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High-performance liquid chromatography-based assay for glutathione transferase theta 2 activity: Application to characterize interindividual variability in human liver fractions

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ABSTRACT

Human glutathione transferase T2-2 (GSTT2-2) is one of the enzymes considered to play a role in inactivation of toxicants and carcinogens. The expression level of this enzyme is determined by genetic and environmental factors, which may lead to differences in susceptibility. As a specific assay for GSTT2-2 so far a spectroscopical assay based on GSH-conjugation of menaphthyl sulfate (MSu) was used. This spectrophotometric assay, however, appeared too insensitive to accurately quantify the GSTT2-2 activities in a panel of 20 human liver samples. More recently, expression levels of GSTT2-2 in biological samples are quantified by measuring mRNA levels. Since mRNA-levels do not always correlate well with enzyme activity, a specific and sensitive assay is required. In the present study a highly sensitive high-performance liquid chromatography (HPLC)-based method was developed. By applying the new method, firstly, the specificity of GSTT2-2 among 15 recombinant human GST isoforms in catalyzing GSH-conjugation of MSu was confirmed. In addition, a 65-fold inter-individual variation of GSTT2-2 activity was found from the individual liver fractions. By applying the method to individual liver fractions, a 65-fold inter-individual variation of GSTT2-2 activity was found. As a second application, the role of GSTT2-2 in GSH-conjugation of the environmental carcinogen 1-methylpyrene sulfate (MPS) was studied by correlation analysis with GSTT2-2-catalyzed MSu conjugation. The relatively poor correlation suggested that other GSTs also contribute to MPS-conjugation, as confirmed by incubations with recombinant GSTs.

1. Introduction

Human glutathione transferases (GSTs) are important phase II enzymes ubiquitously expressed in different tissues. Because they catalyze the inactivation of electrophiles and hydroperoxides using the cofactor glutathione (GSH), they play an important protective role in the elimination of potentially toxic and carcinogenic chemicals, including drugs, pesticides, and environmental pollutants. Based on their subcellular localizations, human GSTs can be divided into three subclasses namely cytosolic GSTs, microsomal GSTs and mitochondrial GSTs [1]. The cytosolic human GSTs are dimeric proteins and are the most extensively-studied subclass regarding their contributions as a protective enzyme system. The human cytosolic GSTs are further divided in seven distinct classes: Alpha (A), Mu (M), Pi (P), Theta (T), Omega (O), Sigma (S), and Zeta (Z). Amongst them, the GST theta class enzymes are relatively less studied compared to the major cytosolic GSTs. However, the genetic polymorphisms of Theta class GSTs have been associated with increased risk for chemical-induced DNA-damage which my lead to increased risk of cancers [2].

The human Theta class GST is composed of two isoforms, GSTTT1-1 and GSTT2-2. These GSTs differ from the other cytosolic enzymes by having a relatively low affinity to GSH, which explains

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; GSH, glutathione; GST, glutathione transferase; $HClO_4$, perchloric acid; HLC, human liver cytosol; HPLC, high-performance liquid chromatography; IDRs, idiosyncratic drug reactions; MP, 1-methylpyrene; MPdA, N^6-(1-methylpyrenyl)-2′-deoxyadenosine; MPdG, N^2-(1-methylpyrenyl)-2′-deoxyguanosine; MPMA, S-((pyrene-1-yl) methyl) N-acetylcysteine; MPS, 1-methylpyrene sulfate; MP-SG, 1-methylpyrene GSH conjugate; MSG, 1-menaphthyl sulfate GSH conjugate; MSu, 1-menaphthyl sulfate; PAHs, polycyclic aromatic hydrocarbons.

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that they cannot be isolated from tissue extracts by GSH affinity chromatography [3]. Furthermore, GSTT1-1 and GSTT2-2 do not conjugate 1-chloro-2,4-dinitrobenzene (CDNB), which is a general substrate for all other GSTs [4]. Substrates for the theta class GSTs are 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) which is selective for GSTT1-1 and 1-menaphthyl sulfate (MSu) which appeared to be a substrate for GSTT2-2 [4,5]. However, the selectivity of GSH-conjugation of MSu for GSTT2-2 has not been studied with the full spectrum of human GSTs yet. So far, the GSH-conjugation of MSu has been performed by a spectrophotometric method [6]. Although this method has advantages of low-cost lab facilities and quick assay duration, disadvantages are the lacking of sensitivity and accuracy with complicated matrices. These disadvantages limit its application to samples with low GSTT2-2 expression levels, such as cell lines. As alternative methods to determine GSTT2-2 expression levels, mRNA levels and western blot assays are used [7,8]. However, it has been demonstrated that mRNA-levels of drug metabolizing enzymes sometimes poorly correlate with protein levels and enzyme activity [9,10]. Furthermore, western blot analysis is not able to distinguish between active and inactive enzymes. Therefore, a more sensitive method for measuring of GSTT2-2-catalyzed GSH-conjugation of MSu is required.

Although it has been demonstrated by immunochemical methods that human GSTT2-2 is expressed in multiple organs, the expression level and variability of GSTT2-2 activity in human tissues is still poorly studied. Inter-individual variability of GSTs is generally considered to have a significant impact on internal exposure to reactive drug metabolites, which may thereby affect susceptibility to toxicity and carcinogenicity [1]. Although in contrast to GSTT1-1, no null genotype has been identified for *GSTT2*, a deletion of the neighboring *GSTT2b* pseudogene was shown to result in a very strong decrease in the enzyme expression level [11]. This deletion has been associated with an increased risk of esophageal squamous cell carcinoma [12]. Furthermore, a G537A mutation in the promotor area of *GSTT2*, which leads to lower transcription of *GSTT2*, has been associated with an increased risk for colorectal cancer [13].

Mutagens that have been identified as substrates for GSTT2-2 include N-acetoxy-PhIP, secondary lipid peroxidation products, organic hydroperoxides, and sulfate esters of methylated polycyclic aromatic hydrocarbons (PAHs) [14]. The selectivity towards sulfate esters can be rationalized by the presence of a sulfate binding pocket which was found in the crystal structure of GSTT2-2 [15]. 1-Methylpyrene (MP) is one of the methylated polycyclic aromatic hydrocarbons and has been detected in cigarette smoke at levels exceeding that of benzo[α]pyrene [16,17]. As the ultimate carcinogen of MP, 1-methylpyrene sulfate (MPS) is formed by sequential benzylic hydroxylation and sulfation by catalysis of sulfotransferases. MPS has been shown to bind covalently to DNA forming N^2-(1-methylpyrenyl)-2′-deoxyguanosine (MPdG) and N^6-(1-methylpyrenyl)-2′-deoxyadenosine (MPdA) in *in vitro* and *in vivo* experiments [18]. The excretion of S-((pyrene-1-yl) methyl) N-acetylcysteine (MPMA) in rat urine [19] indicated an involvement of GSH-conjugation pathway *in vivo*. Although several non-reactive sulfate esters of methylated PAHs have been tested with GSTT2-2, the role of human GSTs in detoxifying MPS has not been characterized yet.

The major aims of the present study were, i) to establish a sensitive HPLC-UV-based method to enable accurate quantification of human GST T2-2 activity using MSu as a substrate; ii) to apply this method for the characterization of the specificity in catalyzing MSu GSH-conjugation by comparing 15 recombinant human GSTs; iii) to determine the inter-individual variation of enzyme activity and to estimate the expression level of GSTT2-2 in a panel of human liver cytosol (HLC) from 20 different donors; iv) to characterize the role of GSTT2-2 in the detoxification of MPS by applying correlation analysis and recombinant human GSTs.

2. Materials and methods

2.1. Materials

Recombinant human GST isoforms A1-1, A2-2, A3-3, A4-4, M1-1, M2-2, M3-3, M4-4, P1-1*A, P1-1*B, P1-1*C, P1-1*D, K1-1, T1-1, and T2-2 were expressed and purified according to the protocols published previously [20]. Pooled HLC was purchased from BD Biosciences (Breda, The Netherlands). Individual HLC was prepared from liver fractions from 20 donors kindly provided by Kaly-Cell (Strassbourg, France). MSu and S-(1-menaphthyl) glutathione (MSG) were synthesized according to previously described methods [21,22]. MPS was kindly provided by Prof. Hansruedi Glatt (German Institute of Human Nutrition, Berlin, Germany). GSH, ammonium acetate, formic acid and ammonium hydroxide were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

2.2. Analysis of GSH-conjugation of MSu by spectrophotometry and by HPLC-UV

The GSH-conjugation of MSu was determined by the spectrophotometric method as described by [8]. Incubations were performed in a final volume of 1 mL 100 mM Kpi buffer pH 7.4 (prepared by mixing 100 mM KH_2PO_4 and 100 mM KH_2PO_4 at ratio of 1.98:8.02) containing recombinant human GSTs or HLC (pooled and individual), 5 mM GSH and 100 μM Msu and at an incubation temperature of 37 °C. After starting the reaction by addition of GSH, the increase in absorbance at 298 nm was recorded for 10 min using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Cambridge, England).

The purposed HPLC-UV method, more accurate and sensitive than the spectrophotometric one, was developed to quantify the enzyme activity in incubations with HLC. To this end, incubations as described above were terminated by the addition of 1% perchloric acid ($HClO_4$) (final concentration) and centrifuged at 14,000 rpm to precipitate the proteins. The supernatants were analyzed on a Shimadzu HPLC system consisting of two LC-20AD binary pumps, a SIL-20AC auto-sampler (cooled at 4 °C), and a SPD-20A UV/VIS detector set at 298 nm. Chromatographic separation of analytes was performed with a Luna 5 μm C8 column (50 mm × 3 mm) and a gradient composed of solvent A (10 mM ammonium acetate, pH 8.0 adjusted with ammonium hydroxide) and solvent B (100% acetronitrile). The flow rate was set at 0.5 mL/min and the gradient was programmed as follows: 0–2 min, isocratic 10% B; 2–12.5 min, linear increase from 10% B to 35%B, 12.5–13 min, linear increase from 35% B to 99% B; 13–13.5 min, linear decrease from 99% B to 10% B; 13.5–20 min isocratic at 10% B.

2.3. Isoenzyme selectivity of GST-catalyzed GSH conjugation of MSu

Previous studies on the specificity of GSH-conjugation by GSTT2-2 were performed with an incomplete set of human GSTs. Therefore, in the present study 15 recombinant human GSTs were incubated at a GST concentration of 50 nM with 100 μM MSu at 37 °C for 10 min. The major hepatic GST isoforms, GSTA1-1, A2-2, and M1-1 were also incubated at an enzyme concentration of 100 μM, which is the highest concentration found in HLC [23,24]. All incubations were performed in 100 mM potassium phosphate (KPi) buffer, pH 7.4, in the presence of 5 mM GSH at a final volume of 200 μL in duplicate. Incubations were initiated by the addition