INVIOLMENT OF CYTOCHROMES P-450 2E1 AND 3A4 IN THE 5-HYDROXYLATION OF SALICYLATE IN HUMANS

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ABSTRACT:

Hydroxylation of salicylate into 2,3 and 2,5-dihydroxybenzoic acids (2,3-DHBA and 2,5-DHBA) by human liver microsomal preparations was investigated. Kinetic studies demonstrated that salicylate was 5-hydroxylated with two apparent Km values, one high-affinity Km of 606 μM and one low-affinity Km greater than 2 mM. Liver microsomes prepared from 15 human samples catalyzed the formation of 2,5-DHBA at a metabolic rate of 21.7 ± 8.5 pmol/min. The formation of 2,3-DHBA was not P-450 dependent. Formation of 2,5-DHBA was inhibited by 36 ± 14% following preincubation of microsomes with diethyldithiocarbamate, a mechanism-based selective inhibitor of P-450 2E1. Furthermore, the efficiency of inhibition was significantly correlated with four catalytic activities specific to P-450 2E1, whereas the residual activity was correlated with three catalytic activities specific to P-450 3A4. The capability of seven recombinant human P-450s to hydroxylate salicylate demonstrated that P-450 2E1 and 3A4 contributed to 2,5-DHBA formation in approximately equal proportions. The Km values of recombinant P-450 2E1 and 3A4, 280 and 513 μM, respectively, are in the same range as the high-affinity Km measured with human liver microsomes. The plasmatic metabolic ratio 2,5-DHBA/salicylate, measured 2 h after ingestion of 1 g acetylsalicylate, was increased 3-fold in 12 alcoholic patients at the beginning of their withdrawal period versus 15 control subjects. These results confirm that P-450 2E1, inducible by ethanol, is involved in the 5-hydroxylation of salicylate in humans. Furthermore, this ratio was still increased by 2-fold 1 week after ethanol withdrawal. This finding suggests that P-450 3A4, known to be also inducible by alcoholic beverages, plays an important role in this increase, because P-450 2E1 returned to normal levels in less than 3 days after ethanol withdrawal. Finally, in vivo and in vitro data demonstrated that P-450 2E1 and P-450 3A4, both inducible by alcohols, catalyzed the 5-hydroxylation of salicylate.

Aspirin (O-acetylsalicylic acid) is widely used as an analgesic drug, often for self-medication, and as an anti-inflammatory agent in the treatment of rheumatoid arthritis in humans. Furthermore, there is also a great interest in the use of aspirin as a prophylactic agent against thrombotic vascular diseases. After ingestion, a substantial amount of aspirin is hydrolyzed to salicylic acid (SA) by esterases in the gastrointestinal tract, in the liver, and to a smaller extent, in serum (Leonards, 1962; Coudray et al., 1995). SA is further metabolized by conjugation to glycine to form salicyluric acid, by microsomal hydroxylases to form gentisic acid [2,5-dihydroxybenzoic acid (2,5-DHBA)] and by conjugation to glucuronic acid. About 60% of SA remains unmodified and can undergo the attack of highly reactive hydroxyl radical (’OH) to produce 2,3-dihydroxybenzoic acid (2,3-DHBA) (Grootveld and Halliwell, 1988; Halliwell et al., 1991; Halliwell and Kaur, 1997), and to a smaller extent, catechol, both of which have not been reported as products of enzymatic metabolism. It has been suggested that formation of 2,3-DHBA from salicylate is a mean of monitoring ’OH formation in vivo (Coudray et al., 1995; Halliwell et al., 1991; Halliwell and Kaur, 1997; Thome et al., 1997).

In contrast, 2,5-DHBA may arise from metabolism of salicylate by enzymes of endoplasmic reticulum (Ingelman-Sundberg et al., 1991). But the cytochrome(s) P-450 involved in the 5-hydroxylation of SA have not been characterized yet in humans. Therefore, this article deals with the demonstration of involvement of both P-450 2E1 and P-450 3A4, which are quantitatively important in the human liver (Guengerich and Turvý, 1991; Shimada et al., 1994) in the 5-hydroxylation of salicylate.

Materials and Methods

All reagents were of analytical grade. SA, 2,3-, and 2,5-dihydrobenzoic acids, 3,4-dihydroxybenzoic acid (3,4-DHBA), NADPH, diethyldithiocarbamate (DEDTC), troleandomycin (TAO), desferrioxamine (DFO), and DEDTC were from Sigma-Fluka-Aldrich (Saint-Quentin Fallavier, France).

In Vitro Studies. Microsomal samples. Human liver microsomes belong to a microsome bank set up in our laboratory for many years (Berthou et al., 1991). Their specific content and monoxygenase activities have been previously characterized, especially for P-450 2E1 (4-nitrophenol-2-hydroxylation
and chlorzoxazone 6-hydroxylation (Zerilli et al., 1997), N-nitrosodimethylamine N-demethylation (Bellec et al., 1996), butanol oxidation (Lucas et al., 1993) and P-450 3A4 [nifedipine oxidation, buprenorphine N-dealkylation (Iribarne et al., 1997), tamoxifen N-demethylation (Jacolot et al., 1991)].

In addition, microsomes from human B-lymphoblastoid cell lines or insect cells baculovirus transfected with human P-450 2E1 (M106k and P206, respectively) and 3A4 (M107r and P202, respectively) cDNAs plus NADPH oxidoreductase (OR) and cytochrome b5 (b5) were purchased from Gentest Corp. (Woburn, MA).

**Hydroxylation of SA by microsomal P-450s.** The Km of P-450 for 5-hydroxylation of salicylate was evaluated in microsomal human liver samples with salicylate concentrations ranging from 100 to 3000 μM and was calculated using the Eadie-Hofstee representation.

To determine salicylate hydroxylation by human liver microsomes, incubations were carried out in a final volume of 1 ml containing potassium phosphate buffer 0.15 M (pH 7.4) microsomal proteins (1 mg), SA adjusted to pH 7.4 (0.6 and 2.5 mM), NADPH (1 mM), and DFO (100 μM). To inhibit the in vivo formation of hydroxyl radical, which can occur in the presence of contaminating iron ions, buffers were pretreated on a Chelex column retaining contaminating metal ions (Chelex 100; Sigma) and reaction mixtures also contained DFO (100 μM), as described by Ingelman-Sundberg et al. (1991). Reaction mixtures were incubated at 37°C for 30 min and the reaction was stopped by adding 75 μl of 12 N HCl. Then, 3,4-DHBA was added as internal standard (500 ng dissolved in HPLC mobile phase), and extraction was carried out using 4 ml diethylether. After evaporation to dryness under nitrogen, samples were reconstituted in mobile phase (200 μl), 2,3-and 2,5-DHBA were measured by HPLC analysis-amperometric detection.

To determine salicylate hydroxylation by human recombinant cytochrome P-450 enzymes, microsomes from human B-lymphoblastoid or insect cells expressing human P-450 2E1 or P-450 3A4 (from Gentest) were used. Recombinant P-450 2E1 and P-450 3A4 from human B-lymphoblastoid were supplemented with cytochrome b5 (Oxford Biomedical Research, Oxford, MI) in the molar ratio of 2:1 for h5P-450 2E1. The reaction was conducted as described for the human liver microsomes with 0.25 mg of microsomal proteins. The rate of formation 2,5-DHBA was linear for up to 30 min and 1 mg of microsomal proteins.

**Chemical inhibitors.** DEDTC and TAO are known to be mechanism-based inhibitors of P-450 2E1 (Guengerich et al., 1991) and P-450 3A (Rodrigues, 1994; Newton et al., 1995), respectively. DEDTC was preincubated at a concentration of 0.3 mM with microsomal proteins and NADPH 1 mM for 10 min before the addition of SA (600 μM), whereas TAO was used in the same conditions at a concentration of 50 μM. Metabolic rates were compared with corresponding controls including preincubation steps.

**In Vivo Studies.** SA 2.3, and 2,5-DHBA were measured in the plasma of control (n = 15) and alcoholic subjects (n = 12) 2 h after a single oral dose of 1 g of lysine acetylsalicylate (Aspegic, Synthélabo, de Plessis-Robinson, France). Alcoholic patients (daily consumption of alcohol 148 ± 46 g) were entering the hospital for a detoxification period. They were tested at the beginning of their stay in hospital after overnight ethanol abstinence (day 0) to avoid the presence of ethanol in blood and at 1 week (day 7) of ethanol withdrawal. This protocol was approved by the ethical committee of the Centre Hospitalier Universitaire of Brest (France) and all subjects gave their informed consent.

Plasma samples (500 μl) were mixed with 100 μl of HCl N and 30 ng of 3,4-DHBA dissolved in the mobile phase. Samples were then extracted by 5 ml of diethylether. After evaporation, the dry residue was dissolved in 200 μl of HCl 0.2 N. SA and DHBA were measured using HPLC-UV or amperometric detection, respectively.

**HPLC Analysis.** Determination of 2,3- and 2,5-DHBA. These compounds were separated by HPLC using a Ultrasphere ODS column (particle diameter 5 μm, 250 × 4.6 mm; Beckman, Gagny, France) after injection of 20 μl of the samples. The mobile phase consisted of 30 mM sodium citrate/27.7 mM sodium acetate, pH adjusted to 3.55 with orthophosphoric acid/methanol (95:5, v/v). The flow rate was 1 ml/min. The HPLC system was equipped with a BAS-IC-4A electrochemical amperometric detector (Bioanalytical Systems, West Lafayette, IN) equipped with a glassy carbon working electrode operating at +0.7 V against a Ag/AgCl reference electrode and a detection range of 10 nA.

**Determination of SA.** Samples (20 μl) were applied on a Nucleosil C18 column (particle diameter 5 μm, 250 × 4.6 mm, Interchim, Montluçon, France). SA was detected using an UV detector at 236 nm. The mobile phase consisted of 30 mM sodium citrate/27.7 mM sodium acetate, adjusted at pH 3.5 with orthophosphoric acid/methanol (82:18, v/v). The flow rate was 1 ml/min. Quantification was achieved using standard curves constructed from measurements of peak area ratios.

**Statistical Analysis.** Correlation coefficients were calculated using an ANOVA table by the least-squares regression analysis from the raw data (Stat-View, Alsyd, Meylan, France). Because a quite normal Gaussian distribution in the panel of 15 human liver microsomal preparations was observed (skewness = 1.00), correlation coefficients were calculated by including all the samples.

**Results**

**Kinetic Studies.** Two hydroxylated metabolites of salicylate were detected by HPLC when incubated in presence of NADPH and human liver microsomes (Fig. 1). These metabolites were identified by their retention times and their electrochemical properties as 2,3- and 2,5-DHBA. The formation of 2,5-DHBA was dependent upon NADPH. In contrast, production of 2,3-DHBA was very low and could not be clearly related to an enzymatic reaction involving NADPH.

**Correlation Studies and Chemical Inhibitions.** Salicylate was hydroxylated into 2,5-DHBA by human liver microsomes in the presence of NADPH, but this hydroxylation was dramatically decreased when NADPH was omitted. Using 2.5 and 0.6 mM salicylate as substrate concentrations, the mean rate formations of 2,5-DHBA were 47 ± 26 and 21.7 ± 8.5 pmol/min/mg, respectively, for the 15 liver microsomal samples. These two metabolic rates were signifi-
Preincubation of human liver microsomes with DEDTC 0.3 mM inhibited 2,5-DHBA formation by 30% and this inhibition was correlated with P-450 3A activities (Fig. 4). Preincubation of microsomes with TAO inhibited 2,5-DHBA formation by 30% and this inhibition was correlated with P-450 3A activities (Fig. 4). Preincubation of microsomes with DEDTC inhibited 2,5-DHBA formation by 30% and this inhibition was correlated with P-450 3A activities (Fig. 4).

P-450 2E1 catalytic activity was very sensitive to the presence of cytochrome b5; b5, added at a ratio of 2:1 b5/P-450-450 increased the 5-hydroxylation of salicylate by approximately 2-fold. The effect of b5 on the ability of P-450 3A4 to hydroxylate salicylate was somewhat complex. Coexpression of b5 with P-450 3A4 provided a superior turnover when compared with reconstituted systems in which addition of purified b5 had no significant effect on catalytic activity.

Recombinant human P-450 3A4 and P-450 2E1 hydroxylated SA following a monophase Michaelis-Menten kinetics, characterized by apparent Kₘ of 513 and 280 μM and Vₘ of 32 and 19 pmol/min/mg, respectively. These Kₘ values are in the same range as the high-affinity Kₘ of 600 μM measured with human liver microsomes. Therefore, P-450 2E1 and P-450 3A4, the most important liver P-450 isozymes, were demonstrated to participate to the hydroxylation of SA into 2,5-DHBA.

In Vivo Studies. Plasma 2,5-DHBA and SA were measured in 15 controls and in 12 alcoholic patients 2 h after intake of 1 g of aspirin. Due to individual variations in the pharmacokinetics of aspirin, the plasma levels of SA vary largely from one individual to another (42–696 μM; 295 ± 165 μM for 15 controls). Consequently, the concentrations of 2,5-DHBA were corrected for this variation and 2,5-DHBA/SA ratios were reported (Fig. 5). No differences were observed for these ratios between men and women (data not shown). The (2,5-DHBA/SA)-1000 ratio (see Materials and Methods, HPLC Analysis) was dramatically increased in alcoholic patients when entering the hospital: 23.9 ± 10.3 versus 7.82 ± 4.2 in controls (p < .001). This 2,5-DHBA/SA ratio decreased significantly after 1 week of withdrawal: 14.9 ± 10.1 versus 23.9 ± 10.3 (p < .02) (Fig. 5). These results confirm the involvement of P-450 2E1, inducible by alcohol, in the 5-hydroxylation of salicylate.

**Discussion**

Salicylate is metabolized by P-450 enzymes of human microsomal preparations to give 2,5-DHBA. Our results confirm previous findings reported by Ingelman-Sundberg et al. (1991), in rat and rabbit liver microsomal fractions. In contrast, formation of 2,5-DHBA was not NADPH dependent, although it was not totally inhibited by DFO, which inhibits iron-dependent OH generation. Therefore, the rate of 2,5-DHBA was not correlated with P-450 activities. The turnover number was below 0.01 min⁻¹ for seven heterologously expressed human P-450s (data not shown). The affinity of human liver microsomes for salicylate was fairly low, Kₘ about 0.6 mM; this value was in agreement with the value reported for rat liver microsomes, Kₘ about 0.4 mM (Ingelman-Sundberg et al., 1991). This value is in the range of plasmatic salicylate concentration (Leonards, 1962; Day et al., 1988). Another low-affinity Kₘ could be measured but its high value was out of the physiological range. Recombinant human P-450 3A4 and P-450 2E1 hydroxylated SA followed a monophasic Michaelis-Menten kinetics, characterized by apparent Kₘ of 513 and 280 μM, respectively. These Kₘ values are in the same range as the high-affinity Kₘ of 600 μM measured with human liver microsomes.

The nature of P-450 isof orm(s) involved in the 5-hydroxylation of salicylate has not been characterized yet in humans. In a previous study (Ingelman-Sundberg et al., 1991), it has been suggested that there was not great specificity in 2,5-DHBA production in rat.
study demonstrated that both P-450 3A4 and 2E1 are involved in this production in humans. Such a conclusion is based on four kinds of results: correlation studies, P-450 3A4 and 2E1-selective inhibitors, metabolism by heterologously expressed humans P-450, and in vivo results. Because the salicylate hydroxylation presented two apparent \( K_m \), the metabolic rates were measured at the high-affinity constant, specifically 0.6 mM, i.e., in the range of \( K_m \) measured with recombinant human P-450 3A4 and P-450 2E1.

The contribution of P-450 2E1 to the 5-hydroxylation of salicylate was suggested by its inhibition by DEDTC, a mechanism-based inhibitor selective to P-450 2E1. The fraction of inhibition, namely 36\( \pm \)14%, was more highly significantly correlated than the total activity with four catalytic activities specific to P-450 2E1. Moreover, the residual activity following DEDTC preincubation was significantly correlated with three P-450 3A4 activities. The fraction of 5-hydroxylation activity inhibited by TAO (30\( \pm \)12%), a mechanism-based inhibitor selective to P-450 3A4, was correlated with nifedipine oxidation. The selectivity of these two inhibitors, DEDTC and TAO, as mechanism-based inhibitors of P-450 2E1 and 3A4 was checked by incubation of recombinant P-450 2E1 and P-450 3A4, respectively. Preincubation of human liver microsomes with 0.3 mM DEDTC plus 50 \( \mu \)M TAO inhibited the formation of 2,5-DHBA by 69\( \pm \)6% (\( n = 4 \) samples), whereas the formation of 2,3-DHBA was not significantly modified. Because this double inhibition represented approximately the addition of DEDTC and TAO inhibitions, the contribution of the two P-450s inhibited by DEDTC and TAO, P-450 2E1 and 3A4, was confirmed.

The use of seven heterologously expressed P-450 enzymes allowed the determination of which isoforms are involved in salicylate 5-hydroxylation. Two P-450s were significantly involved in the reaction, specifically P-450 2E1 and 3A4, with mean turnover numbers of 0.45 and 0.34 min\(^{-1}\), respectively. On the basis of the relative levels of these P-450s in human liver microsomes (22 and 96 pmol/mg protein of P-450 2E1 and 3A4, respectively; Guengerich and Turv, 1991; Shimada et al., 1994), it can be concluded that the mean metabolic rate of salicylate 5-hydroxylation would be approximately 40 pmol/min/mg. This calculated value is in good agreement with the experimental value determined at 0.6 mM substrate, 21.7\( \pm \)8.5 pmol/min/mg.
Furthermore, the contribution of P-450 2E1 and P-450 3A4 can be estimated to be equivalent, confirming the results of chemical inhibitions. Such an extrapolation, however, is limited by some caveats. First, estimates of P-450 isoform content in human liver are based on immunodetectable proteins and not on active P-450 enzyme content (Guengerich and Turvy, 1991; Shimada et al., 1994). Second, it must be borne in mind that the rough estimates of salicylate 5-hydroxylation activity calculated by the formula (turnover number x liver P-450 isoform content) represent only an average.

Finally, the in vivo results confirm the involvement of P-450 2E1 in the 5-hydroxylation of salicylate. Indeed the 2,5-DHBA/salicylate ratio should reflect both P-450 2E1 and P-450 3A4 activities. Consumption of alcoholic beverages has been shown to increase P-450 3A4 activity measured by the formation of 6β-hydroxycortisol (Hoshino and Kawasaki, 1995). Moreover, the finding that 5-hydroxylation activity was still increased by 2-fold after 1 week of alcohol abstinence suggests that the decrease time of P-450 3A4 after alcohol induction was longer than for P-450 2E1. Indeed, the half-lives of P-450 2E1 and 3A4 have been shown to be approximately 6 to 8 h (Roberts et al., 1995) and 40 h (Muntane-Relat et al., 1995) in absence of substrate, respectively. P-450 2E1 activity was shown to return to normal levels in less than 3 days after alcohol abstinence (Lucas et al., 1995).

References


Additional references and data available in the supplementary material.