Pergamon

Biochemical Pharmacology, Vol. 49, No. 2, pp. 141–146, 1995.
Elsevier Science Ltd
Printed in Great Britain
0006-2952/95 $9.50 + 0.00

0006-2952(94)00534-6

RAPID COMMUNICATION

GSH-INDEPENDENT DENITRATION OF THE NITRATE ESTER OF A DIHYDROPYRIDINE DERIVATIVE IN RABBIT HEPATIC CYTOSOL

Naoyoshi Ogawa,* Takuya Hirose, Kiyomi Fukushima, Toshio Suwa and Tetsuo Satoh†

Research Center, Taisho Pharmaceutical Co., Ltd., Saitama 330, Japan; and
†Laboratory of Biochemical Pharmacology and Biotoxicology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 260, Japan

(Accepted 16 November 1994)

Abstract–The denitration of a dihydropyridine derivative having two nitrate ester groups, 2-nitrooxypropyl 3-nitrooxypropyl 1,4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)-3, 5-pyridinedicarboxylate (NND), by rabbit hepatic cytosol was investigated. Sephadex G-150 chromatography of ammonium sulfate precipitate (30-60%) from the cytosol demonstrated the presence of two distinct activities (peak I and peak II) responsible for denitration of [^{14}C]-NND. The first peak, peak I, was observed in the presence of dithiothreitol (DTT), but not in the presence of glutathione (GSH). Moreover, the denitration activity of peak I was not inhibited by *S*-hexyl GSH, an inhibitor of GSH *S*-transferase (GST), indicating that peak I possessed no GST activity. In contrast, the denitration activity of peak II, having GST activity, required GSH and was inhibited by *S*-hexyl GSH. These results strongly suggest that the GSH-independent enzyme system(s), in addition to GST, is responsible for denitration of nitrate esters of NND.

Key words: GSH-independent denitration; organic nitrate esters; GSH *S*-transferase

Organic nitrate esters, such as nitroglycerin and isosorbide dinitrate, are used widely in the treatment of a variety of cardiovascular diseases. Generally, these compounds are rapidly denitrated to corresponding alcohols primarily in the liver but also in the kidney, intestine, lung, heart, blood and blood vessels (1-4). A current hypothesis relating to organic nitrate ester action proposes that these compounds must first undergo metabolic activation (via denitration) to produce nitric oxide, which then induces vascular smooth muscle relaxation (5). However, the biochemical pathways for the formation of nitric oxide from organic nitrate esters are not well understood.

It has been widely accepted that a GSH‡-dependent enzyme system, GST, is an important enzyme system in the denitration of organic nitrate esters (6,7), although other pathways, including hemoproteins such as hemoglobin (8) and cytochrome P450 (9-11), and serum proteins such as albumin (12), have been reported.

NND has two nitrate esters in its molecule. Our previous work§ has shown that the denitration of the nitrate ester of NND in dialyzed rabbit hepatic cytosol is restored by GSH, and that it is potentiated by

* Corresponding author: Naoyoshi Ogawa, Department of Drug Metabolism, Research Center, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-Cho, Omiya, Saitama 330, Japan. Tel.(048)663-1111; FAX(048)652-7254.

‡ Abbreviatons: GSH, glutathione; GST, GSH *S*-transferase; NND, 2-nitrooxypropyl 3-nitrooxypropyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate; DTT, dithiothreitol; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; and PNBC, *p*-nitrobenzyl chloride.

§ Ogawa N, Mizuno K, Itoga H, Fukushima K, Suwa T and Satoh T, GSH-independent denitration of CD-349, a nitrate ester of dihydropyridine derivative, by rabbit hepatic cytosol. *Proceedings of the Third International ISSX Meeting, Amsterdam, The Netherlands, 24-28 June 1991*, p. 247.

DTT. Furthermore, mono-denitrated metabolite profiles (M1/M2) are different between GSH- and DTT-dependent denitration of NND. These data suggest that, in addition to GST, other pathways can be responsible for the denitration of NND.

In the present study, we investigated other characteristics of the enzymes responsible for denitration of NND in rabbit hepatic cytosol.

MATERIALS AND METHODS

Chemicals. [^{14}C]-NND, labeled at the C-4 position of the dihydropyridine nucleus (sp. act. 101 μCi/mg), with radiochemical purity greater than 97%, was synthesized by Amersham International (Buckinghamshire, U.K.).

GSH, DTT, NADPH, CDNB, DCNB, PNBC and *S*-hexyl GSH were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-150 was obtained from Pharmacia LKB (Uppsala, Sweden). Gel filtration standard (blue dextran, thyroglobulin, γ-globulin, ovalbumin) was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Animals and enzyme preparations. Japanese White rabbits (Nippon Institute for Biological Science, Tokyo, Japan; body wt 2-3 kg) were used. Hepatic cytosol fractions were obtained by differential ultracentrifugation of the homogenate (25%, w/v) in 67 mM phosphate buffer. The hepatic cytosol (110 mL) was adjusted to 30% of saturation by the slow addition of solid ammonium sulfate. The mixture was stirred for another 30 min, and the precipitate was removed by centrifugation. The supernatant was adjusted to 60% of saturation with ammonium sulfate, as described above, and the precipitated protein was recovered by centrifugation. The resulting 30-60% precipitate was dissolved in a minimum volume of 10 mM phosphate buffer (pH 7.4). This solution was dialyzed against 10 mM phosphate buffer (pH 7.4) for 16 hr, followed by centrifugation to remove precipitated proteins. A 12-mL sample (984 mg protein) was applied to a Sephadex G-150 column (4.2 x 38 cm) equilibrated with 50 mM phosphate buffer (pH 7.4). Eluate fractions of 11 mL were collected and analyzed for denitration and GST activity. Protein concentrations were determined by the method of Lowry *et al.* (13), using bovine serum albumin as the standard.

Assay of denitration activity. Denitration activity was measured by determining the conversion of [^{14}C]-NND to its denitrated metabolites. Unless otherwise indicated, incubations were conducted aerobically at 37° for 30 min in a 1.0 mL medium containing 0.2 to 1 mg of protein from either fraction, 50 mM phosphate buffer (pH 7.4), 2 mM EDTA, and 20 μM [^{14}C]-NND, in the presence of 2 mM DTT or GSH. The reactions were stopped by adding 80% CH_3CN. Concentrations of NND and its denitrated metabolites were measured by TLC as described previously (14). The denitration rate of NND by DTT or GSH in the absence of enzyme was subtracted from the total enzymatic rates, for calculation of specific activities.

Assay of GST activity. GST activities were assayed using CDNB, DCNB and PNBC as substrate, according to the method of Habig *et al.* (15).

RESULTS AND DISCUSSION

The rabbit hepatic cytosol was divided into three fractions by adding ammonium sulfate (0-30%, 30-60%, 60-90%). The denitration activities in the presence of GSH or DTT were mainly recovered in 30-60% ammonium sulfate precipitate (the recovery of activity from cytosol: DTT, 60%; GSH, 72%).

Sephadex G-150 chromatography of this fraction demonstrated the presence of two distinct activities (peak I and peak II) responsible for the denitration of NND (Fig. 1). The denitration activity in the presence of DTT was localized in peak I at the higher molecular weight region, which was clearly dissociated from the GST activity using CDNB as substrate. The denitration activity in the presence of GSH was eluted as a major peak (peak II), which was completely associated with GST activity, at the lower molecular weight region, and a minor peak, which was associated with peak I. The molecular weight of the enzyme, estimated from standard protein elution from Sephadex G-150, was about 175,000 for peak I and 50,000 for peak II, respectively. The latter is consistent with that of GST (16). Then each peak was pooled and characterized further.

The denitration activity of peak I was potentiated by DTT but not by GSH (Table 1), indicating that this reaction is GSH independent. Therefore, the denitration activity of peak I in the presence of GSH should be due to the basal activity of peak I, and not activation by GSH. In addition, *S*-hexyl GSH, an inhibitor of GST, did not inhibit the activity of peak I. In contrast, the denitration activity of peak II was activated by GSH and was strongly inhibited by *S*-hexyl GSH. These results suggest that peak I has no GST activities, but that peak II does. It has been shown that cytosolic GST has four classes of isozymes (Alpha, Mu, Pi and Theta) and that specific activities towards various substrates differ among these isozymes (16,17). CDNB is a good substrate for Alpha-, Mu- and Pi-class enzymes. Theta-class enzymes display low activity with CDNB, and comparatively high activity with PNBC. Thus, studies were performed to determine GST activity with CDNB, DCNB and PNBC in peak I and peak II. As shown in Table 2, GST activities in peak I were all negligible, whereas peak II had marked GST activities towards all substrates (Table 2). This result indicates that, in contrast to peak II, peak I does not have any GST isozymes.

The denitrations of NND under the present conditions are considered to be enzymatic in nature, since NND degradations by the incubation of NND with boiled peak I and DTT (or boiled peak II and GSH) were negligible compared with denitration in the presence of untreated peak I or peak II.

These results strongly suggest that the GSH-independent denitration enzyme, in addition to GST, may be responsible for denitration of the nitrate esters of NND.

NND was denitrated to two distinct mono-denitrated metabolites, M1 and M2, in rabbit hepatic cytosol.* When determining the M1/M2 ratio during the denitration in peak I and peak II, the M1/M2 ratio in peak I was about 1, indicating that peak I has little regioselectivity for the denitration of nitrate ester groups in positions 2 and 3 of the two propyl ester moieties of NND. In contrast, the ratio in peak II was 0.2, indicating the preferential formation of M2, which is a denitrated metabolite of the nitrate ester in position 2 of the propyl ester moiety. This result provides further evidence of the dissimilarity between peak I and peak II denitration activities.

In our preliminary investigation using nitroglycerin as a substrate, peak I, as well as peak II, had significant denitration activity (data not shown), suggesting that GSH-independent activity (peak I) could also be responsible for the denitration of nitroglycerin. Although the mechanism of denitration by peak I is not clear, DTT should function to reduce the enzyme protein responsible for denitration directly. This explanation is strengthened by our preliminary finding that cysteamine also enhanced the denitration activity of peak I. However, glutathione and cysteine have no such effect. It is conceivable, therefore, that there is a specificity for the reduction of the enzyme. Another explanation for the role of DTT is that it keeps a labile physiological reducing agent in the reduced form. Although we cannot determine the kind of labile reducing agent, it would presumably have to be of high molecular weight, since the cytosol was dialyzed and put on the gel-filtration column. In any case, further study

* Ogawa N, Hirose T, Fukushima K, Suwa T and Satoh T, manuscript submitted for publication.

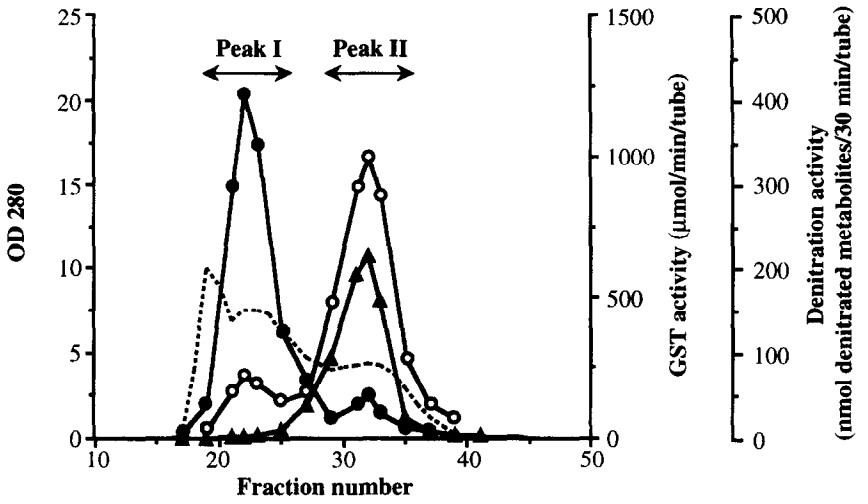

Fig. 1. Fractionation of denitration and GST activities with Sephadex G-150 chromatography. Rabbit hepatic cytosol was concentrated by $(NH_4)_2SO_4$ precipitation and applied to a column (4.2 x 38 cm) of Sephadex G-150 equilibrated with 10 mM phosphate buffer, pH 7.4. GST activity was measured with CDNB (-▲-). The denitration activities towards NND were measured by adding GSH (-○-) or DTT (-●-) as co-factor. Protein was measured at 280 nm (····) using appropriately diluted samples.

Table 1. Denitration activity of peak I and peak II

	Concn (mM)	Denitration activity (nmol denitrated metabolites/30 min/mg)	
		Peak I	Peak II
None		0.97	0.16
DTT		3.53	0.29
DTT + *S*-hexyl GSH	0.1	3.46	ND*
	1	3.60	ND
GSH		0.96	3.80
GSH + *S*-hexyl GSH	0.1	ND	2.36
	1	ND	0.52
DTT + GSH		3.54	3.85

Values are means of three determinations.
*ND : not determined.

Table 2. GST activity of peak I and peak II

	Peak I		Peak II	
	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)
CDNB	5.9	0.150	3844	11.510
DCNB	0.8	0.002	30	0.090
PNBC	0.5	0.001	229	0.696

is needed for the identification of the physiological cofactor, replaced by DTT *in vitro*. Since the molecular size of the denitration activity of peak I and peak II is about 175 and 50 kDa, respectively, there is a possibility that DTT may release GST and GSH from the trimer of GST in peak I. However, the denitration of peak I should not be due to GST, because the GST activity of peak I preincubated with DTT had no significant increase compared with peak I preincubated without DTT (data not shown). Several groups have demonstrated that thiol is intimately involved in the metabolic denitration and pharmacological effects of organic nitrate esters (5,18), although several reports have challenged this sulfhydryl hypothesis (19,20). Thus, it is interesting that, in the present study, the denitration activity of peak I was activated by the thiol compound DTT. However, clarification of the possibility that the denitration activity of peak I plays a role in the pharmacological effects of nitrate esters requires further investigation.

In conclusion, the present study strongly suggests that the GSH-independent denitration enzyme system(s), in addition to GST, is responsible for denitration of the nitrate ester of NND. The purification and identification of GSH-independent denitration enzyme in cytosol from rabbit liver are being carried out currently in our laboratory.

REFERENCES

1. Kabuto S, Kimata H, Yonemitsu M and Suzuki J, Metabolism of nipradilol by liver homogenates from different species. I. Comparative studies on the denitration of nipradilol and other organic nitrates. *Xenobiotica* **16**: 307-315, 1986.
2. Tam GS, Nakatsu K, Brien JF and Marks GS, Determination of isosorbide dinitrate biotransformation in various tissues of the rabbit. *Biopharm Drug Dispos* **8**: 37-46, 1987.
3. Posadas del Rio FA, Juarez FJ and Garcia RC, Biotransformation of organic nitrate esters *in vitro* by human liver, kidney, intestine and blood serum. *Drug Metab Dispos* **16**: 477-481, 1988.
4. Taylor IW, Ioannides C and Parke DV, Organic nitrate reductase: Reassessment of its subcellular and tissue distribution and its relationship to the glutathione *S*-transferases. *Int J Biochem* **21**: 67-71, 1989.
5. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ and Gruetter CA, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide. Evidence for the involvement of *S*-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* **218**: 739-749, 1981.
6. Habig WH, Keen JH and Jakoby WB, Glutathione *S*-transferase in the formation of cyanide from organic thiocyanates and an organic nitrate reductase. *Biochem Biophys Res Commun* **64**: 501-506, 1975.
7. Keen JH, Habig WH and Jakoby WB, Mechanism for the several activities of the glutathione *S*-transferase. *J Biol Chem* **251**: 6183-6188, 1976.
8. Bennett BM, Kobus SM, Brien JF, Nakatsu K and Marks GS, Requirement for reduced, unliganded hemoprotein for the hemoglobin- and myoglobin-mediated biotransformation of glyceryl trinitrate. *J Pharmacol Exp Ther* **237**: 629-635, 1986.
9. Servent D, Delaforge M, Ducrocq C, Mansuy D and Lenfant M, Nitric oxide formation during microsomal hepatic denitration of glyceryl trinitrate: Involvement of cytochrome P-450. *Biochem Biophys Res Commun* **163**: 1210-1216, 1989.
10. McDonald BJ and Bennett BM, Cytochrome P-450 mediated biotransformation of organic nitrates. *Can J Physiol Pharmacol* **68**: 1552-1557, 1990.
11. Schroder H, Cytochrome P-450 mediated bioactivation of organic nitrates. *J Pharmacol Exp Ther* **262**: 298-302, 1992.

12. Chong S and Fung H-L, Thiol-mediated catalysis of nitroglycerin degradation by serum proteins. Increase in metabolism was not accompanied by *S*-nitrosothiol production. *Drug Metab Dispos* **18**: 61-67, 1990.
13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.
14. Ogawa N, Mizuno K, Fukushima K, Suwa T and Satoh T, Metabolic fate of a new dihydropyridine calcium antagonist, CD-349, in rat and dog. *Xenobiotica* **23**: 747-759, 1993.
15. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferase: The first step in mercapturic acid formation. *J Biol Chem* **249**: 7130-7139, 1974.
16. Mannervik B and Danielson UH, Glutathione transferases - Structure and catalytic activity. *CRC Crit Rev Biochem* **23**: 283-337, 1988.
17. Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM and Ketterer B, Theta, a new class of glutathione transferases purified from rat and man. *Biochem J* **274**: 409-414, 1991.
18. Needleman P and Johnson EM, Mechanism of tolerance development to organic nitrates. *J Pharmacol Exp Ther* **184**: 709-715, 1973.
19. Gruetter CA and Lemke SM, Dissociation of cysteine and glutathione levels from nitroglycerin-induced relaxation. *Eur J Pharmacol* **111**: 85-95, 1985.
20. Abdollah A, Moffat JA and Armstrong PW, *N*-Acetylcysteine dose not modify nitroglycerin-induced tolerance in canine vascular rings. *J Cardiovasc Pharmacol* **9**: 445-450, 1987.