Biochemical Pharmacology, Vol. 45, No. 1, pp. 268–270, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

Biotransformation of glyceryl trinitrate by rat aortic cytochrome P450

(*Received* 11 *August* 1992; *accepted* 17 *September* 1992)

Abstract—Denitration of glyceryl trinitrate (GTN) by the microsomal fraction of rat aorta was found to be NADPH dependent and followed apparent first-order kinetics ($T_{1/2}$ 70.1 min). Biotransformation of GTN was regioselective for glyceryl-1,2-dinitrate formation, and was inhibited by carbon monoxide, SKF-525A, and oxygen. In aortic microsomes prepared from phenobarbital-pretreated rats, biotransformation was increased 7-fold, and was regioselective for glyceryl-1,3-dinitrate formation. These data strongly suggest the involvement of aortic cytochrome P450 in the biotransformation of GTN.

It is generally accepted that the biotransformation of organic nitrates to an active species [presumably nitric oxide (NO)*] which then activates guanylyl cyclase is prerequisite for their vasodilator action. Although enzymatic and nonenzymatic pathways for the biotransformation of organic nitrates have been described, the role of these pathways in mechanism-based biotransformation (vascular biotransformation of organic nitrates leading to activation of guanylyl cyclase and vasodilation) is unclear. The biotransformation of organic nitrates by rat hepatic microsomes has been assessed, and the evidence strongly suggests the involvement of cytochromes P450 in this reaction [1, 2] and that this reaction results in the formation of an activator of guanylyl cyclase [3]. Cytochrome P450 is present in vascular tissue both in endothelial and smooth muscle cells and cytochrome P450 activities have been found in aortic microsomes [4]. In addition, Finnen *et al.* [5] have reported that aortic cytochrome P450 activity can be induced by phenobarbital (PB).

The objective of the present study was to assess the biotransformation of glyceryl trinitrate (GTN) by rat aortic microsomes and to compare it with that found in rat hepatic microsomes with respect to kinetic parameters, the regioselectivity of the denitration reaction, the effect of oxygen and cytochrome P450 inhibitors, and inducibility with phenobarbital.

Methods

Experiments were carried out on preparations of the microsomal fraction of pooled, endothelium-denuded rat aortae from untreated animals [200 aortae per preparation; aortae were purchased from Pel-Freez Biologicals (Rogers, AK) and received frozen on dry ice] and from animals that had been pretreated with PB (0.1% in the drinking water for 5 days; 20 aortae per preparation) prior to being killed. Microsomes were prepared using standard differential centrifugation procedures after homogenization in 4 vol. of 10 mM Tris–HCl, pH 7.8, and resuspension of the microsomal pellet in 1.15% KCl, 1 mM EDTA, 0.1 M phosphate buffer (pH 7.4). The cytochrome P450 activity in these microsomes was monitored by following 7-ethoxycoumarin deethylase activity according to the method of Greenlee and Poland [6].

Incubation of microsomes with GTN was performed at 37° in the presence or absence of NADPH (1 mM) and under an air (aerobic) or humidified nitrogen (anaerobic) atmosphere at the concentrations and times indicated. To assess the role of cytochrome P450 in the biotransformation reaction, microsomes were exposed to carbon monoxide (CO) or SKF-525A (1 mM) prior to the incubation with GTN as described [2]. The total volume of the incubation mixture was 1.0 mL. GTN and its denitrated metabolites, glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN), were quantified by megabore capillary column gas–liquid chromatography [2].

All data are expressed as group means ± SD. Data from experiments examining the effects of CO, SKF-525A, and oxygen on GTN biotransformation were analyzed by one-way analysis of variance. Data from experiments comparing biotransformation in microsomes from untreated and PB-pretreated animals were analyzed by Student's *t*-test for unpaired data. P values of 0.05 or less were considered statistically significant.

Results

Aortic microsomal preparations from untreated animals had a low but detectable 7-ethoxycoumarin deethylase activity of 20 ± 10 pmol/mg protein/min (N = 3), and this activity was increased approximately 3-fold in aortic microsomes from PB-pretreated animals. Finnen *et al.* [5] reported comparable activity of 11.4 ± 3.2 pmol/mg protein/min in untreated rats, which was increased approximately 4-fold in PB-pretreated animals. The 7-ethoxycoumarin deethylase activity in rat liver microsomes, by comparison, was 2.56 ± 1.32 nmol/mg protein/min.

Biotransformation of GTN in aortic microsomes from untreated animals was NADPH dependent under anaerobic conditions, since there was no loss of GTN or appearance of GDNs over a 60-min period when NADPH was not added to the reaction mixture. The biotransformation of GTN followed apparent first-order kinetics with a half-life value of 70.1 min (Fig. 1). This half-life value was approximately 140-fold greater than that found using rat hepatic microsomes under the same conditions [2]. The disappearance of GTN was accompanied by the appearance of 1,2- and 1,3-GDN and the ratio of 1,2-GDN/1,3-GDN formed at 60 min was 2.78 ± 0.57 (N = 3). Biotransformation of GTN in aortic microsomes from PB-pretreated animals (anaerobic, 60-min incubation) occurred at a significantly greater rate than in microsomes from untreated animals: 7.0 ± 0.6 vs 1.1 ± 0.1 pmol/mg protein/min ($P \leq 0.05$; N = 3). In contrast to biotransformation in microsomes from untreated animals, biotransformation in aortic microsomes from PB-pretreated animals was regioselective for the formation of 1,3-GDN with a ratio of 1,2-GDN/1,3-GDN formed at 60 min of 0.43 ± 0.03 (N = 3).

CO and SKF-525A, cytochrome P450 inhibitors, under anaerobic conditions, significantly inhibited the biotransformation of GTN ($P \leq 0.05$; Table 1). Furthermore, with the addition of oxygen to the reaction, biotransformation of GTN was not detected.

* Abbreviations: CO, carbon monoxide; 1,2-GDN, glyceryl-1,2-dinitrate; 1,3-GDN, glyceryl-1,3-dinitrate; GTN, glyceryl trinitrate; NO, nitric oxide; and PB, phenobarbital.

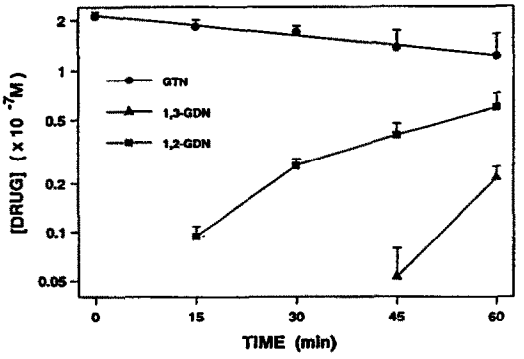

Fig. 1. Time course of GTN (●) disappearance and 1,2-GDN (■) and 1,3-GDN (▲) formation in rat aortic microsomes under anaerobic conditions following exposure to 0.2 μM GTN and 1 mM NADPH. Incubation mixtures contained 0.85 ± 0.10 mg protein/mL. All data points represent means ± SD of three populations of 200 rats each.

Table 1. Inhibition of rat aortic microsomal biotransformation of GTN by carbon monoxide, SKF-525A, and oxygen

Treatment	% Biotransformation
Control	28.0 ± 2.4
Carbon monoxide	2.3 ± 0.5*
SKF-525A	3.4 ± 0.8*
Oxygen	ND

Data represent the means ± SD of three populations of 200 rats each. Incubation mixtures containing 0.85 ± 0.10 mg protein/mL were incubated with 0.2 μM GTN for 60 min under anaerobic conditions except where oxygen was added. The rate of GTN biotransformation in control microsomes was 1.1 ± 0.1 pmol/mg protein/min. ND, not detected.

* $P \leq 0.05$ when compared with control (one-way ANOVA).

Discussion

The observed NADPH-dependent biotransformation of GTN by rat aortic microsomes suggests the involvement of the cytochrome P450–cytochrome P450 reductase system. To explore the nature of this interaction, the effects of oxygen and the cytochrome P450 inhibitors CO and SKF-525A on microsomal GTN biotransformation were assessed. The resulting high degree of inhibition of GTN biotransformation by all of these agents suggests that the denitration of GTN by this system primarily occurs by a direct interaction with the heme moiety of cytochrome P450. Thus, these characteristics of aortic microsomal GTN denitration parallel those previously reported to occur during hepatic microsomal biotransformation of GTN [1, 2].

Biotransformation of GTN by aortic microsomes occurred at a rate considerably less than that seen using hepatic microsomes ($T_{1/2}$ 70.1 min and approximately 30 sec, respectively; [2]). The cytochrome P450 content of aorta is low and we have been unable to measure cytochrome P450 levels using CO binding spectral data. Thus, a comparison of aortic and hepatic biotransformation corrected for cytochrome P450 content has not been possible. However, aortic microsomes do possess 7-ethoxycoumarin deethylase activity, which we have measured to be approximately 100-fold less than that in hepatic microsomes. In addition, Juchau *et al.* [7] reported that the aryl 4-monooxygenase activity of rabbit aorta is 97-fold less than in rabbit hepatic microsomes. These differences in aortic versus hepatic cytochrome P450 activities are thus similar to the 140-fold difference in the half-life of GTN reported here.

An interesting aspect of GTN biotransformation is the relative formation of its two dinitrate metabolites, 1,2-GDN and 1,3-GDN. The regioselective 1,2-GDN formation seen in hemoglobin- and myoglobin-mediated GTN biotransformation [8] and in vasular biotransformation of GTN in several species, and in several cultured cell lines, led to the proposal that there is a vascular pathway for GTN biotransformation which is hemoprotein-mediated [9]. In the current study, the observed regioselective biotransformation of GTN to 1,2-GDN in aortic microsomes from untreated animals is consistent with the above proposal. However, in microsomes from PB-pretreated animals, biotransformation of GTN was regioselective for 1,3-GDN formation. The change in regioselectivity of the denitration reaction after PB treatment suggests that multiple cytochrome P450s are involved in vascular GTN biotransformation and that different isoforms vary in their preference for denitration of a particular nitrate ester group. Presumably, the change in the cytochrome P450 isoenzyme profile following induction by PB results in an overall preference for the formation of 1,3-GDN rather than the selective 1,2-GDN formation resulting from biotransformation by the constitutive cytochrome P450 isoforms.

Aortic microsomes from PB-pretreated animals biotransformed GTN at a rate which was approximately 7-fold greater than that seen in microsomes from untreated animals. This increased biotransformation of GTN by aortic microsomes from PB-pretreated animals is similar to that seen in hepatic microsomes from PB-pretreated animals [2]. We have found previously that there was a small, but significant decrease in the EC_{50} for GTN-induced relaxation of aortic strips prepared from PB-pretreated rats [3]. In addition, we have found that the vascular biotransformation of GTN is increased under low oxygen conditions and that this increase is prevented by pretreatment of the tissues with carbon monoxide [3]. These data suggested that at least a portion of the vascular biotransformation of GTN was mediated by hemoproteins. The results of the present study suggest that cytochrome P450 is one such hemoprotein. A remaining question is whether vascular cytochrome P450-mediated biotransformation of GTN results in NO formation. We have found that hepatic microsomal biotransformation of GTN results in the formation of an activator of aortic guanylyl cyclase [3]. However, when similar experiments were performed using aortic microsomes rather than hepatic microsomes, no activation of guanylyl cyclase was observed. While this would argue against a role of aortic cytochrome P450 in NO formation from GTN, it should be appreciated that the overall rate of GTN biotransformation by aortic microsomes is markedly less than that by hepatic microsomes and thus may not generate sufficient quantities of NO to activate the enzyme under the *in vitro* conditions employed. On the other hand, we have found recently that the cytochrome P450 substrate, 7-ethoxyresorufin, markedly inhibits relaxation and cyclic GMP accumulation induced by GTN and also inhibits the aortic biotransformation of GTN [10]. Furthermore, depletion of cytochrome P450 by pretreatment of animals with cobalt protoporphyrin resulted in inhibition of GTN-induced relaxation of rat aortic strips (Long PG and Bennett BM, unpublished observations).

In summary, we have provided strong, albeit indirect, evidence for the cytochrome P450-mediated biotransformation of GTN in aortic tissue. This biotransformation was NADPH dependent, inhibited by CO, SKF-525A, and oxygen, and increased in microsomes obtained from PB-pretreated animals. Thus, the characteristics of aortic microsomal GTN denitration are similar to those previously reported to occur in hepatic microsomes. Taken together, these data suggest a role for cytochrome P450 in the vascular biotransformation of GTN.

Acknowledgements—This work was supported by a grant from the Heart and Stroke Foundation of Ontario (A1608). B. M. Bennett is a recipient of a Scholarship from the Heart and Stroke Foundation of Canada. B. J. McDonald is a recipient of a Studentship from the Medical Research Council of Canada.

Department of Pharmacology and Toxicology
Queen's University
Kingston, Ontario
Canada K7L 3N6

BERNARD J. MCDONALD
BRIAN M. BENNETT*

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* Corresponding author. Tel. (613) 545-6473; FAX (613) 545-6412.