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NITROGLYCERIN AND ISOSORBIDE DINITRATE STIMULATION OF GLUTATHIONE DISULFIDE EFFLUX FROM PERFUSED RAT LIVER

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Abstract—Nitroglycerin (GTN) and isosorbide dinitrate (ISD) are metabolized by glutathione *S*-transferase to nitrite with production of GSSG from GSH. Infusion of organic nitrates into perfused rat liver led to efflux of GSSG in the bile and nitrite in the perfusate. Biliary GSSG increased more rapidly than did nitrite release as GTN infusion rate was increased, indicating that GSSG reducing capacity was being exceeded. Rapid GTN-induced oxidation of GSH may be the mechanism of tissue GSH depletion by GTN and other alkylnitrates. Such depletion of glutathione may reduce nitrite production from organic nitrates and underlie tolerance to these drugs.

The initial step of organic nitrate metabolism in the liver is mediated by an organic nitrate reductase activity [1] which is a property of several isoforms of the glutathione *S*-transferase family [2]. These enzymes have been postulated to catalyze the formation of an intermediate, glutathione sulfenyl nitrite (equation 1), which reacts directly with GSH‡ to yield nitrite and GSSG (equation 2) [3]. The formation of nitrite is thought to be obligatory for the vasodilatory effect of organic nitrates in vascular tissue, and this reaction has been studied in liver for insight into the mechanism of action of these compounds.

$$GTN + GSH \xrightarrow{\text{GSH } S\text{-transferase}} GDN + GSNO_2 \quad (1)$$

$$GSNO_2 + GSH \xrightarrow{\text{non-enzymatic}} H^+ + NO_2^- + GSSG \quad (2)$$

Over a decade ago, Needleman and Harkey [4] showed that isolated perfused rat liver removed GTN infused into it and released nitrite into the perfusate. In addition, they noted that GTN infusion caused a fall in liver glutathione concentration. Improvements in the ability to study glutathione effects on drug metabolism in the perfused liver have prompted us to reexamine the metabolism of organic nitrates in this system.

MATERIALS AND METHODS

Male Sprague–Dawley rats (250–450 g) were fed a stock diet (LabBlox, Wayne Feeds, Chicago, IL) *ad lib*. They were anesthetized with pentobarbital (65 mg/kg, i.p.). After cannulation of the bile duct with PE-10 tubing, the portal vein was cannulated and the liver was perfused with oxygenated Krebs–Henseleit buffer as described previously [5]. Bile was collected in 3% meta-phosphoric acid for periods of 5 min. Bile samples were weighed, and the bile flow rate was calculated. Perfusate samples were collected at 5-min intervals. Following a 20-min stabilization period, GTN or ISD was infused for 20 min. The drug infusion was followed by a 20-min recovery period. GSSG was measured in bile as described previously [5]. Total glutathione was measured in bile and perfusate by the method of Tietze [6]. Nitrite was measured in the perfusate by the method of Bell *et al.* [7] as modified by Ignarro *et al.* [8]. Lactic dehydrogenase was measured in the perfusate as a marker of liver damage [5].

RESULTS

Figure 1 shows biliary GSSG release and perfusate nitrite release in response to two different GTN infusion rates. GTN infusion caused prompt increases in biliary GSSG and perfusate nitrite release. At the lower GTN infusion rate these were maintained for 20 min, but at the higher infusion rate they showed a tendency to fall during the infusion. Both returned to baseline after the infusion was stopped. No increase in LDH release was detected (Table 1). These results indicate that the organic nitrate reductase activity of the glutathione *S*-transferases can be monitored in the perfused liver.

Figure 2 shows biliary GSSG release and perfusate nitrite release for four GTN infusion rates. Both values increased with increasing GTN infusion rates. If 1 nitrite ion results from the metabolism of 1 molecule of GTN in this system, 86% of the GTN

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‡ Abbreviations: GSH, reduced glutathione; GSSG, glutathione disulfide; GTN, nitroglycerin; GDN, glyceryl dinitrate; and ISD, isosorbide dinitrate.

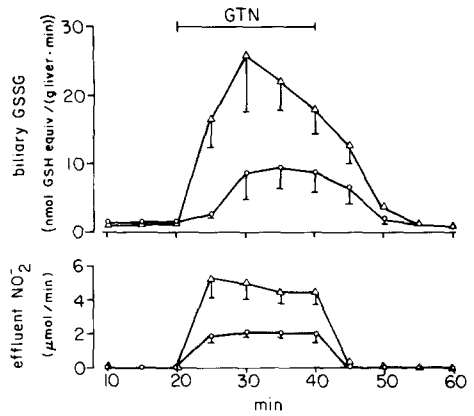

Fig. 1. Effect of GTN on biliary GSSG release (upper panel) and caval nitrite (NO_2^-) release (lower panel) with time. GTN was infused at a rate of 2.2 μmol/min (○) and 7.7 μmol/min (△) from 20 to 40 min of the 60 min perfusion (denoted by the bar). Values are means ± SD for four livers.

was metabolized when 1.1 μmol was infused per min. This fell to 64% at an infusion rate of 7.7 μmol/min, suggesting a saturation of the metabolism capacity.

Table 1 shows that GTN infusion increased caval glutathione release slightly at the highest infusion rate. There was also a tendency for GTN infusion to cause increased biliary GSH release and bile flow rate.

ISD was infused into livers by the same protocol as that used for GTN. The results are summarized in Table 2. Infusion of 4.7 μmol ISD/min caused a 2.8-fold increase in biliary GSSG compared with the 16.5-fold increase measured when 4.4 μmol GTN/min was infused. Biliary GSSG release reached a maximum at a perfusion time of 35 min when ISD was infused, whereas a maximum in biliary GSSG release occurred at 30 min when GTN was infused (Fig. 1). Infusion of a higher dose of ISD (19.4 μmol/min) increased nitrite and biliary GSSG release further but not to the same degree as GTN. ISD infusion resulted in nitrite release but the magnitude of the release was significantly less than with GTN infusion. When 4.4 μmol GTN was infused per min, the nitrite released was 68% of the infused dose. However, infusion of 4.7 μmol ISD/min resulted only in 17% of the infused dose being released as nitrite. These results are consistent with slower metabolism of ISD by glutathione *S*-transferase isozymes as compared to GTN [3].

Table 1. Effect of GTN infusion on bile flow, caval glutathione release and biliary GSH release

GTN dose (μmol/min)	Rat wt (g)	Liver wt (g)	Bile flow [mg/(g liver·min)]		Caval glutathione release [nmol GSH equivalents/(g liver·min)]		Biliary GSH release [nmol GSH equivalents/(g liver·min)]		Caval LDH release [(nmol NADH oxidized/min)/(g liver·ml of perfusate)]	
			15–20 min	25–30 min	At 20 min	At 30 min	15–20 min	25–30 min	15–20 min	25–30 min
1.1	276 ± 35	8.1 ± 0.8	1.0 ± 0.1	1.0 ± 0.2	11 ± 2	13 ± 2	1.6 ± 0.6	1.4 ± 0.9	6.7 ± 1.4	<5
2.2	317 ± 13	10.9 ± 0.5	1.0 ± 0.2	1.1 ± 0.1	10 ± 3	12 ± 1	2.6 ± 0.7	4.4 ± 3.0	5.2 ± 3.1	6.5 ± 2.1
4.4	320 ± 48	9.8 ± 1.4	1.2 ± 0.04*	1.6 ± 0.3*	11 ± 2	13 ± 6	1.2 ± 0.2†	6.6 ± 2.0†	<5	5.3 ± 1.7
7.7	408 ± 8	12.6 ± 1.2	0.8 ± 0.1‡	1.2 ± 0.2‡	7 ± 1§	10 ± 2§	1.5 ± 0.4	6.3 ± 5.5	<5	<5

Values are means ± SD, N = 4.
*–§ Values with the same superscript are significantly different, $P < 0.05$ (Student's *t*-test).

DISCUSSION

These experiments show that metabolism of organic nitrates in the liver leads to the formation of GSSG as would be predicted from knowledge of the *in vitro* reaction. GSSG has two major fates in the hepatocyte. One is reduction to GSH by glutathione reductase and the other is export into the bile by the mechanism that transports glutathione conjugates. Assuming that the formation of each nitrite ion is accompanied by the formation of 1 GSSG, 1.2% of the GSSG formed from GTN metabolism at the

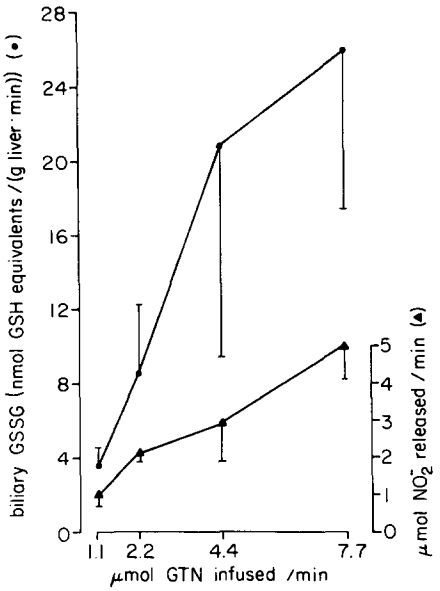

Fig. 2. Biliary GSSG release (●) and caval nitrite (NO_2^-) release (▲) in reponse to infusion of GTN at four rates. GSSG was measured in bile collected from 25 to 30 min during the perfusion, and nitrite was measured in the caval effluent at 30 min. Values are means ± SD for four livers.

lowest infusion rate appeared in the bile. This increased to 3.2% at the highest GTN infusion rate. Thus, these experiments show that transport into bile becomes more efficient at higher organic nitrate infusion rates, suggesting that reduction of GSSG becomes limiting under these conditions. Recent work has shown that up to 50% of the GSSG released into bile is broken down and therefore not detected [9]. Considering these findings, it seems likely that export of GSSG could account for the depletion of glutathione by GTN observed in earlier work [4].

Organic nitrates are the first-line treatment for angina pectoris. However, the development of long-acting preparations of these drugs has led to the recognition that tolerance to their vasodilatory effect develops within hours to days when they are administered in a constant manner [10]. This problem has spurred renewed efforts to understand their metabolism with the hope that a treatment can be devised to prevent the development of tolerance.

The organic nitrate reductase reaction is postulated to have a mechanistic feature which could underlie tolerance. The enzymatic portion of the reaction (equation 1) is suggested to produce a glutathione conjugate which then reacts non-enzymatically with GSH (equation 2) to yield nitrite and GSSG [3]. It is possible that, at high reaction rates or at low GSH concentrations, the non-enzymatic reaction could be slower than the formation of the conjugate. This would allow the conjugate to accumulate and compete with GSSG for transport into bile. Such an occurrence could explain the fall in biliary GSSG release noted in the last 10 min of infusion of the highest GTN dose (Fig. 1). The increase in biliary GSH noted under these conditions (Table 1) could result from dissociation of the

Table 2. Perfused liver response to ISD infusion

ISD dose (μmol/min)	Rat wt (g)	Liver wt (g)	Bile flow [mg/(g liver·min)]		Caval nitrite release (μmol/min)	Biliary GSSG release [nmol GSH equivalents/(g liver·min)]		Biliary GSH release [nmol GSH equivalents/(g liver·min)]	
			15–20 min	30–35 min	At 35 min	15–20 min	30–35 min	15–20 min	30–35 min
4.7	299 ± 35	11.8 ± 1.9	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.4	0.9 ± 0.1*	2.5 ± 0.2*	1.0 ± 0.3	1.1 ± 0.5
19.4	307 ± 77	10.8 ± 2.5	1.0 ± 0.1†	0.7 ± 0.2†	1.9 ± 0.5	0.6 ± 0.2‡	3.8 ± 1.5‡	1.2 ± 0.7	1.9 ± 1.9

Values are means ± SD, N = 4.
*–‡ Values with the same superscript are significantly different, $P < 0.05$ (Student's *t*-test).

glutathione sulfenyl nitrite in the bile in a manner analogous to the postulated dissociation of *S*-hydroxymethyl glutathione [11]. Thus, these results are consistent with the formation of the conjugate and excretion of it in the bile. Direct proof of its existence, however, will require its isolation and identification.

Metabolism by the liver blocks the vasodilatory effects of GTN [12], and, therefore, nitrite produced there has no role in the action of the drug. To cause vasodilatation, GTN must probably be metabolized to nitrite in vascular tissue. Nitrite is an essential precursor of NO which is the active form [8]. Blood vessel cells have lower glutathione concentrations than liver cells [13] and thus may be more easily depleted of this substance than liver by organic nitrate metabolism. If that occurs, equation (1) might continue with production of the conjugate, but equation (2) might be impaired because of its non-enyzmatic nature. This would lead to export of the conjugate from the cell and would keep cellular glutathione concentration low without producing the active nitrite. By this mechanism, continuing administration of organic nitrate could maintain low glutathione concentrations in vascular tissue without producing nitrite. This hypothesis requires further investigation as the mechanism of tolerance development. These results illustrate the extensive involvement of glutathione in organic nitrate metabolism and indicate that further studies may provide additional insight into the mechanism of organic nitrate tolerance.

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