degree of organic nitrate decomposition, nitrite formation and guanylate cyclase stimulation

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We continuously studied the quantitative formation of nitric oxide (NO), nitrite and nitrate ions from several organic nitrate esters in the presence of various thiol-containing compounds by spectroscopy and HPLC. The results indicate that there are different pathways of decomposition depending on the chemical nature of the mercaptan tested. The amino acid cysteine is known to function as an essential cofactor for guanylate cyclase activation by organic nitrates in vitro. *For comparison we investigated several structural analogues with respect to their nitric oxide or nitrite ion releasing potency. Both were found to represent the main products resulting from nitrate ester breakdown besides the respective alcohols. We found that only those compounds were able to activate the enzyme in the presence of nitroglycerin (GTN) which induce the release of NO as well. On the other hand, nearly all other thiols tested caused an* in vitro *decomposition of organic nitrates by producing excess nitrite and the corresponding disulfide without the formation of NO. Thus, the decomposition of organic nitrates to nitrite ions does not contribute at all to activation of guanylate cyclase. Our results confirm that the liberation of nitric oxide is the common principle of action for all nitrovasodilators. In addition, our results suggest that the thiol consuming transformation of organic nitrates into nitrite ions (ratio NO/nitrite 1:10) may lead to a depletion of cysteine stores, resulting in a decreased formation of NO and, consequently, in a decrease of guanylate cyclase activation, clinically arising as nitrate tolerance.*

Introduction

Organic nitrates have remained useful agents for the management of several cardiovascular diseases since they were introduced into clinical therapy over a hundred years ago. Their therapeutic effect is due mainly to a preload reduction caused by venous pooling. Today, there is clear evidence that the pharmacodynamic action is mediated by the second messenger cyclic GMP, following the activation of the enzyme guanylate cyclase. Subsequently, the increase in cyclic GMP activates cyclic GMP-dependent protein kinases which may result finally in a decreased phosphorylation status of myosin light chains, followed by relaxation of vascular smooth muscle[1]. The exact mechanism, however, is still a matter of some debate. Previous investigations have shown that activation of guanylate cyclase by organic nitrates in a cell-free system requires the presence of the amino acid cysteine[2].

Organic nitrates are known to be rapidly degraded into nitrite ions non-enzymatically in the presence of glutathione or cysteine[3]. The enzymatic pathway of metabolism is catalyzed by 'glutathione-S-transferase'[3] and the so-called 'nitrate-forming enzyme'[4]. During the course of these reactions nitrite and nitrate ions, respectively, are released as well. It is, therefore, generally accepted that organic nitrates have to be metabolized before they can become effective. The metabolites generated such as thionitrites, nitroso-haem-complexes, nitrite ions and nitric oxide free radicals are thought to be potential mediators of enzyme stimulation[5,6,7,8]. A scheme of possible intracellular pathways is given in Fig. 1.

The objective of our study was to find out which one of these metabolites may be the common mediator responsible for guanylate cyclase activation. We, therefore, examined the enzymatic and non-enzymatic pathways of nitrate degradation.

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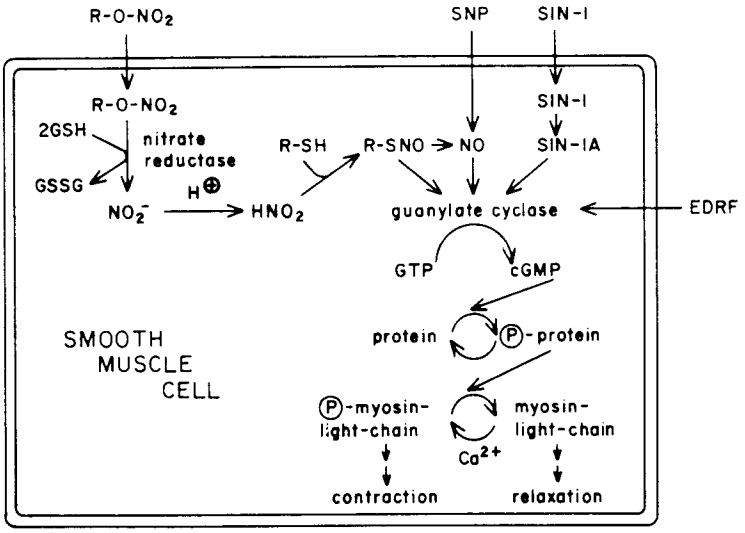

Figure 1 Scheme of possible intracellular pathways leading to smooth muscle relaxation, ($RONO_2$—organic nitrates; SNP—sodium nitroprusside; SIN-1—active metabolite of molsidomine; EDRF—endothelium derived relaxing factor).

Materials and methods

GC ASSAY

Preparation of soluble guanylate cyclase from rat liver and assay conditions have already been described[9].

NO MEASUREMENT

The method is based on the instantaneous oxidation of an oxyhaemoglobin solution to methaemoglobin by the nitric oxide formed. The continuous registration is performed at two different wavelengths given by the maximum of the extinction difference between oxyhaemoglobin and methaemoglobin and the isobestic point in dependence on the time. The gradient of the resulting straight-line is a measure of the velocity of NO-liberation. This method is highly sensitive of all nitrogen oxides specific for NO and is not disturbed by molecular oxygen and nitrite ions. For more detailed information refer to Feelisch and Noack[10]. Reaction selectivity had recently been confirmed by chemiluminescence[11].

NITRITE/NITRATE-MEASUREMENT

We used a modified high pressure liquid chromatography (HPLC) method described by Leuenberger *et al.*[12]. The simultaneous determination of nitrite and nitrate ions was performed on an amino column using an acidic mobile phase (all conditions given in ref. [10]).

MATERIALS

All compounds used were of analytical grade or were purified to HPLC purity prior to use. Organic nitrates tested were a kind gift of the respective pharmaceutical manufacturers.

Results

Recently, we were able to demonstrate that the non-enzymatic pathway of organic nitrate metabolism plays a much larger role in their physiological action than has been supposed up to now[9,13]. During this study, we measured the time-dependent decomposition of various nitrates in the presence of cysteine by means of HPLC and compared these results to the subsequent activation of isolated guanylate cyclase. We found a good correlation between the velocity constants of nitrate decomposition and the degree of enzyme activation. This correlation, however, held true only in the presence of cysteine.

When we measured the decomposition of nitroglycerin in the presence of other thiol compounds the results were quite different as shown in Fig. 2. In the case of glutathione, for example, we found a rapid degradation of nitroglycerin which could be increased even further by adding 30 μg of partially purified enzyme. Surprisingly enough, there was no corresponding activation of guanylate cyclase in the case of glutathione, whereas the effect of N-acetyl-

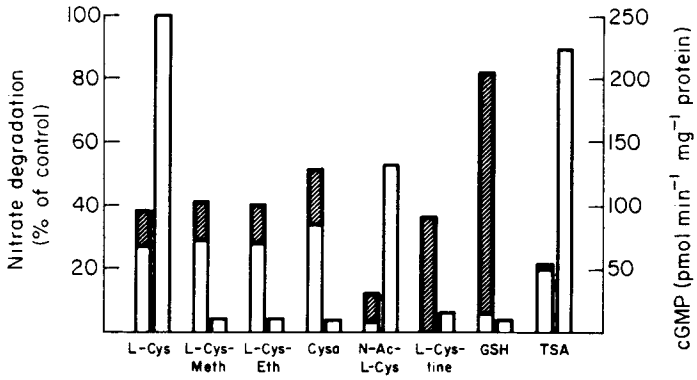

Figure 2 Comparison of both decomposition of nitroglycerin (left columns) and guanylate cyclase activation (right columns) in the presence of various thiol compounds: left columns—percentage degradation of 50 μM nitroglycerin solution in the presence of the thiols indicated on the abscissa and after further addition of 30 μg soluble guanylate cyclase (hatched portion); right columns—activation of guanylate cyclase in the presence of 50 μM nitroglycerin and 5 mM of the thiols indicated (cys—cysteine; cys-meth—cystein methyl ester; cys-eth—cysteine ethyl ester; cysa—cysteamine; N-Ac-Cys—N-acetyl-cysteine; GSH—reduced glutathione; TSA—thiosalicylic acid).

cysteine was quite the opposite: we only saw a minor nitrate degradation, but at the same time there was a substantial activation of guanylate cyclase. This suggested that degradation alone may not be a parameter sufficient enough to characterize the enzyme-activating potency of organic nitrates. Thus, to achieve further clarity it was necessary to measure directly the generated metabolites nitrite and nitrate ions and nitric oxide. By choosing the same conditions for both assays, guanylate cyclase and nitrate degradation, we were able to directly compare both processes.

At first, we determined the velocity of nitric oxide release from various organic nitrates in the presence of cysteine. The concentrations for the nitrates and cysteine were 1 mM and 5 mM, respectively. For the same nitrates we determined the median effective concentration (EC_{50}) of guanylate cyclase activation (Fig. 3, Table 1). By plotting the EC_{50} values versus the reciprocal values for the nitric oxide liberation of the corresponding nitrate, we found a linear relationship with a correlation coefficient of 0·98. This means that for each organic nitrate the potency of guanylate cyclase activation increases with its ability to release NO. As should be expected from these results, we found the same degree of NO liberation for each organic nitrate when tested at its median effective concentration (EC_{50}). We were surprised to

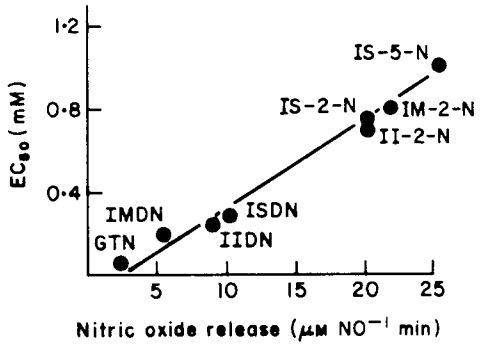

Figure 3 Correlation between nitric oxide release and enzyme activity of various organic nitrates. Conditions for NO-measurement: organic nitrates 1 mM, L-cysteine 5 mM, oxyhaemoglobin 2·5 μM; pH 7·70, 37°C, N = 8. Assay conditions for EC_{50} determination: pH 7·5, $MgCl_2$ 4 mM, GTP 1 mM, 30 μg protein, L-cysteine 5 mM, incubation time 15 min at 37°C, N = 3 (GTN—glyceryl trinitrate; IMDN—isomannide dinitrate; IIDN—isoidide dinitrate; ISDN—isosorbide dinitrate; II-2-N—isoidide 2-nitrate; IM-2-N—isomannide 2-nitrate; IS-2-N—isosorbide 2-nitrate; IS-5-N—isosorbide 5-nitrate).

find this true even in the case of SIN-1, the active metabolite of molsidomine, which so far was thought to stimulate guanylate cyclase as an intact molecule rather than by liberating NO[14]. Our results, however, clearly demonstrate that this compound

Table 1 Correlation between nitric oxide release and enzyme activity of various organic nitrates—same conditions as for Fig. 3, except that organic nitrates were tested at their EC_{50} concentrations indicated

Organic nitrate + cysteine (5 mM)	EC_{50} value (mM)	Nitric oxide release ($\mu M\ min^{-1}$)
ETN	0·145	0·046 ± 0·002
GTN	0·069	0·050 ± 0·005
IMDN	0·200	0·045 ± 0·003
IIDN	0·242	0·047 ± 0·004
ISDN	0·280	0·046 ± 0·002
IS-2-N	0·750	0·047 ± 0·004
IS-5-N	1·000	0·045 ± 0·003
SIN-1 (without cyteine)	0·0028	0·054 ± 0·007

decomposes rapidly into NO and other metabolites even in the absence of cysteine. This further indicates that the formation or liberation of nitric oxide is the common principle of action for all nitrovasodilators. When we added oxyhaemoglobin, which is known to function as an effect and rapid scavenger of nitric oxide, to our GC assays we noticed a substantial, dose-dependent rightward shift of the concentration–response curves for nitroglycerin, SIN-1 and sodium nitroprusside. Activation was completely inhibited at 10μM oxyhaemoglobin.

When investigating the thiol-induced decomposition of organic nitrates additionally into nitrite ions, we surprisingly found two mutually independent pathways depending on the structure of the thiols tested: nearly all of the 54 thiol-compounds examined were able to decompose organic nitrates to nitrite ions and the corresponding alcohols, but only three compounds were able to release nitric oxide as well. These were the same compounds which were also effective as costimulators at the enzyme guanylate cyclase, namely cysteine, N-acetyl-cysteine and thiosalicylic acid[9]. Hence, it becomes clear that the decomposition of organic nitrates to nitrite ions does not contribute at all to the activation of guanylate cyclase by organic nitrates. Moreover, many of the thiols tested were even more effective in nitrite liberation than cysteine, but at the same time they did not show any effect on guanylate cyclase activation. On the other hand, the release of nitric oxide compared to cysteine correlated with enzyme activation in each case. For example, cysteamine or thiophenol induced a substantially higher release of nitrite ions from nitroglycerin, whereas the nitrite releasing potency of N-acetyl-cysteine, for example, is only 16% of that found in the case of cysteine. Yet both compounds produce about the same amount of NO when incubated with nitroglycerin. These observations are in good agreement with studies performed by Axelsson and Karlsson in 1984[15], who showed that the induction of tolerance by nitroglycerin does not change the rate of nitrite production. Thus, it seems plausible that tolerance is associated with a decrease in NO release rather than with a decrease in formation of nitrite ions.

We systematically examined different structural analogues of cysteine and thiosalicylic acid and found that the potency of both the nitric oxide and the nitrite releasing properties were connected with the pKa value of the mercapto group and also correlated with the relative nucleophilicity of the thiolate anions. Obviously, a free mercapto group is essential for both pathways. An additional carboxylic function in a distinct spatial orientation towards the sulphur has to be present for a significant nitric oxide liberation. The distance of the two carbon atoms between carboxylic function and mercapto sulphur alone does not seem to be sufficient enough to cause a considerable NO release, for 3-mercaptoproprionic acid and 2-mercaptonicotinic acid are both ineffective. Apart from certain sterical requirements, electrochemical parameters also seem to be important. The splitting-up into two decomposition pathways, the nitrite and the nitric oxide pathway, could also be observed when the dependence of both processes on the concentration of cysteine was investigated. In the case of nitrite ion liberation from nitroglycerin a linear relationship was found between the concentration of cysteine and the amount of nitrite ions released. The correlation was linear at a concentration between 10 μM and 100 mM cysteine.

For the release of nitric oxide we could determine a maximal effect which was obtained at about 20 mM, flattening at higher concentrations. Thus, the kinetics of nitric oxide liberation in the presence of cysteine are very similar to the kinetics of guanylate cyclase activation. In both systems the threshold concentration of cysteine is 0·2 mM and the maximal effect is reached at 20 mM. Even the calculated EC_{50} values for both effects are almost identical (Fig. 4).

Our results, furthermore, suggest that methylene blue inhibits activation of guanylate cyclase by decreasing the release of nitric oxide or by a possible interaction with the haem moiety of the target enzyme. This can also be demonstrated as rightward shift of the dose–response curves for each organic nitrate[10].

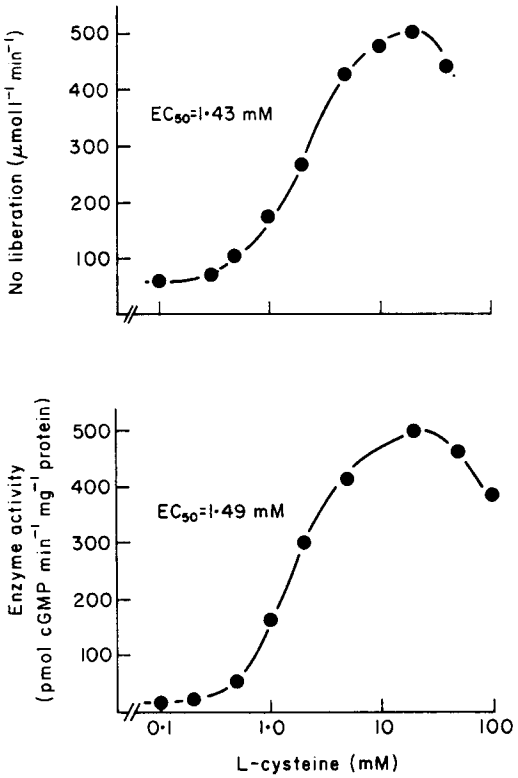

Figure 4 Dependence of enzyme activity and nitric oxide release from the concentration of cysteine in the presence of nitroglycerin: enzyme assay: pH 7·70, $MgCl_2$ 4 mM, GTP 1 mM, 30 μg protein, nitroglycerin 50 μM, L-cysteine 1–100 mM, incubation time 15 min at 37°C; $N = 3$; NO measurement: pH 7·70, nitroglycerin 1 mM, L-cysteine 1–40 mM, oxyhaemoglobin 2·5 μM, 37°C; $N = 4$.

Discussion

Our results clearly demonstrate the importance of non-enzymatic NO liberation as the main pathway of guanylate cyclase activation by organic nitrates *in vitro*. On the other hand we have shown that the reductive hydrolysis to nitrite ions, although it is the main pathway of decomposition, is not linked with the activation of guanylate cyclase and, therefore, cannot be responsible for the vasodilating action of this class of compounds. It is conceivable that the non-enzymatic degradation of organic nitrates may play an important role in guanylate cyclase activation even in intact tissue. However, we cannot preclude that the *in vivo* enzymatic metabolism of organic nitrates may result in the formation of nitric oxide as well. Recently Bennett *et al.*[16] and Schröder *et al.*[17] showed that in isolated blood vessels and cultured cells organic nitrates increased cGMP levels in a stereoselective manner. However, stereoselectivity was lost when guanylate cyclase activation was measured in a broken cell system. This suggests that *in vivo* nitrate metabolism and subsequent guanylate cyclase activation may be mediated by an as yet unidentified stereoselective enzyme or influenced by a stereoselective membrane penetration. But, whatever pathway of metabolism is more important *in vivo*, our studies have shown finally that activation of guanylate cyclase requires the formation of NO free radicals. In the light of these facts the formation of postulated thionitrites from nitrite ions and free cysteine[3] seems unlikely, for it is difficult to believe that nanomolar amounts of intracellularly released nitrite ions would cause any significant change in enzyme activity, when the basal level in the cell is in the micromolar range.

In addition, some of our findings may shed new light on nitrate tolerance. It is possible that the rapid and thiol-consuming transformation of organic nitrates into nitrite ions causes a severe but transient depletion of intracellular cysteine stores which have to be regenerated. During this period of regeneration the decreased level of cysteine would affect the NO pathway of nitrate metabolism as well. This would result in a decreased formation of NO, followed by a decrease in guanylate cyclase activation and smooth muscle relaxation. Kukovetz and Holzmann in 1986[18] postulated two different mechanisms of tolerance development, since coronary strips, made tolerant by nitroglycerin pretreatment, did not reveal any cross-tolerance to sodium nitroprusside and SIN-1. According to our results, this different behaviour may be due to the fact that, in contrast to organic nitrates, NO-formation from sodium nitroprusside and SIN-1 does not require the presence of thiol-containing compounds. According to the data presented we would propose the scheme of guanylate cyclase activation for nitrovasodilators shown in Fig. 5.

All nitrovasodilators have to penetrate through the membrane of smooth muscle cells as intact molecules. Organic nitrates will then be denitrated, enzymatically or thiol-mediated, into the alcohols, nitrite and nitrate anions, which can interchange with extracellular compartments. Only cysteine, either in its free form or bound to the enzyme, is able to induce an additional liberation of nitric oxide. Sodium nitroprusside releases NO spontaneously and SIN-1 by a radical separation from its ring-opened form SIN-1 A. The nitric oxide formed subsequently activates

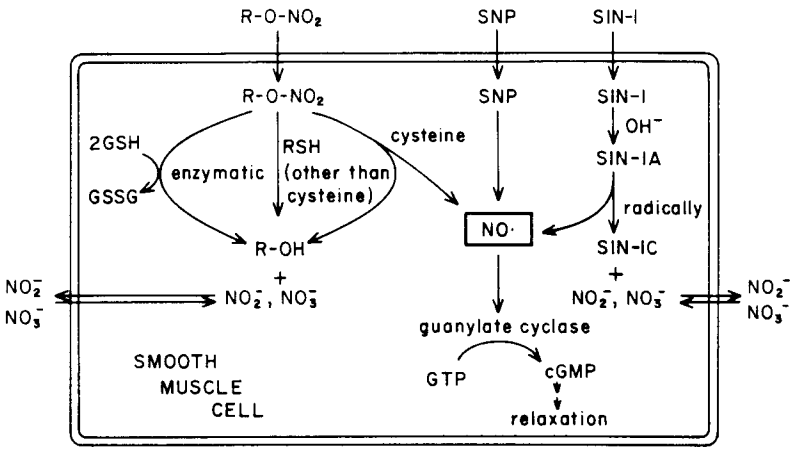

Figure 5 Proposed scheme for the activation of soluble guanylate cyclase.

the enzyme guanylate cyclase, finally resulting in smooth muscle relaxation.

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