Biochemical Pharmacology, Vol. 53, pp. 117–120, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00602-8

SHORT COMMUNICATION

Subunit Specificity and Organ Distribution of Glutathione Transferase-Catalysed *S*-Nitrosoglutathione Formation from Alkyl Nitrites in the Rat

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ABSTRACT. Glutathione transferase (GST)-catalysed *S*-nitrosoglutathione (GSNO) formation from alkyl nitrites was determined with the homodimers 1–1, 2–2, 3–3, and 4–4 isolated from rat liver. The 4–4 isoform showed a high specificity for the alkyl nitrites. Total GST activities were studied in homogenates from different organs. The liver showed highest GST activity both with amyl nitrite and with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the activity ratio of amyl nitrite over CDNB being 3.8. In lung and heart, these ratios were 6.2 and 5.7, respectively, indicating a selectivity of these organs for alkyl nitrite metabolism and GSNO formation. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:117–120, 1997.

KEY WORDS. *S*-nitrosoglutathione; glutathione transferase; amyl nitrite; *n*-butyl nitrite

Amyl nitrite has been used medically for more than a century as an angina pectoris relieving agent. Recently, it was reported that human liver cytosolic and microsomal GSTs† catalyse the formation of GSNO from organic nitrites at high rates [1, 2]. GSNO exhibits many NO-like properties, such as vasodilation and inhibition of platelet aggregation [3, 4]. In human liver, highest activities were observed with the A1–1 and M1a–1a isoforms [1]. In this study, we investigated the subunit specificity with four major GST isoenzymes—1–1, 2–2, 3–3, and 4–4—isolated from rat liver. Because alkyl nitrites are volatile drugs administered via inhalation, the heart being the target organ, we studied GSNO formation in different organs, including lung, heart, and erythrocytes.

MATERIALS AND METHODS

Chemicals

Amyl nitrite, *n*-butyl nitrite, and CDNB were purchased from Merck (Darmstadt, Germany), and GSH came from Boehringer (Mannheim, Germany).

Preparation of Tissue Homogenates

Male Wistar rats, weighing 200–250 g, were sacrificed under pentobarbital anaesthesia. Organs were removed, weighed, and homogenized in a Waring blender with a 10-fold volume of a medium containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4). Homogenates were centrifuged for 15 min at 500 × *g* and the supernatants used for enzyme determinations.

Erythrocytes were isolated by centrifugation of heparinized blood samples at 400 × *g* for 10 min. Plasma and buffy coat were discarded. The erythrocytes were washed in 0.9% NaCl three times and then lysed with three volumes of distilled water for 1 hr at 4°C. The hemolysate was centrifuged at 5000 × *g* for 30 min, the supernatant diluted 10 times, and a sample treated with one volume CM-Sepharose equilibrated in 20 mM potassium phosphate buffer, pH 6.4, to remove hemoglobin before assay.

Preparation of GST Isoenzymes

A GST-enriched fraction obtained by *S*-linked GSH-agarose affinity chromatography as described by Meyer *et al.* [5] was used for the purification of the different GSH isoenzymes from rat liver and heart. Isoenzymes 2–2, 3–3, and 4–4 from liver were isolated by subsequent CM-Sepharose chromatography at pH 6.7 as described by Habig *et al.* [6], and isoenzyme 4–4 from heart was prepared by CM-Sepharose chromatography at pH 6.0 as described previously [7]. Isoenzyme 1–1 was isolated by chromatofocusing on a Mono P column (Pharmacia) with diluted Pharmalyte (8–10.5), according to Ålin *et al.* [8]. GST subunits were identified by reverse phase HPLC using a Dynamax C18 column essentially as described previously [5].

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† *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; GSNO, *S*-nitrosoglutathione; GST, glutathione transferase (EC 2.5.1.18).
Received 2 May 1996; accepted 6 August 1996.

Assays

GST activity was measured with CDNB as described by Habig *et al.* [6], and GST-catalysed GSNO formation from alkylnitrites was measured using 1.5 mM GSH and 1.0 mM alkyl nitrite as described previously [1]. Reaction rates were determined at 25°C. Stock solutions of alkylnitrites (60 mM) were prepared in acetone; the final concentration of the vehicle was less than 2%. GSNO was measured at 334 nm using an extinction coefficient of 767 $M^{-1} \cdot cm^{-1}$ [9]. Protein was determined according Bensadoun and Weinstein [10].

RESULTS AND DISCUSSION

The expression of the multiple forms of GST differs during development and from one tissue to another [11–13]. Soluble GSTs are a mixture of homo- and heterodimers, which in rat liver almost exclusively consist of the alpha subunits 1 and 2 and the mu subunits 3 and 4. After isolation by affinity chromatography [5] and further purification, we estimated that the different subunits amounted to 12%, 22%, 27%, and 36%, respectively, in accordance with data presented by Ketterer *et al.* [13]. Activity measurements of the different homodimers show that the mu 4–4 isoform is most effective in the metabolism of alkyl nitrites to form GSNO (Table 1). From the subunit composition and the data presented, it can be calculated that subunit 4 may be responsible for 85% of the activity of soluble GST for GSNO formation in rat liver. For CDNB, the different subunits showed no marked preference (Table 1) [13]. Similarly, the mu 4–4 isolated from rat heart shows high specific activity with alkyl nitrites. In this organ, we measured the relative amount of subunit 4 to be higher than in liver (63%; *cf.* [7]). Although the activities determined for the liver and heart isoenzymes differ to some extent (Table 1), both enzymes have been shown to be identical [7]. In this earlier study, the transferase 4–4 from heart and liver exhibited similar activity towards a large variety of different substrates [7].

Table 2 shows GST activities with CDNB and amyl nitrite as the substrates in different organs of the rat. The liver, the major xenobiotic-metabolizing organ, shows the highest activity with both substrates. Lung and heart have considerable activity with amyl nitrite compared to GST activity with CDNB, the activity ratios being 6.2 and 5.7, respectively. In kidney, intestine, and brain, these values are much lower, and in erythrocytes, enzyme-catalyzed GSNO formation is below detection levels.

TABLE 1. Specificity of different GST isoenzymes towards CDNB and alkyl nitrites in rat liver and heart

	GST activity (μmol/min · mg)		
Isoenzyme	**CDNB**	**Amyl nitrite**	***n*-Butyl nitrite**
Liver			
1–1	25 ± 1.2	13 ± 1.0	16 ± 2.6
2–2	17 ± 4.6	11 ± 2.6	13 ± 3.3
3–3	20 ± 9.0	10 ± 1.9	11 ± 1.4
4–4	7 ± 1.4	100 ± 4.7	106 ± 11.0
Heart			
4–4	11 ± 0.3	55 ± 4.6	51 ± 4.6

Values are mean ± SEM (N = 3–5).

TABLE 2. GST activities towards CDNB and amyl nitrite in different organs of the rat

	GST activity (nmol/min · mg)		
Organ	**CDNB**	**Amyl nitrite**	**Amyl nitrite/ CDNB**
Liver	610 ± 30	2320 ± 320	3.8
Kidney	165 ± 10	274 ± 10	1.7
Intestine	135 ± 7	91 ± 9	0.7
Lung	78 ± 5	486 ± 46	6.2
Heart	68 ± 7	390 ± 30	5.7
Brain	54 ± 7	131 ± 5	2.4
Erythrocyte	6 ± 1	ND	ND

Values are mean ± SEM (N = 4). ND, not detectable.

In addition to rat heart, GST subunit 4 is a major component of soluble GSTs in rat lung, but only a minor component in kidney [13, 14], brain [15], or intestine [16]. Concurrently, GSNO formation is higher in lung and heart than in the other organs (Table 2), although GST activity with CDNB in kidney is 2–3-fold higher than in lung and heart. The major GST components in rat kidney are the alpha subunits 1 and 2 [13, 14], which are active towards CDNB but contribute less to alkyl nitrite metabolism (Table 1). A further constituent of rat kidney is the pi subunit 7 [13, 14, 17]. This isoform is of minor importance for amyl nitrite metabolism, as demonstrated by the low activity ratio found in rat intestine (Table 2), in which GST 7-7 is the major isoenzyme, representing approximately 65% of cytosolic GST [16].

No detectable GST activity with amyl nitrite is seen in rat erythrocytes (Table 2). Since subunit 8 predominates in rat erythrocytes [5], it seems that this alpha form is also not very active in GSNO formation.

Despite the similarities between different subunits within one class, their substrate specificity may be at great variance. This is shown here for the rat mu isoforms 3–3 and 4–4, which have an amino acid sequence similarity of approximately 80% (see [18]) but differ by a factor of 30 in their relative activities towards amyl nitrite and CDNB (Table 1). A similar phenomenon has been observed with human GST isoenzymes. Meyer *et al.* [1] showed that GSTM1a-1a and GSTA1-1 purified from human liver exhibit high activities with alkyl nitrites. GSTA2-2, however, is 15–100-fold less active than GSTA1-1, although the amino acid sequences of the two alpha forms are identical to approximately 96% [19]. Moreover, the rat alpha subunits 1 and 2, which have 76% similarity to human GSTA1-1 [18, 19], exhibit low activity towards alkyl nitrites compared to human GSTA1-1. Thus, the metabolism

of test compounds in human and rat organs containing these GST forms should be distinguished.

In human heart, the GSTM1a form is of importance for alkyl nitrite metabolism, since the GST alpha level is negligibly low in this target organ [20]. Three mu forms were described to be present and were identified as M1a–1a, M1a–2, and M2–2 on the basis of their isoelectric points and N-terminal amino acid sequences [20]. Whether the GSTM2 form is active towards alkyl nitrites is not known at present. The pi isoenzyme, the predominant GST in human heart (60–80%) [20], shows no activity towards amyl nitrite [1].

In rabbit aorta, the pharmacological effect of the vasodilator glyceryl trinitrate was described to be dependent on GST activity [21, 22] and correlated with the mu class enzyme [23]. Tsuchida *et al.* [20] investigated the GST-catalysed denitrification of glyceryl trinitrate [24] with different human isoenzymes isolated from liver, aorta, and heart. Human liver GSTM1a-1a and GSTA1-1 showed the highest activity towards glyceryl trinitrate (1.08 U/mg and 0.85 U/mg, respectively), with GSTA2-2 exhibiting much lower activity (0.17 U/mg); for GSTA1-2, an intermediate activity was measured (0.48 U/mg). No detectable activity was found with GSTP1-1 from human placenta (<0.01 U/mg). Thus, while metabolic rates are lower than those recently described for alkyl nitrites [1], the subunit specificity for glyceryl trinitrate is similar, in spite of structural differences between the nitrate and nitrite esters. Furthermore, as for alkyl nitrites, the GSTM1a form is important for glyceryl trinitrate metabolism in heart tissue, because glyceryl trinitrate is not metabolised by GSTM2-2 [20]. In human aorta, two mu class GSTs different from the liver and heart enzymes were found to be present. Interestingly, both showed enzymic activity towards glyceryl trinitrate. These data indicate that other mu forms exist that are able to metabolise glyceryl trinitrate and possibly exhibit alkyl nitrite metabolising activity.

Considering the GST composition in human lung, the mu class enzyme could also be of major importance in this organ [14, 25]. The question arises as to whether lung tissue may form GSNO upon inhalation of alkyl nitrites and release NO as a nitrosothiol into the circulation, or whether amyl nitrite, at least in part, escapes lung metabolism and reaches the heart, generating GSNO at this site. It has been reported that GSNO is endogenously formed in human airways [26] and that S-nitrosoalbumin and S-nitrosohemoglobin may act as important stable transport molecules for NO in blood [27–29].

A polymorphism of the GST mu in humans has been described, which may be of relevance for the metabolism of the alkyl nitrites. Approximately half of the population lacks GSTM1. Administration of amyl nitrite to heart-diseased patients belonging to the null phenotype could lead to retarded metabolism of this drug. Whether its therapeutic effect is dependent on GST activity is not known. If so, higher doses would be required, thereby potentiating side effects. On the other hand, lack of GSTM1 could be favorable if the drug functions via spontaneous nonenzymatic release of NO [30].

Spontaneous release of NO may have toxicological implications in the erythrocyte [29], in which amyl nitrite is not readily metabolised due to low GST levels. In these cells, the nonenzymatic release of the highly reactive NO radical could become detrimental by reaction with molecular oxygen or superoxide anion to form toxic agents such as peroxynitrite. Such a process offers an additional explanation for the oxidation of hemoglobin to metHb by amyl nitrite, currently attributed to elevated nitrite levels in blood after application of this drug.

We thank Therese Ringwelski for technical assistance. This work was supported by Deutsche Forschungsgemeinschaft grant Ak 8/2-1 and by a fellowship from Krebsforschung International e.V., Düsseldorf, to Y. J.

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