RESEARCH COMMUNICATION

The human phenolsulphotransferase polymorphism is determined by the level of expression of the enzyme protein

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We have examined the expression of platelet phenolsulphotransferase (PST) in 60 individuals. Using an antibody which recognizes both forms of PST present in man (P-PST and M-PST), we determined that the polymorphism of platelet P-PST

activity is determined by the level of expression of the enzyme protein. The implications for susceptibility to adverse drug reactions and chemical carcinogenesis are discussed.

INTRODUCTION

Sulphation is one of the major drug-metabolizing pathways and is mediated by a cytosolic enzyme family, the sulphotransferases (STs). STs conjugate a structurally diverse group of both endogenous and exogenous catechols, phenols and alcohols [1,2], including many drugs (e.g. paracetamol, methyl dopa), other xenobiotics (e.g. food additives, carcinogens) and neurotransmitters (e.g. dopamine, noradrenaline and 5-hydroxytryptamine). In fact, approx. 80 % of total plasma catecholamines are sulphotransferase conjugated [3].

Phenolsulphotransferase (PST, EC 2.8.2.1) catalyses the transfer of the sulphate moiety from the active donor molecule 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to an appropriate acceptor compound, forming a sulphate ester. The addition of this charged sulphate group in general decreases the biological activity and increases the polarity relative to the parent compound, thereby facilitating excretion [4]. PST is present in many human tissues, including the easily accessible blood platelets. Platelets and other tissues contain at least two isoenzymes of PST, forms that have been designated the 'phenol-sulphating' (P-PST) and the 'monoamine-sulphating' (M-PST) forms [5–8]. M-PST catalyses the sulphation of micromolar concentrations of dopamine and other catechol or phenolic monoamines, and is resistant to inhibition by 2,6-dichloro-4-nitrophenol (DCNP), whereas P-PST catalyses the sulphate conjugation of micromolar amounts of phenol, 4-nitrophenol and other 'simple' phenols, and is sensitive to inhibition by DCNP [9,10]. M-PST has a subunit molecular mass of 34 kDa, and P-PST has a subunit molecular mass of 32 kDa. Both exist as homodimers *in vivo* [7,11].

Studies in our, and other, laboratories [12,13] have demonstrated that PST activity, and P-PST activity in particular, varies widely within the normal human population and is the subject of a genetic polymorphism. The availability of an antibody [14] that recognizes human PSTs has enabled us to study the molecular basis of this important polymorphism. Consequently, we report here the novel and significant finding that this wide variation in human platelet P-PST activity is determined by the level of P-PST enzyme protein expression.

EXPERIMENTAL

Materials

PAP[³⁵S]S (1.5–2.54 Ci/mmol) was purchased from du Pont/NEN, Stevenage, Herts., U.K. Unlabelled PAPS, barium acetate, Ba(OH)₂, ZnSO₄ and peroxidase-conjugated goat anti-(rabbit IgG), adsorbed with human serum proteins, were from Sigma Chemical Co., Poole, Dorset, U.K., and vanillin was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. 4-Nitrophenol was from Fluka Chemicals, Glossop, Derbys., U.K., and ECL Western blotting detection reagents were obtained from Amersham, Aylesbury, Bucks., U.K. F.p.l.c. instrumentation and columns were from Pharmacia, Milton Keynes, U.K. Scintillation fluid (Emulsifier Safe) was from Canberra Packard, Pangbourne, Reading, Berks., U.K. All other reagents were of analytical grade and were purchased from commonly used local suppliers.

Subjects and platelet preparation

ST activities were measured in platelet homogenates from 60 unrelated individuals. Of these subjects, 41 were female and 19 were male. The mean age was 39.1 ± 16.7 years (range: 16–83 years). Ethical approval was obtained from the Committee on Medical Ethics, Tayside Health Board.

A 50 ml sample of blood was collected from subjects (with their informed consent) by venepuncture into 1 ml of 5 % sodium EDTA. Samples were maintained at room temperature, and platelets were processed within 2 h. Blood was centrifuged at 300 *g* for 10 min, and the platelet-rich plasma was carefully aspirated and re-centrifuged at 4000 *g* for 20 min. The resulting platelet pellet was washed three times with 0.9 % (w/v) NaCl and resuspended in 5 mM potassium phosphate buffer, pH 7.5, prior to homogenization by freezing and thawing three times. Platelet homogenates were stored at –70 °C until assayed [within 2 months; enzyme activity was stable within this period (results not shown)].

PST enzyme assay

PST activity was measured using a modification of the radio-

Abbreviations used: ST, sulphotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; PST, phenolsulphotransferase; P-PST, phenol-sulphating phenolsulphotransferase; M-PST, monoamine-sulphating phenolsulphotransferase; DCNP, 2,6-dichloro-4-nitrophenol; TBS, 154 mM NaCl/10 mM Tris/HCl, pH 7.4; TBST, TBS containing 0.1 % Tween 20.

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enzymic method of Foldes and Meek [15], with PAP[³⁵S]S as the sulphate donor. All assays were carried out in duplicate, and a blank reaction, where substrate was replaced by 20 μ l of water, was performed for each pair of assays. Dopamine, tyramine, 5-hydroxytryptamine, vanillin and 1-naphthol were employed as substrates. The incubation mixture comprised 20 μ g of platelet homogenate protein and potassium phosphate buffer (10 mmol/l, pH 7.4) to a volume of 110 μ l and either 20 μ l of substrate solution or 20 μ l of water for blanks. The reaction was initiated by adding 20 μ l of PAP[³⁵S]S and non-radioactive PAPS (dissolved in distilled water). After incubation for 10 min at 37 °C the reaction was terminated by the addition of 200 μ l of 0.1 M barium acetate. PAPS that had not reacted was removed by precipitation with 200 μ l of 0.1 M Ba(OH)₂ and 200 μ l of 0.1 M ZnSO₄. After centrifugation at 15000 *g* for 5 min, 500 μ l of supernatant was removed to a scintillation vial. After the addition of 4 ml of scintillation fluid and thorough mixing, radioactivity in the reaction product was quantified by liquid-scintillation spectrometry. Results are expressed as pmol of product formed/min of incubation per mg of protein.

F.p.l.c. analysis of platelet cytosol

Human platelet cytosols were subjected to anion-exchange chromatography using F.p.l.c. (Pharmacia) for the purpose of resolving different the M- and P-PST isoenzymes. The anion-exchange column (MonoQ; HR5/5) was equilibrated with 20 mM triethanolamine/HCl, pH 7.4, containing 3 mM 2-mercaptoethanol, at a flow rate of 1 ml/min.

From a given subject, 50 ml of blood was collected and platelets prepared as described. The platelet pellet was resuspended in equilibration buffer prior to homogenization and centrifugation at 105000 *g* for 30 min. The resulting platelet cytosol (2 ml; approx. 6 mg of protein) was injected on to the MonoQ column and, after the elution of unbound material with 10 ml of equilibration buffer, 10 ml of 100 mM NaCl in the equilibration buffer was applied. A linear gradient between 100 and 250 mM NaCl in the equilibration buffer was then applied to the column, and the 1 ml fractions collected were immediately placed on ice after elution from the column. Total protein was monitored continuously by measuring the absorbance of the column eluate at 280 nm and fractions were assayed for P-PST and M-PST activities as described above.

Immunoblot analysis

In order to assess the levels of expression of PST isoenzyme in platelets, immunoblot analysis was employed. The antibody used was an anti-(rat oestrogen ST) antibody (IgG fraction) [14] which had been adsorbed with human serum proteins. Samples (100 μ g) of human platelet homogenates and f.p.l.c. fractions were subjected to SDS/PAGE using the method of Laemmli [16] in an 11.5 % polyacrylamide (monomer) gel. Proteins were then electrophoretically transferred to nitrocellulose (Schleicher and Schuell) [17]. The nitrocellulose was blocked with 1 % BSA in TBS containing 0.1 % Tween 20 (TBST) for 1 h. Incubation with both primary and secondary antibodies was performed in TBST containing 1 % BSA at room temperature. Anti-(rat oestrogen ST) IgG was incubated with the nitrocellulose for 1 h and washes between antibody incubations were in TBST as previously described [18]. The nitrocellulose membranes were incubated for 1 h with the peroxidase-conjugated secondary antibody (adsorbed against human serum proteins) at a dilution of 1:2000. Enhanced chemiluminescence detection was employed to reveal antigen-antibody interactions and was performed as described

by the manufacturer (Amersham International). To facilitate quantification of enzyme protein, pre-flashed X-ray film was used.

PST enzyme protein on immunoblots was quantified as follows: a scanned image of individual immunoblots was made using a desktop scanner (Hewlett-Packard ScanJet IIc) and the images analysed using the QuantiScan densitometric software (Biosoft, Cambridge, U.K.) on an MJN PC486 personal computer. This process has been validated, as described previously [18a].

Protein determination

The protein content of platelet homogenates was estimated using the Lowry method, with BSA as standard [19].

RESULTS AND DISCUSSION

Platelet M-PST and P-PST enzyme activities did not show any significant change with age, and mean values did not differ significantly between males and females, observations which agree with those of others [20,21] (results not shown). Platelet M-PST and P-PST activities from different individuals demonstrated an approx. 15-fold and 6-fold variation in specific activity respectively. The frequency distribution of platelet P-PST enzyme activity (Figure 1) did not fit a normal distribution, and appeared rather to be at least trimodal. If one considers there to be three groups within the population comprising individuals with low specific activity (< 5 pmol/min per mg), intermediate (6–12 pmol/min per mg) and high (> 12 pmol/min per mg) P-PST activity, then the proportions of individuals within these three groups approximate to 1:2:1, suggesting that this may be due to the occurrence of two equally frequent alleles which code for low- and high-activity proteins, with the three groups corresponding to the two groups of homozygotes and the middle group with intermediate enzyme activity being the heterozygotes.

An anti-(rat oestrogen ST) antibody preparation that recognizes human PST proteins on immunoblot analysis (the 32 kDa P-PST and the 34 kDa M-PST) has been prepared in our laboratory. Using this antibody, immunoblot analysis of platelet homogenates from individuals in this study was carried out. The results confirmed the wide inter-individual variation in PST,

Figure 1 Frequency distribution of human platelet P-PST enzyme activity

Platelet homogenates from 60 unrelated individuals were assayed for P-PST enzyme activity with 4-nitrophenol as substrate and the data were plotted as a frequency distribution.

Figure 2 Immunoblot analysis of inter-individual variation in human platelet PST

A representative immunoblot from a series of experiments conducted on 43 subjects. Platelet homogenates (100 μ g protein) from 12 unrelated individuals were subjected to immunoblot analysis with anti-(rat oestrone ST); antibody-antigen interactions were revealed using enhanced chemiluminescence detection (ECL, Amersham). Pre-flashed X-ray film containing the images was photographed and the resulting print scanned using a desktop scanner connected to an IBM-compatible personal computer. The image density of the P-PST-immunoreactive bands was quantified using the Quantiscan software (Biosoft, Cambridge, U.K.), and plotted against the enzyme activity. The correlation coefficient (r^2) for the data was 0.88.

particularly P-PST, expression (Figure 2) and indicated that certain individuals had essentially no P-PST enzyme protein. Figure 2 is a representative blot from a series of experiments on 43 individuals. Upon densitometric analysis of the immunoblots, we demonstrated a strong correlation ($r^2 \cong 0.8$ for each of four blots) between the P-PST enzyme activity as determined with 4-nitrophenol as substrate and immunologically detected P-PST enzyme protein. This strong relationship between P-PST activities and the immunoreactive 32 kDa P-PST polypeptide indicates that variations in platelet P-PST activity between individuals are not due to differences in biochemical properties of P-PST or the presence of endogenous inhibitors, but are a direct consequence of differences in P-PST protein expression. This is the first time that the direct correlation between human platelet P-PST enzyme activity, and in particular the P-PST polymorphism, and enzyme protein expression in a large number of individuals has been reported. Additionally, we found no correlation in human platelet homogenate between P-PST and M-PST activities and enzyme protein levels (results not shown), indicating that these two isoenzymes are under distinct regulatory control.

The dramatic difference in P-PST expression between those with high and low P-PST activity is illustrated in Figure 3, which shows the f.p.l.c. anion-exchange chromatography and subsequent immunoblot analysis of platelet cytosol from individuals with high (Figure 3a) and low (Figure 3b) P-PST activity. The individual with low P-PST has almost no P-PST enzyme protein, suggesting that the residual P-PST enzyme activity (3.0 pmol/min per mg in this individual; normally less than 5 pmol/min per mg

Figure 3 F.p.l.c./anion-exchange analysis of PST enzyme activity in individuals with high and low P-PST

Platelet cytosols from individuals with high (a, specific activity 23 pmol/min per mg with 4-nitrophenol as substrate) and low (b, specific activity 3 pmol/min per mg with 4-nitrophenol as substrate) P-PST enzyme activity were resolved on a MonoQ column attached to an f.p.l.c. system as described in the Experimental section. Fractions from both f.p.l.c. runs were assayed for P-PST (4-nitrophenol as substrate) ($\circ$) and M-PST (vanillin as substrate) ($\bullet$). ST activity and selected fractions from each run were subjected to immunoblot analysis with our anti-(rat oestrone ST) antibody preparation to show the distribution of the M-PST (34 kDa) and P-PST (32 kDa) isoenzymes.

in individuals with essentially no P-PST enzyme protein) may in fact be principally due to the overlapping substrate specificity of another PST isoenzyme, possibly M-PST. We routinely see two peaks of both P-PST and M-PST activity when this analysis is performed, and all peaks react with our anti-(rat oestrogen ST) antibody. The question of whether or not these peaks of enzyme all represent distinct gene products will have to await the results of molecular-biological studies currently underway in several laboratories, including our own.

Human platelet P-PST activity correlates with levels of P-PST in other tissues such as liver, small intestine and cerebral cortex [22–24]. Polymorphism of P-PST may therefore be associated with individual susceptibility to toxicity after exposure to xenobiotics conjugated by this enzyme, including many drugs and

environmental carcinogens. It has been previously suggested that individual differences in the sulphate conjugation of drugs are related to pharmacogenetic variations in PST activity [25–27]. However, sulphation is not just a detoxication process; many procarcinogens, such as aromatic hydroxylamines and hydroxamic acids, and the benzylic alcohols of polycyclic aromatic hydrocarbons, form sulphate esters which are highly reactive, adduct to DNA and are mutagenic [28–30]. Recent work has demonstrated that in man, P-PST is one of the major ST isoenzymes involved in the sulphation of aromatic hydroxylamines and hydroxamic acids (R. A. H. J. Gilissen, K. J. Bamforth, J. F. C. Stavenuiter, M. W. H. Coughtrie and J. H. N. Meerman, unpublished work). In this case, individuals with high levels of P-PST may be at significantly higher risk of forming reactive, mutagenic metabolites after exposure to such chemicals.

The findings described here demonstrate that the basis for the P-PST polymorphism is the level of the corresponding enzyme protein. This work paves the way for studies into the molecular basis of this polymorphism; examination of the inheritance of P-PST in extended families will form an important part of such investigations, and these studies are underway in our laboratory. More importantly, however, these findings will allow the assessment of the clinical significance of the level of p-PST expression in determining individual susceptibility to, for example, adverse reactions to phenolic drugs and chemical carcinogenesis after exposure to aromatic amines.

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