Biochemical Pharmacology, Vol. 38, No. 11, pp. 1749–1753, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

ANTAGONISM OF GLYCEROL TRINITRATE ACTIVITY BY AN INHIBITOR OF GLUTATHIONE *S*-TRANSFERASE

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(*Received* 21 *March* 1988; *accepted* 29 *November* 1988)

Abstract—The *in vitro* spasmolytic activity of glycerol trinitrate was measured on the KCl-contraction of aorta strips from the rabbit. In the presence of sulphobromophthalein, a known inhibitor of glutathione *S*-transferase, the dose–activity curve for the nitrate was displaced to the right. Much smaller displacements were obtained with the control spasmolytic substances—papaverine and *S*-nitroso-*N*-acetylpenicillamine. It was confirmed that sulphobromophthalein inhibits glutathione *S*-transferase activity in aorta homogenates. Aorta extracts did not detectably catalyze the reaction between glutathione and sulphobromophthalein and the glutathione level was not decreased by treating the intact aorta with sulphobromophthalein. It is concluded that sulphobromophthalein acts as a specific antagonist of the spasmolytic activity of glycerol trinitrate, probably as a result of its inhibition of glutathione *S*-transferase. It thus seems probable that glutathione and glutathione *S*-transferase are involved in the pharmacological activation of the organic nitrates.

It now seems certain that the vasodilatory activity of organic nitrates is largely or totally related to increases in the levels of cyclic guanosine monophosphate (cGMP),† which are in turn caused by activation of the enzyme guanylate cyclase by nitric oxide or by *S*-nitrosothiols (reviewed in Ref. 1). The mechanisms by which organic nitrates are activated to form nitric oxide or *S*-nitrosothiols have not been studied in depth, although it has been suggested [2] that glutathione *S*-transferase could play a role. It was reasoned that, if this is indeed the case, inhibitors of glutathione *S*-transferase should act as specific antagonists of organic nitrates. In the present study this possibility has been examined, using sulphobromophthalein, a well characterized inhibitor of glutathione *S*-transferase (e.g. [3, 4]).

MATERIALS AND METHODS

1. *Materials*. Glycerol trinitrate (GTN) was purchased as a 5% solution in ethanol from Nobel (Schlebusch, F.R.G.). Sulphobromophthalein (SBP) was obtained from Sigma as the sodium salt. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was synthesized in our laboratories [5].

2. *Spasmolysis*. Helical strips were cut from the thoracic aorta of female New Zealand rabbits (2.5–3 kg) obtained from Thomae GmbH (Biberach/Riß). Four strips were suspended isotonically in Krebs–Henseleit solution at 1 g tension. After equilibration the strips were contracted with 20 mM KCl. When the state of contraction was constant SBP (10^{-4} M) was added to two of the four strips. SBP itself had no consistent effect on the state of contraction. After a further 10 min the strips were relaxed by the addition of increasing concentrations of GTN, papaverine or SNAP.

Eight to twelve strips were examined for each experiment. Levels of significance were determined with the unpaired *t*-test. Inhibition of relaxation was analysed with the help of the null equations developed by Mackay [6], which require a double reciprocal plot of each concentration of agonist against the concentration of agonist which produces the same effect in the presence of antagonist. The dissociation constant of the inhibitor is then given by $K = [I]/(S-1)$, where $[I]$ is the inhibitor concentration and S the slope. The intercept is zero for competitive inhibition and non-zero for non-competitive inhibition.

3. *Biochemical measurements*. Rabbit aorta was homogenized in 50 mM Tris–Cl, 4 mM $MgCl_2$, 10^{-4} M EDTA, 1 mM dithiothreitol, pH 7.6 buffer, using an Ultra-thorax® homogenizer (Janke and Kunkel AG). The resulting homogenate was successively centrifuged at 800 *g* and twice at 8000 *g*, each time the supernatant being used for the next step. The protein concentration in the final supernatant was then determined by dye binding [7].

Glutathione *S*-transferase activity was measured by following the increase of absorption at 340 nm, using glutathione and 1-chloro-2,4-dinitrobenzene as substrate [8]. Guanylate cyclase activity was measured by radioimmunoassay, as described in [9], with the modification that the reaction was quenched by adding an equal volume of ethanol (10 μl), followed by heating to 100° for 20 sec.

In some experiments the guanylate cyclase was activated by preincubation for 10 min at 25° with 10^{-5} M protoporphyrin IX [10].

Glutathione–SBP conjugase activity was measured as described in [8]. Glutathione levels were depleted as described in [11] with an intraperitoneal dose of DL-buthionine-*SR*-sulphoximine of 440 mg/kg.

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† Abbreviations used: cGMP, cyclic guanosine monophosphate; GTN, glycerol trinitrate; SBP, sulphobromophthalein; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

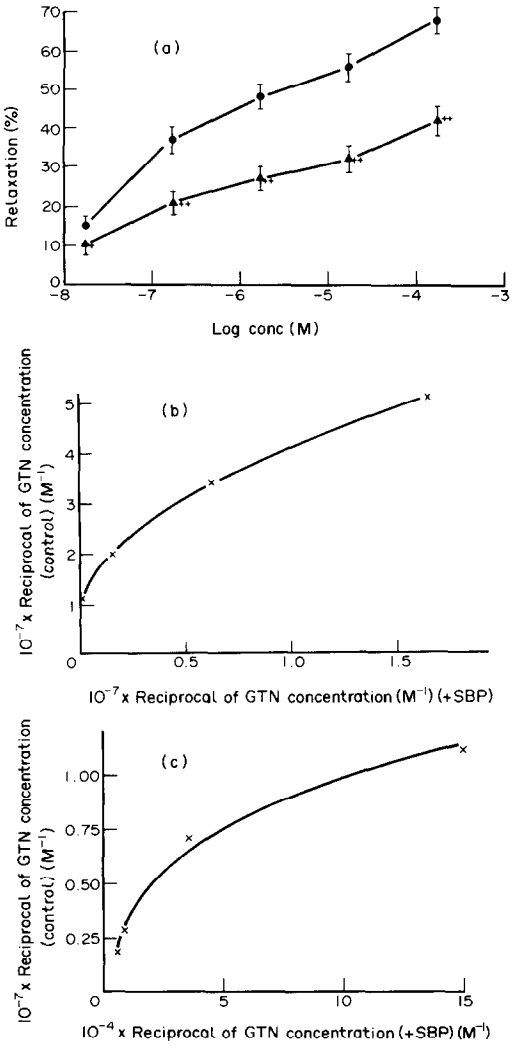

Fig. 1. (a) Relaxation induced by GTN in the presence (-▲-) and absence (-●-) of 10^{-4} M SBP (N = 12). Mean values and SEs are given $^{+}P < 0.05$, $^{++}P < 0.01$. (b) Double reciprocal plot of the concentrations of GTN which produce the same relaxation with and without the addition of 10^{-4} M SBP. (Derived from the data of Fig. 1a.) (c) As in (b), but with expanded scale.

Glutathione levels in arterial homogenates were measured with a spectrophotometric method described in [12].

RESULTS

Effect of sulphobromophthalein on the relaxation induced by GTN. From Fig. 1a it can be seen that SBP (10^{-4} M) inhibits the relaxation induced by GTN. In Figs. 1b and 1c the data are further analysed according to the method of Mackay [6], with two different scales. The plots are quite clearly non-linear, suggesting that the inhibition by SBP is neither simply competitive nor non-competitive in nature. Dissociation constants for the bound inhibitor can be estimated to range between about 150 μM (at low GTN) to about 0.5 μM (at high GTN). Significant, but less marked, inhibition was also observed with Cibachron blue, another known

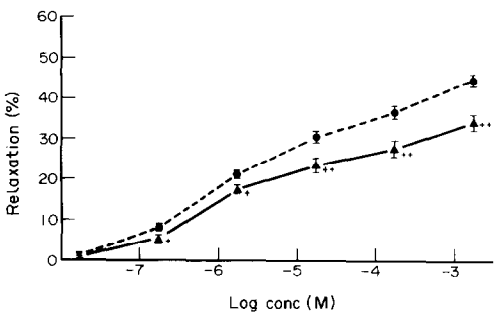

Fig. 2. Relaxation induced by GTN in the presence (-▲-) and absence (-●-) of 10^{-4} M Cibachron blue (N = 8). Mean values and SEs are given $^{+}P < 0.05$, $^{++}P < 0.01$.

inhibitor of glutathione *S*-transferase (Fig. 2) [4].

Effect of sulphobromophthalein on the glutathione S-transferase activity of rabbit aorta homogenates. Under standard conditions (1 mM 1-chloro-2,4-dinitrobenzene, 5 mM glutathione) [8] the glutathione *S*-transferase activity in the homogenates was measured as 0.217 ± 0.042 μmoles substrate converted per min per mg protein (SE; N = 6). Figure 2 shows a double reciprocal plot of the inhibition by SBP of this activity at various substrate concentrations. It can be concluded that the inhibition is not simply competitive or non-competitive, and that it is greater at lower substrate concentrations. No glutathione–SBP conjugase activity could be detected in the aorta extracts (less than 0.001 μmoles substrate converted per min per mg protein).

Measurement of glutathione levels. The glutathione content of the aorta strips was determined by a specific spectrophotometric method [12] to be 0.58 ± 0.08 nmoles/mg (SE; N = 6). After pretreatment of the intact strips with SBP the glutathione content was essentially unchanged—0.55 ± 0.07 nmoles/mg (SE; N = 6). Pretreatment of the rabbits with buthionine sulfoximine depleted the glutathione levels by about 38% to 0.36 ± 0.11 nmoles/mg (SE; N = 6), but did not significantly charge the sensitivity of the strips to GTN (not shown).

Control experiments. As the first control substance we selected papaverine, a vasodilator which is unrelated to the organic nitrates. Figure 3 shows that SBP has virtually no effect on the relaxation induced by papaverine.

Organic nitrates are believed to act by activating the enzyme guanylate cyclase [1]. An inhibitor of organic nitrate activity could therefore act by inhibiting guanylate cyclase—either the basal activity or through the specific activating site to which nitric oxide, *S*-nitrosothiols (formed from the nitrates) and the unrelated compound protoporphyrin IX are believed to bind [10]. Both of these possibilities could be eliminated. The basal activity of guanylate cyclase in the homogenates was measured as 1.45 ± 0.14 pmoles substrate formed per min per mg protein (N = 6; SE), which was not significantly different from the value in the presence of 10^{-4} M SBP—1.47 ± 0.12 pmole/min/mg (N = 6; SE). In the presence of 10^{-5} M protoporphyrin IX the guanylate cyclase activity rose to 9.93 ± 1.66 pmole/

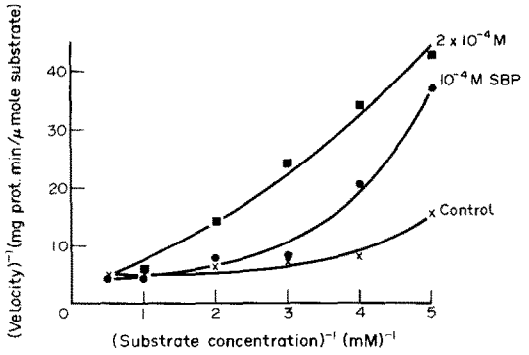

Fig. 3. Double reciprocal plot of the velocity of the glutathione *S*-transferase activity against substrate concentration (1-chloro-2,4-dinitrobenzene), and in the presence of SBP. Control (–×–); 10^{-4} M SBP (–●–); 2×10^{-4} M SBP (–■–) (N = 6).

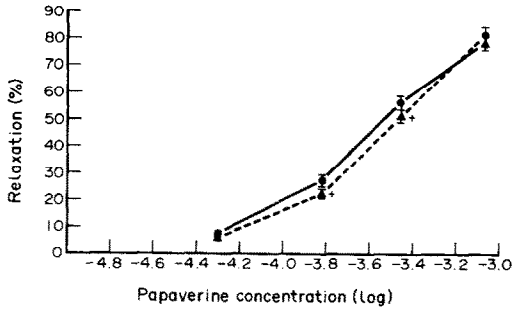

Fig. 4. Relaxation induced by papaverine in the presence (–▲–) and absence (–●–) of 10^{-4} M SBP (N = 10). Mean values and SEs are given $^{+}P < 0.05$.

min/mg, which was not significantly different from the value measured in the presence of 10^{-4} M SBP— 9.50 ± 1.10 pmole/min/mg (both N = 5; SE).

If SBP is a specific inhibitor of the steps involved in the pharmacological activation of GTN it would be predicted that it would have much less or no effect on relaxation induced by the stable *S*-nitroso derivative, SNAP, which has been suggested to act directly on the activating site of guanylate cyclase. This was indeed found to be the case, as seen by a comparison between Figs. 1a and 4a. The weak inhibition of SNAP activity can be analysed as a strict non-competitive inhibition (Fig. 4b), with an inhibitor binding constant of 145 μM (130–164 μM, 95% confidence limit). The presence of an intercept shows that the inhibition is non-competitive, rather than competitive (greater than 95% confidence limit). A comparison between Figs. 4b and 1b and 1c shows that the tight binding component of the inhibition by SBP of GTN activity is quite clearly lacking with SNAP and that the two inhibitions are evidently different in character.

DISCUSSION

It has been shown that the activity of GTN is inhibited by SBP, a known inhibitor of glutathione *S*-transferase (Fig. 1, [3, 4]). It has been confirmed that SBP inhibits the glutathione *S*-transferase activity in rabbit aorta extracts (Fig. 3). SBP had little or no effect on the activity of papaverine, a vasodilator which is unrelated to organic nitrates (Fig. 4). Organic nitrates act by forming *S*-nitrosothiols or nitric oxide which then bind to the activating site of the enzyme guanylate cyclase [1]. It has been shown that SBP does not affect the activity of guanylate cyclase, nor of its activation by protoporphyrin IX, which is thought to bind to the same site as NO and the *S*-nitrosothiols [10]. It has been shown that SBP inhibits the activity of a stable *S*-nitrosothiol, SNAP (Fig. 5). However, this inhibition is much weaker than with GTN and seems to consist of only a single phase (Figs 1 and 5). This leads to the conclusion that SBP inhibits a step in the activation of GTN which lies before guanylate cyclase, and which is not involved in the activity of SNAP. It therefore seems probable that a glutathione and glutathione *S*-transferase dependent step is present in the activation of GTN, and probably of other organic nitrates. This step could involve the reaction of glutathione with the organic nitrate itself, or with a reduced intermediate such as organic nitrite or nitric oxide. One of the several possible mechanisms could be summarized as follows

$R\text{-}ONO_2$ →	R-ONO →	Gl-SNO	→ NO
organic nitrate	organic nitrite	*S*-nitroso-glutathione	nitric oxide

It is also in principle possible that SBP acts, not by inhibiting the transferase, but by itself acting as substrate and thus depleting the levels of glutathione. This explanation would in itself also point to an involvement of glutathione and glutathione *S*-transferase in the activation of GTN, but is in any case improbable, as no SBP-glutathione conjugase activity could be detected in the aorta extracts. It is known that the rabbit has limited ability to conjugate SBP [14]. It was also established that treatment of aorta strips with SBP produced no significant decrease in the level of glutathione.

Depleting the glutathione levels by 38% produced no change in sensitivity to GTN, which suggests that an excess of glutathione is normally present.

The present results also suggest the interesting possibility that activation of SNAP involves a glutathione-dependent reaction, although this must be the subject of further research.

This is apparently the first time that glutathione *S*-transferase has been detected in blood vessel walls. Comparison with the data in [13] for other organs from the rat suggest that the specific activity of glutathione *S*-transferase in artery walls is relatively high. Data for other organs from the rabbit are unfortunately not available.

Although literally nothing is known about the isoenzymes of glutathione *S*-transferase present in blood vessel walls, it seems reasonable to assume that, as with better studied tissues, there is a complex mixture of forms with different kinetic properties. The non-linear dependencies found in Figs 1 and 3 can then be explained by the participation of at least two isoenzymes. Thus the kinetic constants for the substrate can vary between isoenzymes by at least a

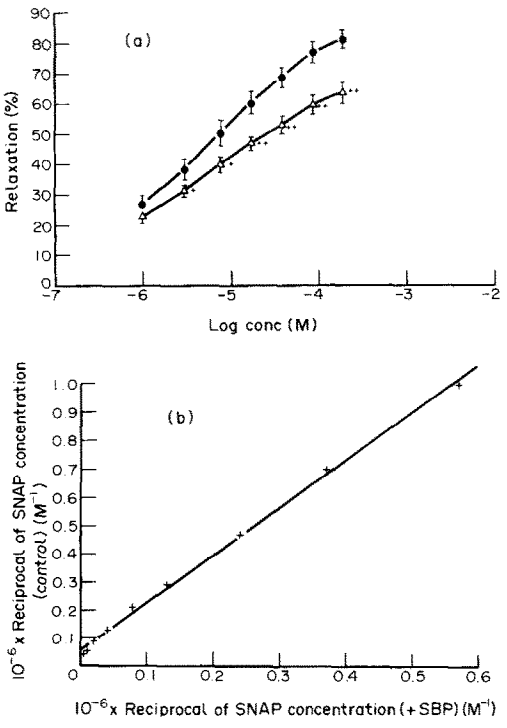

Fig. 5. (a) Relaxation induced by SNAP in the presence of (-△-) and absence (-●-) of 10^{-4} M SBP (N = 8). Mean values and SEs are given. $^{+}P < 0.05$, $^{++}P < 0.01$. (b) Double reciprocal plot of the concentrations of SNAP which produce the same relaxation with and without the addition of 10^{-4} M SBP. Derived from the data of Fig. 3 (a). Coefficient of correlation = 0.997.

factor of 10 (e.g. [15]) (Fig. 3). Published inhibition constants with SBP range between less than 1 μM and greater than 100 μM, depending on the isoenzymes examined [15, 16]. It is also known that the ability of glutathione *S*-transferase to *denitrate* organic nitrates depends by more than a factor of 100 on the isoenzyme examined [15]. Thus one possible explanation of the data in Fig. 1 would be that the isoenzymes which are most active against GTN are least susceptible to inhibition by SBP. At higher GTN concentrations isoenzymes which have lower activity against GTN begin to play a role and these are more sensitive to inhibition by SBP. It has indeed been demonstrated for glutathione *S*-transferase from dog liver that the isoenzymes with high activity against isosorbide dinitrate are those which are relatively insensitive to inhibition by SBP [15]. The inhibition of SNAP activity by SBP may involve an isoenzyme which is relatively insensitive to inhibition by SBP.

Inhibition of glutathione *S*-transferase activity by SBP and similar dyes is described in the literature as either competitive or non-competitive [17]. The data with SNAP (Fig. 5) must be interpreted in terms of a non-competitive inhibition, but no conclusion can be reached for the data with GTN (Fig. 1).

The glutathione *S*-transferase inhibitors which could be examined in this study were severely restricted by the requirement that the initial KCl-contraction remain unaffected. Thus the alkyl tin inhibitors [3] all suppressed the contraction. *S*-Hexylglutathione [4] affected neither the initial contraction nor the nitrate-induced relaxation, perhaps because it was not taken up by the arteries. On the other hand, Cibacron blue [4] also inhibited GTN activity, but to a lesser extent (Fig. 2).

Scientific opinion has been much influenced by the finding [18] that cysteine is much more effective (×34) than an equal concentration of glutathione in promoting the activation by nitroglycerin of guanylate cyclase in arterial extracts. This suggests a role for cysteine, rather than for glutathione, in *in vivo* activation of nitrates. On the other hand much more (×45) glutathione than cysteine is present in arterial extracts [19]. It is also perfectly possible that the *in vitro* system used in [18] was an inadequate model, perhaps because of the lack of a preincubation of the GTN with the sulphydryl compounds. Be that as it may, the present study provides the first real evidence that glutathione and glutathione *S*-transferase play a role in the *in vivo* activation of organic nitrates. More work is needed to establish whether cysteine takes any part in this process.

In a recent study [20], it was suggested that the chemical reaction between organic nitrates and sulphydryl compounds represents an important path for activation. The results presented here indicate that nitrate activation is at least partially enzymic. Moreover it seems very doubtful that the chemical reaction is sufficiently rapid to be useful as more than a model for the enzymic activation.

It is possible to calculate the percentage conversion of the nitrates resulting from the chemical reaction, using realistic nitrate concentrations (ED_{50}), realistic concentrations of glutathione and cysteine [19], rate constants taken or extrapolated from [21], and a reaction time of 15 min. In every case the result is that less than 0.1% of the nitrate is converted, strongly suggesting that nitrate activation is totally enzymic.

In summary, the present work demonstrates that SBP acts as a specific antagonist of organic nitrates by inhibiting a step in their activation, which probably employs the enzyme glutathione *S*-transferase. Other steps in the activation of the nitrates must be the subject of further study.

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