SULFHYDRYL REACTIVITY OF ORGANIC NITRATES: BIOCHEMICAL BASIS FOR INHIBITION OF GLYCERALDEHYDE-P DEHYDROGENASE AND MONOAMINE OXIDASE*

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SUMMARY: Glyceryl trinitrate caused a concentration dependent inhibition of the SH rich enzymes, glyceraldehyde-3-phosphate dehydrogenase and monoamine oxidase. Ethacrynic acid, an SH alkylating agent also inhibits both of these enzymes. The disulfide reducing agent, dithiothreitol, could protect against the inhibition of these enzymes by organic nitrates but is ineffective against ethacrynic acid.

The known reactivity of organic nitrate vasodilators with reduced glutathione in the presence or absence of liver organic nitrate reductase, (1-3) has stimulated investigation of the possibility that the mechanism of the pharmacologic action of nitrates might involve either direct or enzymatic reaction with -SH groups. SH groups play a role in determining mitochondrial structure and are essential for oxidative phosphorylation (4). Organic nitrates have been demonstrated to uncouple oxidative phosphorylation by liver and heart mitochondria (5, 6). Nitrates also have been shown to inhibit (by an unknown mechanism) the mitochondrial SH-rich enzyme monoamine oxidase (7, 8). Furthermore, organic nitrates that are potent vasodilator compounds in dogs are the nitrates which are readily denitrated in the presence of reduced glutathione and nitrate reductase (9).

When rabbit thoracic aorta strips are incubated at pH 7.4 with high concentrations of glyceryl trinitrate (GTN) they do

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not become desensitized to the vasodilator activity of the drug; however, incubation of aortic strips with GTN at a more alkaline pH produces a profound tolerance (10). The reaction of organic nitrates with sulfhydryl containing molecules proceeds according to the following reaction:

$$RONO_2 + 2\ R'SH \longrightarrow R'SSR' + ROH + NO_2^-$$

This reaction proceeds much more rapidly at alkaline pH. Incubation of GTN (100 μg/ml) with glutathione (1 mM) yielded 8 times (15.6 ± 2.8 (n = 8) μmoles/liter/15 min) as much nitrite at pH 9.4 than at pH 7.4 (1.7 ± 0.2 (n = 8) μmole/liter/15 min). The pH dependency of the tolerance induction suggested that the mechanism of tolerance may involve oxidation of a sulfhydryl in the GTN receptor. Upon incubation of GTN with rabbit thoracic aortas at pH 9.4, more NO_2^- is formed and the tissue shows a net loss of titrable SH groups not observed at pH 7.4 (10).

In the present investigation we have employed two enzyme systems to determine if the primary reaction of GTN involves oxidation of critical sulfhydryl groups and if these systems can be regenerated by disulfide reduction.

METHODS: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was assayed by a modification of a previously described method (11). The reaction mixture consisted of: 0.1 M phosphate buffer (pH 7.4), 10 mM Na_2HAsO_4, 10 mM EDTA, 1 mM NAD and 1.0 μg/ml GAPDH (Sigma, isolated from rabbit skeletal muscle). The reaction mixture was preincubated with glyceryl trinitrate (GTN) or ethacrynic acid (EA) at 37° C for 30 min and then assayed for GAPDH activity by adding 1 mM glyceraldehyde-3-phosphate.

Monoamine oxidase assay was assayed by a radiometric technique (12) which utilized the reaction between C^{14}-tryptamine and MAO to yield ^{14}C-indole acetic acid which is soluble in

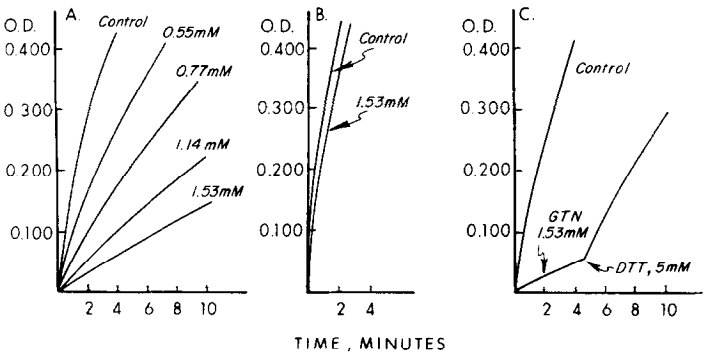

Fig. 1A. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition by GTN. GAPDH was incubated alone and with GTN (concentrations are shown on the Fig.) for 30 minutes at 37° C. The reactions were started by adding glyceraldehyde-3-P and recorded continuously.

Fig. 1B. Dithiothreitol (DTT) protection of GAPDH inhibition by GTN. GAPDH was incubated with 0.2 mM DTT without and with 1.53 mM GTN for 30 minutes at 37° C.

Fig. 1C. DTT reversal of GAPDH inhibition. GAPDH was incubated without and with 1.53 mM GTN. At the time indicated DTT, 5 mM, was added to the reaction mixture containing 1.53 mM GTN.

toluene. Therefore, the labeled metabolite was easily extracted from the aqueous phase with toluene and an aliquot counted. Rat liver mitochondria were prepared as previously described (5).

RESULTS AND DISCUSSION: GAPDH inhibition: Figure 1A shows that GTN caused a dose-dependent inhibition of GAPDH. Ethacrynic acid (EA) in equimolar concentrations caused approximately the same inhibition as GTN. Dithiothreitol (DTT, 0.2 mM) was able to protect the enzyme fully from the inhibition by GTN when DTT and GTN were preincubated together (Fig. 1B). When DTT, was added to a reaction inhibited by 1.53 mM GTN, an immediate reversal occurred (Fig. 1C). At pH 7.4 the direct interaction between GTN and DTT proceeds very slowly and there is minimal loss due to this non-enzymatic redox reaction. At a more alkaline pH (greater than pH 8) the non-enzymatic reaction between organic nitrates and SH compounds is greatly accelerated.

Treatment	Conc. μM	PERCENTAGE INHIBITION Without DTT	With DTT
GTN	50	30	9
	100	41	16
	200	62	28
Ethacrynic acid	50	22	20
	100	33	35
	200	51	54

Table 1. Effect of GTN and ethacrynic acid on monoamine oxidase activity. Rat liver mitochondria was incubated with tryptamine and the agents being tested for 15 min at 37° in 0.5 M phosphate buffer (pH 7.4). When ethacrynic acid (EA) was present in the assay, it was preincubated with the mitochondria for 15 min at 37° before the addition of the tryptamine. The dithiothreitol (DTT) concentration was 1 mM.

MAO inhibition: Incubation of GTN (or EA) with liver mitochondria caused a concentration dependent inhibition of monoamine oxidase (MAO) (Table 1). The disulfide reducing agent, dithiothreitol (DTT) partially protected against the MAO inhibition caused by GTN but was ineffective against EA. Preincubation of EA with either DTT or cysteine abolished EA induced MAO inhibition. In comparable experiments (n = 4) other nitrates (erythrityl tetranitrate, isosorbide dinitrate, and mannitol hexanitrate at 25 μM concentrations) caused a 50 % inhibition of MAO and this inhibition was partially overcome when the incubation mixture included 1 mM DTT. These results indicate the organic nitrates inactivate MAO and GAPDH by SH oxidation.

The concentration of GTN required to produce a detectable stimulation of rat liver mitochondrial respiration (5) is the same as that required to inhibit MAO (Table 1). It would be interesting to determine if DTT can protect mitochondrial res-

piration from the effects of organic nitrates. Ethacrynic acid (EA) an SH alkylating agent, inhibits the oxidation of NAD-linked substrates and succinate by rat liver mitochondria at low concentrations (13). EA has also been shown to inhibit glycolysis in rat or rabbit kidney extracts and the inhibition was abolished by prior addition of DTT (14). EA acts in these cases as a thiol reagent and covalently links to SH groups (15).

At pH 7.4 EA caused a marked reduction in SH concentration when incubated with either cysteine, or thoracic aorta (not shown). EA at pH 7.4 markedly reduced NO_2^- formation (useful measure of the redox reaction of organic nitrates with SH) when GTN was incubated in the various SH systems, indicating that EA has access to SH groups which react with organic nitrates. Incubation of EA with aortic strips at pH 7.4 blocks the activity of vasodilators whereas incubation of EA and aortas at pH 9.5 produces no change in vasodilator responsiveness (not shown).

In the present study the concentrations of organic nitrate required to inhibit MAO or GAPDH are greater than those required to dilate vascular smooth muscle. However, high concentrations are useful in exaggerating a critical reaction which is readily masked by a large pool of SH which is not essential for the organic nitrate reaction. Furthermore, the concentrations of organic nitrates employed in these studies are of the order of magnitude required for the induction of *in vitro* or *in vivo* tolerance (10). In fact, the mechanism of organic nitrate tolerance appears to be the result of the oxidation of critical SH groups in the GTN receptor site in vascular smooth muscle (10). Thus, under conditions that favor induction of GTN tolerance, tissue SH levels decrease and the SH dependent denitration was enhanced. In addition, the disulfide reducing agent dithiothrei-

tol reversed GTN induced (*in vivo* or *in vitro*) tolerance in aorta strips (10).

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