

Olive Oil Phenols Inhibit Human Hepatic Microsomal Activity

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ABSTRACT We have examined the inhibition of human hepatic microsomal androstenedione 6 β -hydroxylation and both reductive and oxidative 17 β -hydroxysteroid dehydrogenase (17 β -HSD) activity by complex phenols found in olive oil. Structurally similar compounds were also examined for comparison. Androstenedione 6 β -hydroxylase activity was inhibited by oleuropein glycoside, hydroxytyrosol and gallic acid. Oleuropein glycoside, hydroxytyrosol, gallic acid and dihydroxybenzoic acid also inhibited reductive 17 β -HSD activity. Oxidative 17 β -HSD activity was not inhibited by any of the compounds tested; however gallic acid stimulated activity by ~30%. Androstenedione 6 β -hydroxylase activity showed atypical kinetics. For oleuropein glycoside, hydroxytyrosol and gallic acid the apparent K_i values were determined to be 80, 77 and 70 μ mol/L, respectively. Analysis of structural features of inhibitory compounds established that a 3,4-dihydroxyphenyl ethanol structure was required for inhibition of androstenedione 6 β -hydroxylase for this group of compounds. *J. Nutr.* 130: 2367–2370, 2000.

KEY WORDS: • humans • cytochrome P450
• 17 β -hydroxysteroid dehydrogenase • CYP3A

Phenols, widely distributed in vegetables, are found in high concentrations in the typical components of the Mediterranean diet. The complex phenols found in olive oil include the glycoside oleuropein, and its hydrolysis product hydroxytyrosol (3,4-dihydroxyphenyl ethanol). The amounts of these olive oil “minor components” or “nonnutrients” vary, depending on a number of factors including production and storage (Brenes et al. 1999); however concentrations of 2.3–9 mg/L of oleuropein and 1.4–5.6 mg/L of hydroxytyrosol have been reported. (Montedoro et al. 1992). The possible beneficial effects of these complex phenols have been examined in a number of studies. It has been suggested that they may play a role with respect to the inhibition of platelet aggregation and arachidonic acid metabolism (Petroni et al. 1994 and 1995) and also in the prevention of oxidation of LDL (Visioli et al. 1995).

Hepatic microsomal enzymes, in particular the cytochromes

P450 (CYP)², are important in the metabolism of xenobiotic and endogenous substrates. Most xenobiotics, such as drugs, are metabolized and generally inactivated along pathways that involve different CYP. However, if only one CYP is involved in the clearance of a drug, then inhibition will have a major impact on the therapeutic effects of the drug (reviewed, Murray 1999). Inhibition of CYP by nonnutrients has been demonstrated (Guengerich et al. 1994) potentially altering therapeutic effects (Kupferschmidt et al. 1995). Evidence now indicates that there are three CYP3A subfamily proteins in humans. Of these CYP3A4 and CYP3A5 are expressed in adult liver. (Nebert et al. 1991). Androstenedione 6 β -hydroxylase activity is regarded as being a specific marker for the CYP3A proteins (Waxman et al. 1991). Aside from the CYP, there are many other enzymes that participate in xenobiotic and endobiotic metabolism. The enzyme 17 β -hydroxysteroid dehydrogenase (17 β -HSD), also known as 17 β -hydroxysteroid oxidoreductase, controls the important final step in the biosynthesis of androgens and estrogens. At least five isoenzymes, that exhibit different patterns of expression in cells, substrate specificity and regulatory mechanisms, have been described (Labrie et al. 1997). Reduction (e.g., androstenedione to testosterone) utilizes NADPH and NADH as cofactors whereas NADP and NAD are cofactors for the oxidative (dehydrogenation) pathway.

The present study was undertaken to examine the effects of some complex phenol compounds on androstenedione 6 β -hydroxylase, and both oxidative and reductive 17 β -HSD activity. Inhibition of androstenedione 6 β -hydroxylase, or oxidative or reductive 17 β -HSD activity, could potentially alter the metabolism of xenobiotics or endobiotics by olive oil phenols.

MATERIALS AND METHODS

Materials. A sample of pooled human liver microsomes was purchased from Human Biologics International (Scottsdale, AZ). NADP, NADPH, NADH, isocitrate dehydrogenase, unlabeled androst-4-ene-3,17-dione (androstenedione), 17 β -hydroxy-4-androsten-3-one (testosterone) and 3 β -hydroxysteroid dehydrogenase, were purchased from the Sigma Chemical (St. Louis, MO). [4-¹⁴C] Androstenedione (sp. act. 2.0 GBq/mmol) and [4-¹⁴C] testosterone (sp. act. 1.9 GBq/mmol) were purchased from Amersham (Sydney, Australia). 16 β -Hydroxyandrostenedione was prepared enzymatically by the action of 3 β -hydroxy steroid dehydrogenase on 3 β , 16 β -dihydroxyandrost-5-ene-17-one (Prof. D. N. Kirk and MRC steroid Reference Collection, Queen Mary's College, London, United Kingdom) as described (Sheets and Estabrook 1985). Other hydroxylated testosterone and androstenedione standards were obtained from the MRC Steroid collection or Steraloids, (Wilton, NH). Oleuropein was purchased from Indofine Chemicals (Somerville, NJ); gallic acid and *p*-coumaric acid were purchased from ICN (Aurora, OH); 3,4-dihydroxybenzoic acid was purchased from BDH (Poole, United Kingdom); 2-(4-hydroxyphenyl) ethanol was purchased from Fluka (Buchs, Switzerland); 3,4-dimethoxyphenethyl alcohol and 3,4-dihy-

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²Abbreviations used: CYP, cytochrome P450; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase

droxyphenylacetic acid alcohol were purchased from Aldrich (Milwaukee, WI). Hydroxytyrosol was synthesized by the LiAlH_4 reduction of 3,4-dihydroxyphenylacetic acid (Baraldi et al. 1983). All solvents and other miscellaneous chemicals were at least analytical reagent grade.

Assays. Microsomal androstenedione hydroxylase and reductive 17β -HSD activity were assayed essentially as previously described (Waxman et al. 1983). For incubations utilizing NADPH, microsomal fractions were incubated in a 1 mL reaction mixture containing isocitrate (4 mmol/L), isocitrate dehydrogenase (0.4 I.U.), MgCl_2 (8 mmol/L), 1 mmol/L NADP and androstenedione (43.75–87.5 $\mu\text{mol/L}$, 4.8 MBq/mmol) for 10 min at 37°C , after which the incubation mixtures were extracted twice with ethyl acetate. Testosterone oxidation was assayed essentially as described for microsomal androstenedione hydroxylase and reductive 17β -HSD activity except that testosterone (4.8 MBq/mmol) was used as substrate. One mmol/L NAD was used in place of the NADPH-generating system. Inhibitors, where used, were added in water, and appropriate control incubations were performed concurrently. Application to, and development of, TLC plates was carried out as previously described (Stupans and Sansom 1991). Zones corresponding to hydroxylated androstenedione standards and to testosterone were visualized under UV light and scraped into vials for scintillation spectrometry (ACS; Amersham, Sydney, Australia).

All assay conditions were optimized with respect to time, protein concentration and substrate concentrations to ensure linearity.

Data analysis. For each experiment, data are expressed as the mean of triplicate observations. Kinetic parameters were determined using a range of inhibitor concentrations and substrate concentrations. The method of Dixon was used to calculate inhibition constants (K_i) (Dixon and Webb 1979). As this method cannot distinguish the type of inhibition, the Cornish-Bowden method was used (Cornish-Bowden 1974). Eadie-Hofstee plots were used to visually detect deviations from linearity.

RESULTS

Several compounds, shown in **Figure 1**, were examined as inhibitors of androstenedione 6β -hydroxylase, reductive and oxidative 17β -HSD activities. Data for inhibition of these activities are shown in **Table 1**. There was inhibition of androstenedione 6β -hydroxylase activity by three of the selected test compounds, i.e., oleuropein glycoside, hydroxytyrosol and gallic acid. These same compounds, and also dihydroxybenzoic acid, also inhibited reductive 17β -HSD activity, with little inhibition shown by the other test compounds. Oxidative 17β -HSD activity was not inhibited by any of the compounds tested; however, gallic acid stimulated activity by $\sim 30\%$.

The type of inhibition of androstenedione 6β -hydroxylase activity was determined using varying concentrations of oleuropein glycoside, hydroxytyrosol and gallic acid and varying concentrations of androstenedione. **Figure 2** shows the Dixon plot obtained for oleuropein glycoside. The apparent K_i was determined to be 80 $\mu\text{mol/L}$. Similarly for hydroxytyrosol and gallic acid, apparent K_i values of 77 and 70 $\mu\text{mol/L}$, respectively, were determined; however the method cannot distinguish between competitive and mixed-type inhibition (Dixon and Webb 1979). The Cornish-Bowden plot did not indicate typical kinetic inhibition (Cornish-Bowden 1974) (not shown). The atypical nature was confirmed by Eadie-Hofstee plots (**Fig. 3**).

DISCUSSION

In the present study a range of complex phenols was examined for their ability to inhibit androstenedione 6β -hydroxylase and reductive and oxidative 17β -HSD activities in human liver microsomes. As discussed above, androstenedione 6β -

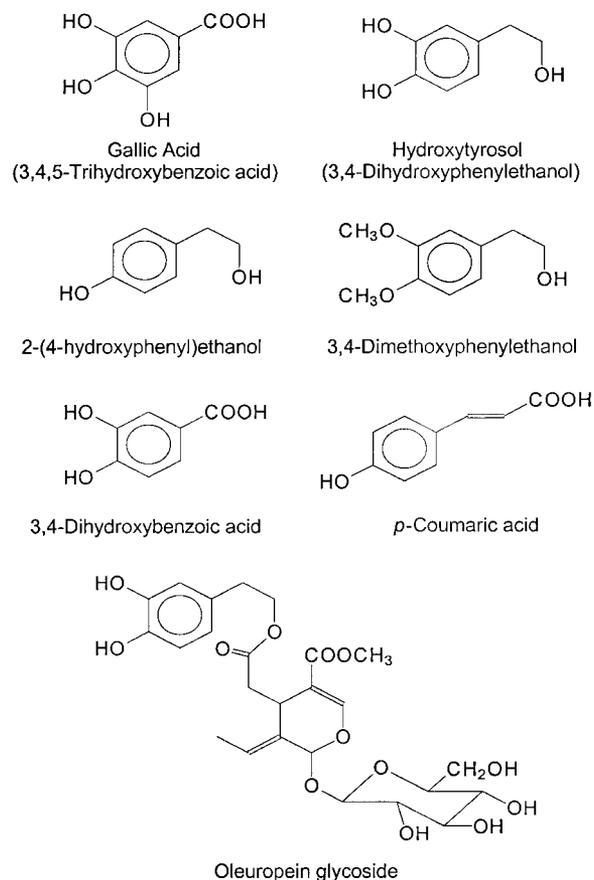


FIGURE 1 Structures of complex phenol compounds and other test compounds used as potential inhibitors.

hydroxylase activity is regarded as being a specific marker for the CYP3A proteins. (Waxman et al. 1991). This assay was therefore used to determine CYP3A inhibition. In humans, both oxidative 17β -HSD C_{19} (i.e., testosterone to androstenedione) and reductive 17β -HSD C_{19} activity in hepatic microsomes are principally mediated by 17β -HSD type 2 (Labrie et al. 1997).

Inspection of the molecular structure of the test compounds revealed several interesting results (Table 1). Hydroxytyrosol was found to be a good inhibitor of androstenedione 6β -hydroxylase and reductive 17β -HSD activities, whereas a compound which differs only by the absence of the 3-hydroxy function [2-(4-hydroxyphenyl) ethanol] was observed to be a poor inhibitor. The test compound 3,4-dihydroxybenzoic acid was found to be an inhibitor of reductive 17β -HSD activity inhibitor and yet was a poor inhibitor of androstenedione 6β -hydroxylase activity. It differs from hydroxytyrosol only by the absence of a two-carbon side chain and the presence of a carboxylic acid function. The addition of another hydroxy group to 3,4-dihydroxybenzoic acid as in gallic acid markedly increased inhibitory potency with respect to androstenedione 6β -hydroxylase activity. The addition of this hydroxy group also changed the compound from one that had no effect with respect to oxidative 17β -HSD activity to one that stimulated activity. The addition of methyl ether functions to hydroxytyrosol as in 3,4-dimethoxyphenyl ethanol decreased inhibitory potency. The test compound *p*-coumaric acid was a very poor inhibitor. These observations taken together with the observation that inhibitory potency with respect to androstenedione 6β -hydroxylase was not markedly different be-

TABLE 1

Percentage inhibition of human liver microsomal androstenedione 6 β -hydroxylase, reductive and oxidative 17 β -HSD activities by the test compounds (100 μ mol/L)¹

Test compound (100 μ mol/L)	Androstenedione ² 6 β -hydroxylase	Oxidative 17 β -HSD	Reductive 17 β -HSD
Oleuropein glycoside	42 \pm 3	NI	40 \pm 4
Hydroxytyrosol	50 \pm 4	NI	26 \pm 3
Gallic acid	50 \pm 4	32% Stimulation	50 \pm 5
3,4-Dihydroxybenzoic acid	20 \pm 3	NI	40 \pm 5
2-(4-Hydroxyphenyl) ethanol	16 \pm 3	NI	10 \pm 3
3,4-Dimethoxyphenyl ethanol	7 \pm 2	NI	20 \pm 4
p-Coumaric acid	5 \pm 4	NI	NI

¹ Values are means \pm SD, $n = 3$. NI, no inhibition detected. Control activities were 0.55 nmol/(mg microsomal protein \cdot min) (androstenedione 6 β -hydroxylase), 0.13 nmol/(mg microsomal protein \cdot min) (reductive 17 β -HSD) and 4.65 nmol/(mg microsomal protein \cdot min) (oxidative 17 β -HSD). For comparison 50 μ mol/L triacetyloleandomycin inhibited 99% androstenedione 6 β -hydroxylase activity.

² The androstenedione or testosterone concentration was 43.75 μ mol/L. NADPH cofactor was used for assay of reductive 17 β -hydroxysteroid dehydrogenase (17 β -HSD) activity and NAD for oxidative 17 β -HSD activity.

tween oleuropein glycoside and hydroxytyrosol suggests that the presence of a 3,4-dihydroxyphenyl ethanol structure is required for inhibitory activity of androstenedione 6 β -hydroxylase. It is interesting to note that olive oil phenol antioxidant activity is also associated with the requirement for a 3,4-dihydroxyphenyl 2 carbon side chain structure (de la Puerta et al. 1999)

The apparent K_i for the inhibition of androstenedione 6 β -hydroxylase activity by oleuropein glycoside was found to be 80 μ mol/L. This value is similar to that observed for the inhibition of CYP3A4 in human liver microsomes by another food-derived compound, tangeretin (K_i 72 μ mol/L) (Obermeier et al. 1995) but is higher than that reported for naringenin (K_i 23 μ mol/L) (Ubeaud et al. 1999) or 6',7'-dihydroxybergamottin (25 μ mol/L concentration required to inhibit 6 β -hydroxytestosterone formation by 50%) (Edwards et al. 1996). However it is worth pointing out that at the present stage the compounds actually responsible for the effects of grapefruit juice are still unidentified (Bailey et al. 1998).

The atypical kinetics observed for CYP3A activities have been reported previously (Schwab et al. 1988), (Stupans and

Sansom 1991) and recently also for other CYP activities (Ekins et al. 1998). Interpretation of inhibition kinetics in these circumstances is inappropriate.

In view of the important role that steroidal hormones have in the etiology of hormone-dependent diseases, design of inhibitors to the 17 β -HSD isoenzymes has received attention (Tremblay and Poirier 1998). Flavonoids have been reported to inhibit reductive 17 β -HSD type 1 (Le Bail et al. 1998), (Makela et al. 1998) and oxidative type 2 (Makela et al. 1998). In this study inhibitors were identified for reductive microsomal human 17 β -HSD type 2 activity, i.e., androstenedione to testosterone. Potential inhibitors for this pathway have not been described to date.

This study is the first report of the potential inhibition of xenobiotic and endobiotic metabolism by complex phenols derived from olive oil and other related compounds. We have identified oleuropein glycoside, hydroxytyrosol and gallic acid as inhibitors of CYP3A and reductive 17 β -HSD activity. The role of food-derived CYP inhibitors has been highlighted by studies of 6',7'-dihydroxybergamottin (Edwards et al. 1996), and of their therapeutic importance (Kupferschmidt et al. 1995). The physiological role of such inhibition is speculative; however, it deserves further investigation.

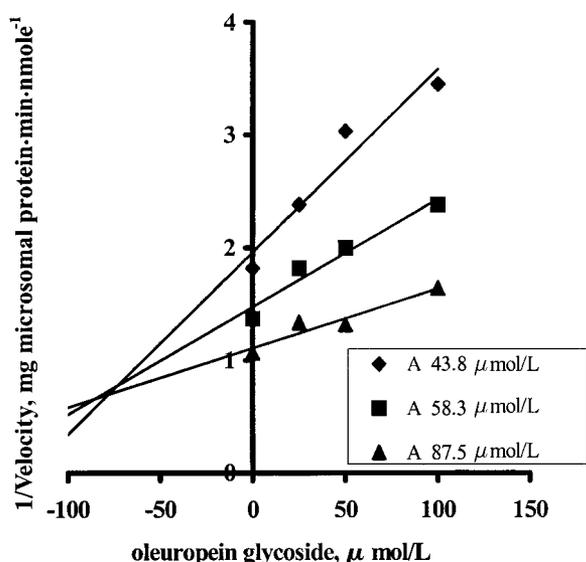


FIGURE 2 Dixon plot showing the inhibition of androstenedione (A) 6 β -hydroxylase activity by oleuropein glycoside.

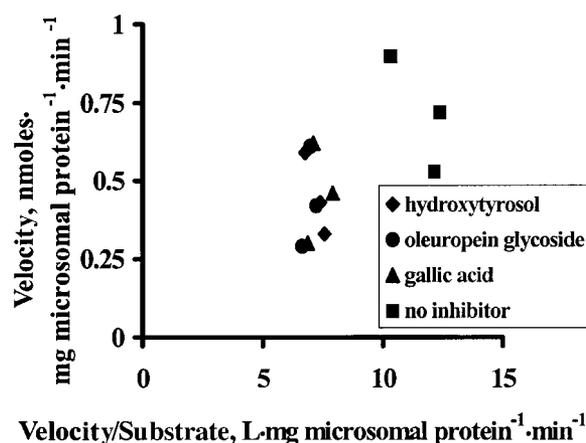


FIGURE 3 Eadie-Hofstee plot showing the atypical kinetics for androstenedione 6 β -hydroxylase activity determined in human hepatic microsomes. Curves in the presence of inhibitors are also shown.

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