CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol

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A B S T R A C T

The dietary phenol tyrosol has been reported to be endogenously transformed into hydroxytyrosol, a potent antioxidant with multiple health benefits. In this work, we evaluated whether tyrosine hydroxylase (TH) and cytochrome P450s (CYPs) catalyzed this process. To assess TH involvement, Wistar rats were treated with α-methyl-L-tyrosine and tyrosol. Tyrosol was converted into hydroxytyrosol whilst α-methyl-L-tyrosine did not inhibit the biotransformation. The role of CYP was assessed in human liver microsomes (HLM) and tyrosol-to-hydroxytyrosol conversion was observed. Screening with selective enzymatic CYP inhibitors identified CYP2A6 as the major isoform involved in this process. Studies with baculosomes further demonstrated that CYP2D6 and CYP3A4 could transform tyrosol into hydroxytyrosol. Experiments using human genotyped livers showed an interindividual variability in hydroxytyrosol formation and supported findings that CYP2D6 and CYP2A6 mediated this reaction. The dietary health benefits of tyrosol-containing foods remain to be evaluated in light of CYP pharmacogenetics.

1. Introduction

Hydroxytyrosol [HT, 2-(3,4-dihydroxyphenyl)ethanol], the main phenolic compound found in olives, virgin olive oil and red wine, is also a product of dopamine oxidative metabolism (DOPET) (Fig. 1A). The health benefits attributed to HT bioactivities include antioxidant, anti-inflammatory, cardioprotective, antitumor, antimicrobial, anti-diabetic and neuroprotective affects (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012; Rodríguez-Morató et al., 2015).

Based on evidence that the phenolic compounds in olive oil are protective against LDL oxidation (Covas, de la Torre, & Fitó, 2015), the European Food Safety Authority (EFSA, 2011) released a claim regarding the beneficial effects of the daily ingestion of phenolic compound-rich virgin olive oil on cardiovascular disease risk factors. To achieve such benefits a daily dose of 5 mg HT and its derivatives (including tyrosol (Tyr) and oleuropein) in olive oil is...
required which has opened up the possibility of employing HT and Tyr as nutraceuticals.

The addition of a hydroxyl group at position 3 of the phenol ring provides HT with an o-diphenol moiety, a key factor for its presenting higher antioxidant activity than Tyr (Carrasco-Pancorbo et al., 2005). Tyr content in red wine has been reported to be more than 5-fold greater than that of HT (Piñeiro, Cantos-Villar, Palma, & Puertas, 2011), nevertheless, a significant amount of HT has been observed to be endogenously produced. De la Torre, Covas, Pujadas, Fitó, and Farré (2006) described that urinary HT excretion was 1.4-fold higher after the consumption of 250 ml of red wine compared to 25 ml of virgin olive oil, despite the HT content being ~5-times lower (0.35 vs 1.70 mg, respectively). Moreover, urinary recovery of HT following red wine administration was greater than the amount of HT present in the wine itself, suggesting an endogenous HT production. An ethanol dose-related increase of urinary HT excretion in 24 healthy men has been observed, probably through the induction of endogenous HT production (Pérez-Mañá, Farre, Pujadas et al., 2015; Pérez-Mañá, Farré, Rodríguez-Morató et al., 2015). Such findings are consistent with previous work (Tank & Weiner, 1979) in which ethanol up-regulated dopamine oxidative metabolism, generating HT in a dose-dependent manner (Fig. 1A).

Nevertheless, this ethanol-induced mechanism does not completely explain the amount of HT recovered in urine after wine intake. In a rat study, it was reported that Tyr was converted in vivo to HT, and that Tyr urinary excretion was augmented by ethanol (Pérez-Mañá, Farre, Pujadas et al., 2015; Pérez-Mañá, Farré, Rodríguez-Morató et al., 2015). Despite the fact that Tyr has been identified as a substrate of HT production, the enzyme responsible for this biotransformation remains as yet unknown. Two theoretical possibilities have been considered: the involvement

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**Fig. 1.** (A) Dopamine biosynthesis and oxidative metabolism. Hydroxytyrosol is produced as a metabolite of dopamine (DOPET) and this formation is increased after ethanol intake in humans. (B) Schematic representation of the two hypotheses considered: (i) tyrosine hydroxylase converts tyrosol into hydroxytyrosol as it converts tyrosine to L-DOPA; (ii) cytochrome P450 is responsible for the hydroxylation.
of [i] tyrosine hydroxylase and/or [ii] cytochrome P450 (CYP) isoenzymes (Fig. 1B).

Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine biosynthesis and it catalyzes the aromatic hydroxylation that converts L-tyrosine to L-DOPA (Nagatsu, Levitt, & Udenfriend, 1964). Taking into account that the difference between Tyr and HT is an aromatic hydroxylation, our first hypothesis was that tyrosine hydroxylase was involved in the conversion of Tyr to HT. Alternatively, we considered CYPs which are responsible for catalyzing the oxidation of a wide variety of xenobiotic chemicals and endogenous substrates.

In addition to identifying the enzymes taking part in Tyr-HT biotransformation, we aimed to evaluate the effects of a combined administration of ethanol and Tyr on the urinary recovery of both Tyr and HT. To this end, the effects of the tyrosine hydroxylase inhibitor α-methyl-L-tyrosine (αMT) on this biotransformation was assessed in rats; the involvement of CYPs was examined in human liver microsomes (HLM) and human recombinant proteins (baculosomes); and Tyr hydroxylation was investigated in human genotyped livers.

2. Material and methods

2.1. Drugs and chemicals

HT and HT acetate were supplied by Seprox Biotech (Madrid, Spain). HT-D₃ was purchased from Synfine Research Inc (Ontario, Canada). Tyr, 3-(4-hydroxyphenyl)-1-propanol, homovanillic acid, fluoroxamine maleate, montelukast sodium hydrate, ticloropidine hydrochloride, methoxsalen, tryptamine hydrochloride, (Toronto, Ontario, Canada). HT glucuronide conjugates were synthesized according to a previously described method (Khymenets et al., 2010; Kotronoulas & Tyndale, 2015). Liver donors were then grouped as normal or reduced CYP2A6 and CYP2D6 metabolizers based on predicted activity according to genotype. The CYP2A6 and CYP2D6 normal groups included donors of genotypes CYP2A6*1/*1, *1/*1X2 and CYP2D6*1/*1 only for each gene. CYP2A6 protein levels in these human liver tissues were semi-quantified with Western blotting, as previously described (Al Koudsi, Hoffmann, Assadzadeh, & Tyndale, 1997; Wassenaar, Zhou, & Tyndale, 2015).

2.2. Human liver microsomes and recombinant enzymes

Human liver microsomes (HLM) pooled from 50 donors were purchased from Life Technologies Europe. According to the manufacturer, total protein and P450 content were 20.0 mg/ml and 0.239 nmol/mg, respectively. Microsomes produced from baculovirus-infected insect cells expressing human CYP2A6, CYP3A4, CYP2B6, CYP2C9, and CYP2D6 (Baculosomes ™) were also purchased from Life Technologies Europe with the corresponding protein concentrations and CYP contents.

The characteristics and sources of the 15 human livers used in this study have been previously described (Messina, Tyndale, & Sellers, 1997). Human hepatic microsomes were prepared and stored in 1.15% KCl at −80 °C according to previously established techniques (Tyndale, Inaba, & Kalow, 1989). Human liver tissues were derived from the K- and M-series liver banks provided by Dr. T. Inaba (University of Toronto, Toronto, ON, Canada) and Dr. U. Meyer (Biocentre in Basel, Switzerland), respectively, as previously published (Al Koudsi, Hoffmann, Assazadeh, & Tyndale, 2010; Messina et al., 1997). Cytosolic fractions were collected during microsomal membrane preparation and used as a source of aldehyde oxidase for all in vitro nicotine metabolism assays. Total protein was quantified with a Bio-Rad protein assay kit based on the Bradford dye-binding method (Bio-Rad Laboratories Ltd.).

DNA was extracted from liver tissues for CYP2A6 and CYP2D6 genotyping with phenol/chloroform extraction (Invitrogen, Canada) and ethanol precipitation. DNA was genotyped for the CYP2A6 alleles *2, *4, *5, *6, *9, *12, *17, *20, *21, *23, *24, *25, *26, *27, *28, *35, +1 × 2, and the CYP2D6 alleles *3, *4, *10, as previously described (Tyndale, Droll, & Sellers, 1997; Wassenaar, Zhou, & Tyndale, 2015). Liver donors were then grouped as normal or reduced CYP2A6 and CYP2D6 metabolizers based on predicted activity according to genotype. The CYP2A6 and CYP2D6 normal groups included donors of genotypes CYP2A6*1/*1, *1/*1X2 and CYP2D6*1/*1 only for each gene. CYP2A6 protein levels in these human liver tissues were semi-quantified with Western blotting, as previously described (Al Koudsi et al., 2010).

2.3. Animal studies

Seventy-two male Wistar rats (Charles River) weighing 140–200 g were used in the experiments. They were housed two per cage (temperature 22 ± 2 °C/humidity 55 ± 15%) in a controlled room with a 12-h light/dark cycle (lights on at 08:00 am). The experiments were performed during the light period. Food and water were given ad libitum in the home cages, but only water was available in the metabolic cages (Harvard Apparatus, 48 cm × 28 cm × 36 cm) during the four hours of the experiment. Each cage was provided with a support grid for the animals, separate urine and feces collection funnels, and a drinking tube. On the day prior to the experimental session, the rats were habituated to the metabolic cages for two hours. They were assigned to twelve groups (N = 6/group) (Supplementary Table 1). The rats were first injected with either αMT (50 mg/kg) or saline, and thirty min later received either 0.5 g/kg ethanol (30% ethanol in saline, v/v) or vehicle (saline). One hour later, they were injected with 10 or 20 mg/kg of Tyr (prepared in saline) or vehicle. All treatments were administered intraperitoneally. Following the last administration, the rats were immediately placed in the cages for 4 h and urine produced during this period collected. On terminating the experiment, the rats were euthanized under isoflurane anesthesia. Urine samples were weighed and preserved with 6 N HCl (20 μl/ml urine), and stored at −20 °C until analysis. HT, Tyr, and their corresponding metabolites were quantified using a LC-MS/MS method as previously described (Khymenets et al., 2010; Kotronoulas et al., 2013; Pérez-Mañá, Farre, Pujadas et al., 2015; Pérez-Mañá, Farré, Rodríguez-Morató et al., 2015). Animal procedures were approved by the local ethical committee (CEEA-PRBB; ref. PRM-13-1525) and performed in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research.

2.4. Experiments in human liver microsomes (HLM)

2.4.1. Tyrosol metabolism to hydroxytyrosol

The evaluation of HT formation was carried out at 37 °C in a shaking bath for 0–60 min. The final incubation mixtures (250 μl) contained Tyr (10–100 μM), HLM (0.5 mg protein/ml), and NADPH (1 mM) in 100 mM sodium phosphate buffer (pH 7.4). The
reactions were initiated by the addition of the NADPH solution after a 3-min pre-incubation, and stopped at each corresponding time by the addition of 250 μl ice-cold methanol. A negative control in the absence of NADPH was employed to verify the NADPH-dependent reaction. To each reaction mixture, 20 μl IS solution (10 μg/ml each of HT-D₃ and 3-(4-hydroxyphenyl)-1-propanol) was added. The resulting mixture was centrifuged (13,000 rpm, 5 min, 4 °C). The supernatant was transferred to an amber screw-top glass tube, diluted with 4.25 ml of Milli-Q water, acidified to pH 2 with 250 μl 4% H₃PO₄, and subjected to a solid-phase extraction (SPE) procedure (Oasis HLB® 3 cc, 60-mg cartridges; Waters Corporation, Dublin, Ireland). The SPE cartridges were preconditioned sequentially with 2 ml methanol and 2 ml water. After sample loading, the cartridges were washed with 2 ml water. The compounds of interest were then eluted with 3 ml methanol. After methanol removal (25 °C, 10–15 psi), the dry residues were reconstituted in a 100 μl mixture of mobile phases (90%A/10%B, v/v), centrifuged at 10,000 rpm for 3 min, transferred to HPLC vials, and analyzed by LC-MS/MS. This procedure was employed for both the mix of HLM and the individual genotyped microsomes.

Identification and quantification of HT and Tyr were performed using an Agilent 1200 series HPLC system (Agilent technologies) coupled to a triple quadrupole (6410 Triple Quad LC/MS; Agilent) mass spectrometer with an electrospray interface. Chromatographic separation of HT and Tyr was carried out on an Acquity UPLC® BEH C₁₈ column (100 mm × 3.0 mm i.d., 1.7 μm particle size) (Waters Corporation) at 40 °C in an isocratic mode using 75% mobile phase A (0.01% ammonium acetate, pH 5) and 25% mobile phase B (100% methanol). Injection volume was 10 μl. Tyr, HT, and the IS were eluted with the flow rate at 0.25 ml/min in 5 min and monitored in negative ionization using the multiple reaction mode. HT was quantified employing an isotope dilution method by comparing its peak area ratio with HT-D₃.

2.4.2. Nicotine metabolism to cotinine
The rates of nicotine, coumarin, and dextromethorphan metabolism were assessed in vitro in the HLMs from the 15 donors. The velocity of nicotine metabolism to cotinine was determined by incubating micromolar protein (0.5 mg/ml) with nicotine (30 μM), Tris-HCl buffer (pH 7.4, 50 mM), NADPH (1 mM), and 10 μl of human liver cytosol (as the aldehyde oxidase source) in the final volume of 100 μl. The reactions were carried out for 20 min at 37 °C and stopped with the addition of 20 μl of 20% Na₂CO₃. The IS, cotinine-D₃, was added. Samples were extracted and analyzed by LC-MS/MS as previously described (Tanner et al., 2015).

2.4.3. Coumarin metabolism to 7-hydroxycoumarin
Coumarin metabolism to 7-hydroxycoumarin was evaluated by incubating micromolar protein (0.05 mg/ml) with coumarin (2 μM), Tris-HCl buffer (pH 7.4, 50 mM), and NADPH (1 mM) in the final volume of 200 μl. The rate of 7-hydroxycoumarin formation was linear from 2 to 15 min for fast CYP2A6 livers, according to the rate of cotinine formation from nicotine (highest tertile), shorter incubation times were avoided to substrate depletion. The reactions were carried out at 37 °C and stopped with the addition of 40 μl trichloroacetic acid (20% v/v). Following the addition of the IS, 4-hydroxycoumarin, the resulting reaction mixtures were extracted and analyzed by HPLC as previously described (Li, Li, & Sellers, 1997), with minor modifications. Briefly, after the addition of 3 ml ethyl acetate, the mixtures were vortexed for 10 s, mechanically shaken for 10 min, and centrifuged at 3000 rpm for 10 min. The upper ethyl acetate layer was then transferred to a 10-ml tube and evaporated to dryness at 37 °C under nitrogen stream. The dry residue was re-dissolved with 110 μl mobile phase, and 100 μl of the solution was injected into the HPLC system (HP 1200 Separation Module). Coumarin and 7-hydroxycoumarin were separated on the ZORBAX SB C18 Column (5 μm. 250 × 4.6 mm; Agilent Technologies, Mississauga, ON) using a mobile phase of acetonitrile, water and acetic acid (25:75:0.1, v/v) at 1 ml/min flow rate. The retention time of 7-hydroxycoumarin, 4-hydroxycoumarin, and coumarin was 7.9, 11.8, and 17.7 min, respectively.

2.4.4. Dextromethorphan metabolism to dextrorphan
The metabolism of dextromethorphan to dextrophan was measured by incubating micromolar protein (0.25 mg/ml) with dextromethorphan (5 μM), 100 mM potassium phosphate buffer (pH 7.4), and NADPH (1 mM) in the final volume of 250 μl. The reactions were carried out for 15 min at 37 °C and stopped with the addition of 250 μl hexane-butanol (95:5 v/v). The IS, 2-benzoazoxolinone, was added. The resulting mixtures were extracted and analyzed by HPLC as previously described (Flores-Pérez, Flores-Pérez, Juárez-Olguín, Lares-Asseff, & Sosa-Maclas, 2004; Hendrickson, Gurley, & Wessinger, 2003), with minor modifications. Specifically, 5 ml hexane-butanol (95:5 v/v) was first added, and the same procedure used for coumarin (see subheading 2.4.2) was followed. Separation of dextromethorphan and dextrophan was carried out with a ZORBAX Bonus-RP column (5 μm, 250 × 4.6 mm; Agilent Technologies, Mississauga, ON) under a gradient elution condition using solvent A (methanol and 0.05 M phosphate buffer, 45:55 v/v, pH 5.8) and solvent B (water) at room temperature. The linear gradient from 100% to 70% solvent A was applied from 0 to 14 min at a flow rate of 0.8 ml/min. From 14 to 27 min, the mobile phase was kept constant at 100% solvent A and 1.2 ml/min flow rate. The eluents were monitored by a fluorescence detector set at an excitation/emission wavelength of 230/330 nm. Retention time was 10.52, 13.26, and 22.63 min for dextrophan, 2-benzoazoxolinone, and dextromethorphan, respectively.

2.5. Enzyme kinetics experiments
Tyr, at concentrations ranging from 0.1 to 4000 μM, was incubated with HLM and the procedure described in the subheading 2.4.1 was followed. All kinetic analyses were performed in duplicate. The kinetic parameters (Kₘ and Vₘₐₓ) were estimated using the Michaelis–Menten equation generated by GraphPad Prism (GraphPad Software, CA, USA, version 5.03 for Windows).

Tyr was also incubated in CYP2A6 and CYP2D6 baculosomes for 60 and 15 min, respectively. These time-points were chosen based on preliminary experiments evaluating enzymatic reaction linearity (Supplementary Fig. 1). The curves describing the reaction kinetics were analyzed using non-linear regression analysis (GraphPad Software 5.03, CA, USA).

2.6. Chemical inhibition analyses
The effects of specific CYP inhibitors on tyrosol hydroxylase activity in HLM were investigated. Tyr (final concentration of 100 μM) was incubated with pooled HLM (0.5 mg/ml) and NADPH (1 mM) in the absence (positive control) and presence of selective chemical inhibitors of CYP isoforms. All the chemical inhibition studies were performed in duplicate. The following CYP inhibitors were used: fluvoxamine for CYP1A2, ticlopidine for CYP2B6, montelukast for CYP2C8, sulfaphenazole for CYP2C9, omeprazole for CYP2C19, quinidine for CYP2D6, and ketoconazole for CYP3A4. In the case of CYP2A6, the substrate coumarin was employed (See Supplementary Fig. 2 for the chemical structures). The specific inhibitors were chosen following the European Medicine Agency guidelines on the Investigation of Drug Interactions (European Medicines Agency., 2012). The following additional inhibitors were
also used in confirmatory experiments with tryptamine, tryptophan, methoxsalen as CYP2A6 inhibitors, and paroxetine and fluoxetine as CYP2D6 inhibitors. The range of inhibitor concentrations was 1–100 µM. Initially, stock solutions of CYP inhibitors were prepared in DMSO. The final DMSO concentration in the reactions was 1.0%. Due to a remarked DMSO-induced inhibitory effect on the reaction, working inhibitor solutions were prepared in methanol. In order to avoid any interference from organic solvent, the inhibitors were prepared in methanol to facilitate solvent removal for the assay. Methanol was evaporated in a vacuum concentrator (SpeedVac). The reaction mixture excluding NADPH was added to tubes containing inhibitors. The reactions were started by the addition of NADPH and were stopped at 60 min by the addition of 250 µl ice-cold methanol.

Additional inhibitory experiments were performed to assess the effect of mechanism-based inhibitors (methoxsalen for CYP2A6, paroxetine for CYP2D6) on HT formation. In these cases, the inhibitor was pre-incubated with microsomes and NADPH for 30 min prior to the addition of the substrate. Methanol was evaporated in a vacuum concentrator (SpeedVac). The reaction mixture excluding NADPH was added to tubes containing inhibitors. The reactions were started by the addition of NADPH and were stopped at 60 min by the addition of 250 µl ice-cold methanol.

2.7. HT formation in cDNA-expressing CYP microsomes

Incubations were carried out at 37 °C in a shaking bath for 0–60 min. The final incubation mixtures (250 µl) contained Tyr (40–400 µM), the corresponding cDNA-expressing CYP microsomes (40–80 pmol CYP/ml), and NADPH (1 mM) in 100 mM sodium phosphate buffer (pH 7.4). The reactions were started by the addition of NADPH solution after a 3-min pre-incubation and stopped at selected times (0–60 min) by the addition of 250 µl ice-cold methanol. A negative control in the absence of NADPH was employed to verify the NADPH-dependent reaction. Prior to analysis, the samples were processed as described for HLM samples.

2.8. Statistical analyses

Data analyses of animal and chemical inhibition studies were performed using a three-way ANOVA model (to evaluate effects of Tyr, ethanol, and αMT on HT urinary excretion) as well as a two-way ANOVA (to assess the effects of Tyr and ethanol on Tyr urinary excretion). Post hoc multiple comparisons were performed for statistically significant results in the global analyses with the LSD test. The correlations between (1) HT formation from Tyr, and 7-hydroxycoumarin formation from coumarin, and (2) HT formation from Tyr, and dextrorphan formation from dextromethorphan were determined by Spearman’s coefficient. Associations between HT production and CYP2A6 genotype and CYP2D6 genotype were analyzed with the Mann–Whitney test. Statistical analyses were performed using SPSS Statistics for Windows (Version 21.0; SPSS Inc. Chicago, IL, USA). The level of statistical significance was defined as P < 0.05. The results in rats are expressed as mean ± S.E.M., and the in vitro findings as mean ± S.D.

Data mining for Tyr-to-HT conversion in individual genotyped livers, multiple regression analysis, and 3D graphics were performed with MATLAB software version 7.0. Experimental data were fitted to a quadratic equation including two linear terms and a quadratic one in order to obtain the optimum correlation.

3. Results

3.1. Animal experiments

A dose-dependent increase in total Tyr urinary recovery was found following Tyr administration (Fig. 2A). A two-way ANOVA revealed a main effect of Tyr (F(2,42) = 23.7, p < 0.001) although no significant effect of ethanol or interaction between factors was observed, indicating that ethanol does not modify the excretion of Tyr (Fig. 2A).

A three-way ANOVA evaluating the action of Tyr, ethanol, and αMT on total HT urinary recovery showed a main effect of Tyr (F(1,42) = 23.7, p < 0.001) and ethanol (F(1,42) = 11.1, p < 0.001), but none of αMT. No interactions amongst factors were observed. However, the results in Fig. 2B demonstrate that Tyr increased total urinary HT recovery in a dose-dependent fashion, while αMT did not significantly inhibit this effect at the dose studied. Ethanol slightly inhibited the conversion of Tyr to HT without reaching statistical significance. The combination of αMT and ethanol also decreased HT formation although this effect was mainly due to the inhibitory action of ethanol (Fig. 2B).

3.2. Tyrosol hydroxylase activity in HLM

HT formation from Tyr took place in human hepatic microsomes in an NADPH-dependent manner and increased in a linear
3.3. Kinetic analyses of Tyrosol ortho-hydroxylation in HLM

Fig. 3 shows the Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee plots for the HLM-mediated HT formation from Tyr. The values represent the mean of two separate experiments. The apparent $K_m$ value was $709 \pm 49 \mu M$ and the $V_{max}$ value was $1294 \pm 31$ pmol/min/mg.

3.4. Chemical inhibition studies with specific CYP inhibitors

The effect of 8 CYP inhibitors on HT formation from Tyr was evaluated. Firstly, a primary screening using 8 specific inhibitors at 100 $\mu M$ was performed. Fig. 4 shows the mean activities (from duplicate determinations) in the presence of these inhibitors. Coumarin reduced HT formation from Tyr (100 $\mu M$) by 60%. CYP2D6, CYP3A4, CYP2B6, and CYP2C9 selective inhibitors slightly diminished HT formation (>10% but <20%) (Fig. 4A).

Additional incubations using nicotine as a CYP2A6 substrate and tryptamine, methoxsalen, and tranylcypromine as CYP2A6 inhibitors (Zhang, Kilicarslan, Tyndale, & Sellers, 2001) were performed. Although nicotine was not found to decrease HT formation, three CYP2A6 inhibitors reduced the biotransformation to a larger degree than coumarin (74%, 77%, and 85%, respectively), with tranylcypromine being the strongest (Fig. 4B). It is worth noting that the rate of coumarin metabolism by CYP2A6 is relatively fast compared to the other substrates/inhibitors tested.

Several inhibitors of CYP2D6 (quinidine, fluoxetine, paroxetine) and CYP2A6 (tranylcypromine, methoxsalen) were pre-incubated with NADPH and microsomes for 30 min and, after the addition of Tyr, the reactions took place for 30 additional min. Pre-incubation with the CYP2D6 inhibitors did not lead to a >20% inhibition, whereas the CYP2A6 inhibitors surpassed 90%. It is of interest that the combination of two mechanism-based inhibitors, paroxetine (CYP2D6) and methoxsalen (CYP2A6), inhibited HT formation by 97% (Fig. 4C). No inhibitory effect was observed when a deuterated analog of HT (HT-D$_4$) was added at 1, 10, and 100 $\mu M$ to the reaction mixture, indicating there was no product-initiated inhibition of the reaction.

3.5. Experiments using human recombinant CYP enzymes (Baculosomes)

HT formation from Tyr was evaluated in baculosomes (microsomes from baculovirus-infected insect cells expressing human isoforms). CYP2A6 capacity for hydroxylating tyrosol was confirmed, and HT was found to be produced in a linear fashion ($r^2 > 0.99$) with the amount of administered protein and Tyr (Supplementary Fig. 3).

In order to evaluate whether other CYP isoforms could contribute to HT production, human recombinant CYP3A4, CYP2B6, CYP2C9, and CYP2D6 (as well as CYP2A6) were tested. These isoforms were chosen based on the results of the chemical inhibition

![Fig. 3. Hydroxytyrosol (HT) formation from tyrosol in human liver microsomes (HLM). (A) Time- and dose-dependent formation; (B) Michaelis-Menten plot; (C) Lineweaver-Burk plot; (D) Eadie-Hofstee plot.](image)
The enzyme kinetics of CYP2D6, CYP3A4, and CYP2A6 (the 3 major contributors) were evaluated individually at 6 time points (0, 5, 15, 30, 45, and 60 min). Differing kinetics were noted for each enzyme. CYP2A6-mediated HT formation occurred in a linear time-dependent manner from 0 to 60 min. The linearity in HT formation mediated by CYP3A4 and CYP2D6 was observed only in the first 15 min, and there was a time-dependent reduction in HT content from 15 to 60 min (Supplementary Fig. 1).

The relationship between Tyr concentration and HT formation rate was evaluated in CYP2A6 and CYP2D6 baculosomes with the aim of comparing their kinetic parameters with those obtained using HLM. HT formation kinetics in baculosomes did not follow a typical Michaelis-Menten hyperbolic pattern. In the case of CYP2A6, a biphasic kinetic profile was observed (Supplementary Fig. 5A). This enzymatic behavior has been previously described (Hutzler & Tracy, 2002) and is characterized as a non-asymptotic profile that becomes linear with increasing substrate concentration. With respect to CYP2D6, the kinetics followed a sigmoidal autoactivation profile (Supplementary Fig. 5B).

3.6. Correlation of Tyr hydroxylation by individual HLM

HT formation from Tyr was evaluated in 15 individual genotyped livers, which exhibited a wide range of CYP2D6 and CYP2A6 activities. Table 1 shows the individual genotypes for CYP2D6 and CYP2A6 and the predicted activities of both isoforms according to genotype.

The correlation between HT formation and CYP2A6 and CYP2D6 activities (determined using the metabolism velocity of coumarin to 7-hydroxycoumarin, and dextromethorphan to dextrorphan, respectively) was non-significant (Fig. 5C and D). The impact of genotype on the association between HT formation and CYP2A6 and CYP2D6 genotypes was non-significant (Fig. 5C and D).

In order to evaluate the concomitant involvement of both isoforms on HT formation, we performed a multivariate regression analysis. A three-dimensional scatter plot for the sample-to-sample variation in Tyr hydroxylation of the 15 individual HLMs, and their correlations with the individual CYP2D6 and CYP2A6 activities, is depicted in Fig. 5E. The equation of the adjusted surface that describes the velocity of Tyr hydroxylation is:

\[
\text{Tyr hydroxylation} = 0.01263 + 0.52778X_1 + 0.044483X_2 - 0.57453X_1X_2
\]

where \(X_1\) is dextromethorphan velocity and \(X_2\) is coumarin velocity. The corresponding correlation coefficient \((r^2)\) is 0.50 \((F = 3.69; P = 0.047)\). It should be noted that the regression coefficient values

**Table 1**

<table>
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<th>Liver ID</th>
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<th>CYP2D6 Genotype</th>
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**Fig. 4.** Inhibitory effect of cytochrome P450 (CYP) isoenzyme specific inhibitors on the formation of hydroxytyrosol (HT) from tyrosol; (A) Primary screening including selective inhibitors of 8 different isoenzymes; (B) Confirmatory incubations using nicotine as a CYP2A6 substrate and selective CYP2A6 inhibitors (tryptamine, methoxsalen and tranylcypromine); (C) Additional experiments to evaluate the potential effect of different inhibitors of CYP2A6 and CYP2D6. In this case, microsomes were pre-incubated with inhibitors for 30 min. Values are given as mean ± standard deviation of two independent experiments. \(P < 0.05\).
Fig. 5. (A) Correlation between hydroxytyrosol (HT) formation and CYP2A6 activity (determined using the metabolism velocity of coumarin to 7-hydroxycoumarin). (B) Correlation between HT formation and CYP2D6 activity (determined using the metabolism velocity of dextromethorphan to dextrorphan). \( P \) and \( r \) values are based on Spearman correlation test. (C) Association between CYP2A6 genotype and HT formation (ng). Normal metabolizers are those with +1/+1, +1/+X2, and +14/+14 CYP2A6 genotypes. Reduced metabolizers are those with +1/+2 and +1/+12 genotypes. (D) Association between CYP2D6 genotype and HT formation (ng). Normal metabolizers are those with +1/+1 CYP2D6 genotype. Reduced metabolizers are those with CYP2D6 +1/+3, +1/+4, and +10/+10 genotypes. \( P \) values are based on Mann-Whitney tests. (E) Multivariate correlation analysis of sample-to-sample variation (n = 15) in tyrosol-3-hydroxylation with CYP2D6 and CYP2A6 activities.
were comparable when CYP2A6 velocity toward either coumarin or nicotine was included in the analysis.

4. Discussion

Recent studies have demonstrated that HT is endogenously formed from Tyr in both rats and humans (Pérez-Mañá, Farre, Pujadas et al., 2015; Pérez-Mañá, Farré, Rodríguez-Morató et al., 2015). However, the enzymes that mediated the conversion remained to be characterized. To the best of our knowledge, we demonstrate for the first time that CYPs convert Tyr into HT, and that this reaction is primarily mediated by two polymorphic CYP isoenzymes, CYP2D6 and CYP2A6. Furthermore, we observed that tyrosine hydroxylase was not involved in the conversion.

We hypothesized that the conversion of Tyr to HT was mediated by either tyrosine hydroxylase or CYPs. In order to examine the possible role of tyrosine hydroxylase in the formation of HT from Tyr, rates were pretreated with α-MT, a tyrosine hydroxylase inhibitor, before Tyr administration. The unchanged urinary HT excretion provided strong evidence that tyrosine hydroxylase was not involved in the conversion of Tyr to HT. In the same rat study, it was confirmed that urinary HT excretion occurred in parallel to the administered Tyr doses, while 0.5 g/kg ethanol slightly inhibited this effect. It is noteworthy that ethanol plays a dual role: it alters dopamine oxidative metabolism generating small amounts of HT, whilst appearing to inhibit the conversion of Tyr into HT.

Having demonstrated that tyrosine hydroxylase was not involved in HT formation, we next tested whether CYPs were capable of mediating Tyr hydroxylation. In vitro HLM experiments confirmed that at least one CYP isoform took part in the conversion of Tyr to HT via hydroxylation with a typical Michaelis-Menten kinetic profile. Such a combination of high $K_m$ and $V_{max}$ values indicates that, despite presenting a low specificity, Tyr-3-hydroxylation occurs at a high velocity.

Once Tyr hydroxylation had been demonstrated to occur in human microsomes, it was necessary to identify the specific CYP isoenzyme(s) that catalyze(s) the reaction. Results from the HLM experiments using selective inhibitors suggested that CYP2A6 was the main isoform responsible for the conversion of Tyr to HT, although CYP2D6 and CYP3A4 appeared to be involved to a lesser extent. The use of baculosomes (recombinant baculovirus containing cDNA) confirmed the capacity of CYP2A6, CYP2D6, and CYP3A4 to biotransform Tyr to HT.

The involvement of CYP3A4 baculosomes in the conversion of Tyr to HT is not surprising as it is the most abundant isoform in the liver, and it metabolizes the greatest number of drugs and other xenobiotics (Pelkonen et al., 2008). Nevertheless, according to inhibition experiments with ketoconazole in pooled human liver microsomes, is should be noted that CYP3A4 is not a strong contributor to in vivo HT formation. The role of CYP2A6 and CYP2D6 in the conversion of Tyr to HT appears to be of greater relevance. Findings from HLM experiments using methoxsalen and paroxetine, two mechanism-based inhibitors of CYP2A6 and CYP2D6, respectively, suggest that both isoforms react toward Tyr in a cooperative manner with CYP2A6 as the major contributor and CYP2D6 the minor one. The kinetics of HT formation as mediated by CYP2A6 and CYP2D6 were evaluated in baculosomes. Contrary to what occurred in microsomes, HT formation in baculosomes hindered the comparison of the individual contributions of CYP2A6 and CYP2D6 to HT formation, although at equimolar concentrations CYP2D6 presented a higher velocity of HT formation than CYP2A6. The discrepancy between the kinetic profile observed in microsomes and baculosomes may be due to the fact that recombinant CYP enzymes are usually expressed with much higher levels of NADPH and CYP reductase than those found in HLMs (Rodrigues, 2008). Moreover, Tyr hydroxylation in a mix of 50 HLMs (presenting a more physiological condition than baculosomes) fully coincided with a typical Michaelis-Menten kinetic profile. Taking into account the previous observations, we suggest that in vivo Tyr hydroxylation follows a Michaelis-Menten kinetic profile.

There is high interindividual variability in CYP2A6 and CYP2D6 activities due to genetic polymorphisms (Yokoi & Kamataki, 1998). CYP2D6, which catalyzes more than 25% of commercial drugs, has a gene locus with more than 80 allelic variants, resulting in considerable differences associated with increased or reduced enzymatic activity and clinical consequences (Teh & Bertilsson, 2012). In a similar manner, the CYP2A6 gene is polymorphic, resulting in high interindividual (Rautio, Krauli, Kojo, Salmela, & Pelkonen, 1992) and interethnic divergence in enzyme activity (Piliguian et al., 2014; Rautio, Rautio, Gullsten, & Pelkonen, 2001). Indeed, 20% of Asians are CYP2A6 poor metabolizers, whereas this prevalence is lower in Caucasians (<1%) (Rauino et al., 2001). Regarding the relative abundance of protein in the liver, CYP2D6 accounts for less than 5% whilst CYP2A6 represents approximately 10% of total hepatic CYP protein (Pelkonen et al., 2008).

The specificity of each CYP isoform toward its substrates is another issue that must be taken into account. CYP2D6 and CYP3A4 are responsible for the metabolism of hundreds of therapeutically important drugs (Pelkonen et al., 2008) whilst relatively few are converted by CYP2A6, the enzyme primarily responsible for nicotine metabolism (Messina et al., 1997).

In this study, CYP2A6 and CYP2D6 from the 15 selected livers were classified according to genotype-predicted activities, and their capacity for nicotine-to-cotinine conversion and dextromethorphan-to-dextrorphan conversion, respectively. As expected, a wide interindividual Tyr-to-HT conversion variability was found. It was also observed that the correlations between HT formation and coumarin (a CYP2A6 substrate) and dextromethorphan (a CYP2D6 substrate) velocity were weak (Spearman $r = 0.28$ in both cases) although there were modest associations between HT formation and CYP2A6/CYP2D6 activities. These results, and the previous findings with respect to baculosomes (reporting the involvement of CYP2D6 and CYP2A6) and inhibition studies with microsomes (demonstrating CYP2A6 function), indicate that both CYP2D6 and CYP2A6 are actively involved in HT formation from Tyr. An implication that is further upheld by the larger regression coefficient generated in the multivariate regression analysis with CYP2D6 and CYP2A6 velocities as two independent variables.

Our study has strengths and limitations. One strength is the combination of in vivo (animal models) and in vitro (microsomes and baculosomes) techniques employed, as well as the use of human livers. A limitation is the relatively low number of human livers used due to the difficulty in obtaining these samples.

5. Conclusions

In conclusion, our study demonstrates for the first time that HT (a potent bioactive molecule with multiple health benefits) is formed from dietary Tyr in HLMs via CYP2A6 and CYP2D6, both working in a cooperative manner. The involvement of two CYP isoenzymes in the production of HT is relevant since to date only a few known substrates of CYP2A6 have been reported. Moreover,
the genetic polymorphisms of both CYP2D6 and CYP2A6 could have a potential impact on the magnitude of health benefits associated with the consumption of Tyr-containing food amongst individuals and ethnicities.

Conflict of interest

RFT has consulted for Apotex on unrelated issues. The other authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.026.

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