Oxidation of reduced haloperidol to haloperidol: involvement of human P450IID6 (sparteine/debrisoquine monooxygenase)

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- 1 The conversion of haloperidol (HAL) to reduced haloperidol (RHAL) and then back to HAL has been established *in vivo* and observed in psychiatric patients. The reduction of HAL to RHAL is known to be catalysed by a ketone reductase, while the nature of oxidation back to HAL is the subject of the present study.
- 2 We examined the *in vitro* oxidation of RHAL to HAL in human livers. The activity was microsomal and evidence is presented to suggest that the sparteine/debrisoquine metabolizing isoenzyme P450IID6 contributes to this oxidation.
- 3 Reciprocal inhibition studies between RHAL and sparteine, a specific substrate for cytochrome P450IID6, indicated that both compounds compete for the same binding site. Quinidine, the most specific inhibitor for this cytochrome P450 potentially inhibited the oxidative conversion of reduced haloperidol to haloperidol. A significant correlation ($r_s = 0.62$, $P < 0.01$) was found between RHAL oxidation and sparteine oxidation in a study involving 17 human liver samples.

Keywords haloperidol sparteine debrisoquine polymorphism P450IID6

Introduction

Reduced haloperidol (RHAL), a hydroxy metabolite of haloperidol (HAL) identified by Forsman & Larsson (1978), is produced in humans by a cytosolic ketone reductase (Inaba & Kovacs, 1989; Inaba *et al.*, 1989). Interconversion is known to occur whereby HAL is found in the plasma after administration of RHAL *in vivo* and *vice versa* (Midha *et al.*, 1989). Interconversion of HAL and RHAL has been observed in rat (Korpi *et al.*, 1985a; Straw *et al.*, 1988), guinea pig (Chang *et al.*, 1988; Korpi *et al.*, 1985b; Nemes *et al.*, 1987) and man (Chakraborty *et al.*, 1989; Korpi *et al.*, 1984; Midha *et al.*, 1987; Pape, 1981). RHAL, the resulting metabolite of HAL, is reported to be about 10–25% as active as HAL in an animal model (Ereshefsky *et al.*, 1984). Dopamine receptors (D_1 and D_2) were examined in the canine striatum (Niznik *et al.*, 1988; Seeman & Niznik, 1988) and HAL was found to have K_D values of 1.2 nM for D_2 ($[^3H]$ -spiperone) and 73 nM for D_1 ($[^3H]$ -SCH-23390), while RHAL had K_D s of 353 nM for D_2 and 1.929 μ M for D_1 (Fassos & Niznik, unpublished data). These differences in binding affinity are consistent with other reports of D_2 receptor binding studies. Someya *et al.* (1990) found that RHAL and HAL displaced 0.2 nM $[^3H]$ -spiperone with IC_{50} values of 1600 nM and 17 nM, respectively, while Korpi & Wyatt (1984) found that

RAHL was 400 times less efficient than HAL in displacing 0.5 nM $[^3H]$ -spiperone.

Large intersubject variation has been observed in the pharmacokinetics of HAL and RHAL. It is conceivable, therefore, that subjects with high reductive capacity relative to oxidative capacity may have less therapeutic response from the same dose of HAL than those with a low reductive capacity relative to oxidative capacity. This is significant with respect to ethnic differences observed in RHAL/HAL ratios (Chang *et al.*, 1987; Someya *et al.*, 1990).

In vivo (Derenne *et al.*, 1989; Gram *et al.*, 1989; Spina *et al.*, 1988; Syvalahti *et al.*, 1986) and *in vitro* (Fonne-Pfister & Meyer, 1988; Inaba *et al.*, 1985; Otton *et al.*, 1983) studies have shown that neuroleptics inhibit the polymorphic metabolism of sparteine (SP), debrisoquine (DB) and bufuralol. Although this does not in itself indicate that the neuroleptics are substrates for the polymorphic enzyme it makes them good candidates for investigation. Recent *in vivo* studies have shown that perphenazine is metabolized by the SP/DB monooxygenase, P450IID6 (Dahl-Puustinen *et al.*, 1989).

In preliminary *in vitro* studies we found that reduced haloperidol (RHAL) was a more potent inhibitor of SP metabolism than HAL. The aim of this study was to

investigate whether RHAL is a substrate for cytochrome P450 and in particular, for the isoenzyme, P450IID6.

Methods

Reagents and materials

Haloperidol (HAL), crystalline (–)-sparteine sulphate (SP), quinidine sulphate (QD), quinine sulphate (QN), magnesium chloride, D-glucose 6-phosphate, β -nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase—type X1, crystalline bovine serum albumin—fraction V and potassium chloride (KCl) were purchased from Sigma Chemical Co. (St Louis, USA). Reduced haloperidol (RHAL) was purchased from Janssen Pharmaceutic (Belgium). Priscoline HCl was a gift from Ciba-Geigy while flurazepam and debrisoquine hemisulphate (DB) were from Hoffman-La Roche. 5-Dehydrosparteine (5DHS), as a perchlorate salt, was a gift of Dr Michael Eichelbaum (M. Fischer-Bosch Institute, Stuttgart, FRG). Human liver tissue from kidney donors was obtained through Drs W. A. Mahon and B. Taylor of the Toronto General Hospital and Dr M. Robinette of Metro Organ Retrieval and Exchange (MORE).

Assay conditions

Washed microsomes were prepared from liver tissue from kidney donors (designated with the code K plus a number, e.g. K10) and one biopsy patient (B2) according to the method of Tyndale *et al.* (1989). Protein concentration was measured by the Biuret method (Layne, 1957). Assay conditions were essentially those of Otton *et al.* (1983) as modified by Tyndale *et al.* (1989) and Tyndale & Inaba (1990). Thus, each tube contained 100 μ l microsomes in 1.15% w/v KCl (0.5 mg protein), substrate ranging in concentration from 100 μ M to 400 μ M (SP in water or RHAL in 5 μ l methanol), inhibitors ranging in concentration from 5 nM to 1 mM in 1.15% w/v KCl, and an NADPH-generating system diluted to 0.5 ml with phosphate buffer (0.2 M, pH 7.4). The assay was started by addition of microsomes and incubation was carried out for 30 min at 37°C. Control samples included methanol where appropriate as this solvent can decrease the activity of P450IID6.

When SP was used as the substrate the reaction was terminated by the addition of 300 μ l cold methylene chloride and then priscoline (0.5 μ g in water) was added as internal standard. SP and the dehydrosparteine metabolites (2DHS, 5DHS) were extracted after addition of 50 μ l of 5 N NaOH. Gas chromatography using a packed column system with a nitrogen-selective detector was as described by Vinks *et al.* (1982). Reaction rates were linear with respect to protein concentration and time within their useful limits and the stability of the liver fraction and frozen incubate was assured (Tyndale *et al.*, 1989).

When RHAL was used as the substrate the reaction was terminated by the addition of 50 μ l 5 N NaOH, internal standard (flurazepam in H₂O) and 5 ml hexane: isopropanol (95:5 v/v). The mixture was vortexed

vigorously and centrifuged. The organic layer was then removed and dried under air and the residue was resuspended in 300 μ l methanol just prior to gas chromatography. The assay and chromatographic conditions have been described by Tyndale & Inaba (1990). The retention times were 4.96, 8.32 and 10.07 min for flurazepam, HAL and RHAL, respectively. Coefficients of variation for the assay of HAL and RHAL ($n = 6$) were 5.24% and 4.66%, respectively. Reaction rates were linear with respect to time for 45 min and with respect to protein concentration up to at least 10 mg ml⁻¹ (Tyndale & Inaba, 1990). The liver fraction was stable at –80°C for at least 40 days and the samples were stable in methanol (at least 12 h) or dried (> 5 days). In order to identify the enzyme as a cytochrome P450, SKF525A was used as a prototype inhibitor.

Data analysis

Experimental data represent means of duplicate incubates. The data shown in Figures 1–4 are means of replicate experiments.

The inhibition data were analysed by the methods of Dixon & Webb (1964) and Cornish-Bowden (1974); lines were fitted by eye.

Results

Assay characteristics

Metabolism of RHAL to HAL by microsomes is drastically decreased upon incubation without an NADPH-generating system (97%), by pre-incubation of the microsomes with carbon monoxide (86%), or by exclusion of the microsomes as the enzyme source (87%). Incubation of HAL (20–400 μ M) with microsomes and NADPH resulted in no measurable breakdown of HAL to other compounds. The P450 inhibitor SKF525A inhibited the oxidation of RHAL to HAL (0.1 mM 11%, 1.0 mM 39%).

Reciprocal inhibition studies

A reciprocal inhibition study tested the effect of RHAL on SP metabolism (K21) and of SP on RHAL metabolism (Figure 1). The data are plotted according to the method of Dixon & Webb (1964) which predicts a linear relationship between reciprocal velocity and the inhibitor concentration at constant substrate concentration. The K_i , as estimated from the plots, was 2 μ M for RHAL inhibition of SP metabolism (Figure 1a) and 200 μ M for SP inhibition of RHAL metabolism (Figure 1b).

Quinidine inhibition studies

Quinidine is a prototype inhibitor of P450IID6 (Gut *et al.*, 1986; Otton *et al.*, 1984) and was tested in order to determine if it would inhibit the oxidation of RHAL to HAL. The estimated K_i for QD inhibition of RHAL metabolism was 0.5 μ M and for SP metabolism it was 0.2 μ M (Figure 2). Parallel lines in the Cornish-Bowden

Figure 1 Dixon plots showing reciprocal inhibition by RHAL and SP in human liver microsomes (K21). Velocity is given in nmol metabolite formed mg^{-1} protein 30 min^{-1} . a) SP substrate concentration was 75, 125, 200 and 250 μM . The K_i for inhibition by RHAL was 2 μM . b) RHAL substrate concentration was 50, 100, 200 and 400 μM . The K_i for inhibition by SP was 200 μM .

Figure 2 Dixon plots showing QD inhibition of SP and RHAL oxidation in human liver microsomes (K21). Velocity is given in nmol metabolite formed mg^{-1} protein 30 min^{-1} . a) SP substrate concentration was 75, 125, 200 and 250 μM . The K_i for QD inhibition was 0.2 μM . b) RHAL substrate concentration was 50, 100, 200 and 400 μM . The K_i for QD inhibition was 0.5 μM .

(1974) plots confirmed the competitive nature of the inhibition (Figures 1 and 2).

Stereoselective inhibition studies

The stereoselective nature of the quinidine/quinine inhibition of P450IID6 (quinidine is 100–250 times more potent than quinine) has been used previously to characterize the activity of this enzyme (Otton *et al.*, 1988; Steiner *et al.*, 1988). In microsomes from K10 the metabolism of SP and of RHAL was inhibited by quinidine and quinine (Figure 3). Quinine curves were shifted to the right of those for quinidine (c.f. Otton *et al.*, 1988). For SP metabolism the IC_{50} values were 0.1 μM for QD and 32 μM for QN (Figure 3). The IC_{50} values were 0.1 μM and 6 μM for QD and QN, respectively, for RHAL oxidation (Figure 3). Inhibition was maximal at 3 μM QD and 300 μM QN for SP, 3 μM QD and 60 μM QN for RHAL.

Relative velocity studies

Because SP oxidation is characteristic of P450IID6 a correlation between RHAL oxidation and SP oxidation in various livers would provide further evidence for RHAL being a substrate for this isoenzyme. Both activ-

Figure 3 The effects of quinidine (QD) and quinine (QN) on the rate of appearance of SP metabolite (2DHS) and RHAL metabolite (HAL) from 200 μM substrate incubated for 30 min with human liver microsomes (K10). Each point is the mean value for duplicate experiments.

Figure 4 Correlation between the log velocity of metabolite formation (nmol mg^{-1} protein 30 min^{-1}) of 2DHS from SP ($200 \mu\text{M}$) and HAL from RHAL ($200 \mu\text{M}$), in 17 human liver microsomal preparations ($r_s = 0.62$, $P < 0.01$). Each value is the mean of experiments on two different days. K16 is a genotypically and phenotypically poor metabolizer liver (Gonzalez *et al.*, 1988; Tyndale *et al.*, 1990), the rest are extensive metabolizer livers.

ities were measured in the same human liver microsomal preparations from 17 different donors. Figure 4 shows the correlation between 2DHS and HAL production ($r_s = 0.62$, $P < 0.01$) suggesting that the same P450 isoenzyme is involved in both reactions.

Discussion

The data strongly suggest that RHAL oxidation is catalysed by one or more cytochromes P450 in human liver. This metabolism requires the liver microsomal fraction and NADPH, and is inhibited by SKF525A and by carbon monoxide. It is important to note, however, that conversion of HAL back to RHAL does not occur in the liver microsomal fraction. It has been established that in human liver the conversion of HAL to RHAL occurs in the cytosolic fraction and is catalysed by an enzyme having the characteristics of a ketone reductase (Inaba & Kovacs, 1989). This activity is also present in red blood cells (Inaba *et al.*, 1989).

During a 30 min incubation with various concentrations of RHAL, oxidation to HAL was measured. There was essentially a complete recovery of the drug in the form of RHAL plus its metabolite HAL. Oxidative *N*-dealkylation of HAL (and possibly RHAL) involving cleavage of the carbon-nitrogen bond of the central chain has been established as a route of elimination (Forsman *et al.*, 1977), but this reaction did not appear to occur during the 30 min incubation.

Direct evidence that P450IID6 catalyses the oxidation of RHAL to HAL awaits measurement in a reconstituted system containing the purified polymorphic cytochrome P450IID6. However, strong indirect evidence can be obtained from experiments using selective inhibitors and by comparing relative activities over a number of livers with different monooxygenase capacities. The data presented here suggest that the poly-

morphic monooxygenase which metabolizes SP/DB is also capable of metabolizing RHAL.

Reciprocal inhibition studies have previously been used to suggest that two different compounds are metabolized by the same enzyme site (Otton *et al.*, 1982). HAL is an established inhibitor of the SP/DB monooxygenase (Inaba *et al.*, 1985) as are many of the neuroleptics (Derenne *et al.*, 1989; Spina *et al.*, 1988; Syvalahti *et al.*, 1986; von Bahr *et al.*, 1985). In preliminary studies we found that RHAL, a metabolite of HAL, was five times more potent than the parent drug in inhibiting SP metabolism and that the inhibition was competitive in nature. Reciprocal inhibition between RHAL and SP, was therefore investigated.

RHAL inhibited SP oxidation with an estimated K_i of $2 \mu\text{M}$ and SP inhibited RHAL oxidation with an estimated K_i of $200 \mu\text{M}$ (Figure 1a and 1b). Whereas absence of SP inhibition of RHAL oxidation and RHAL inhibition of SP metabolism would argue against a single form of P450 metabolizing both reactions, the reciprocal inhibition observed is consistent with involvement of a single form of P450, the polymorphic SP/DB monooxygenase.

QD is a prototype P450IID6 inhibitor (Broly *et al.*, 1989; Gut *et al.*, 1986; Otton *et al.*, 1988; Tyndale *et al.*, 1990) and was therefore tested as an inhibitor of RHAL oxidation. QD inhibited both RHAL and SP oxidations competitively having estimated K_i values of $0.5 \mu\text{M}$ for RHAL inhibition and $0.2 \mu\text{M}$ for SP metabolism (Figure 2a and 2b). Potent (nM) QD inhibition of RHAL oxidation further suggests the involvement of P450IID6 in the oxidation of RHAL.

The stereoselective nature of the quinidine inhibition of P450IID6 is well established, whereby the diastereoisomer quinine is 100–250 times less potent (Otton *et al.*, 1984; Speirs *et al.*, 1986). This difference is characteristic of P450IID6 and has been exploited previously to establish substrates for the polymorphic enzyme *in vivo* as well as *in vitro* (Otton *et al.*, 1988; Steiner *et al.*, 1988). The stereoselective inhibition of RHAL oxidation (like that of SP oxidation, Figure 3) by quinidine and quinine further suggests that this reaction is catalysed predominantly by P450IID6 (Figure 3). The 10–20% activity remaining after inhibition is maximal has been observed before for P450IID6 catalysed reactions such as the *O*-demethylation of metoprolol (Otton *et al.*, 1988), the 1'-hydroxylation of bufuralol (Zanger *et al.*, 1988) and codeine *O*-demethylation (Dayer *et al.*, 1988).

The correlation between RHAL oxidation and SP oxidation in 17 different human livers (Figure 4) was significant, suggesting that P450IID6, which is known to metabolize SP, also metabolizes RHAL. K16 which is genotypically and phenotypically a poor metabolizer liver (Gonzalez *et al.*, 1988; Tyndale *et al.*, 1990) was also a relatively poor oxidizer of RHAL (Figure 4). The oxidation of RHAL to HAL was not significantly correlated to other microsomal enzyme activities (diazepam *N*-demethylation, $r = 0.10$; Inaba *et al.*, 1988; 7-ethoxyresorufin *O*-deethylation, $r = 0.25$; Campbell *et al.*, 1987) or to P450 content ($r = 0.10$). As the horizontal range of data points in Figure 4 represents the range of P450IID6 activity the vertical scatter away from a linear correlation suggests that in addition to the P450IID6 catalysed oxidation of RHAL, other enzyme(s) (with a variable contribution in different livers) are involved.

Recently, Brosen & Gram (1989) classified HAL as a drug whose metabolism was not under the control of the polymorphic SP/DB monooxygenase. This conclusion was based on the observation that serum concentrations of HAL did not differ between one poor metabolizer and eleven extensive metabolizers (Gram *et al.*, 1989). They also observed a substantial rise in the SP metabolic ratio (parent/metabolite) during HAL treatment and were able to correlate this rise with serum concentrations of HAL. In view of our findings it would have been useful to have measured RHAL concentrations because the elevation of MR might be related to the concentration of RHAL as it is a potent inhibitor of SP oxidation. The concentrations of HAL are dependent on the relative velocities of RHAL oxidation, glucuronide conjugation, oxidative dealkylation and ketone reduction. Therefore, the finding that a poor metabolizer and eleven extensive metabolizers have similar HAL concentrations does not necessarily eliminate a role for P450IID6 in the metabolism and interconversion of RHAL and HAL.

The conversion of RHAL to HAL in an extensive

metabolizer may vary when HAL is combined with any drug which has a high affinity for P450IID6, for example the tricyclic antidepressants and other neuroleptics. As multiple neuroleptic treatment is common as well as cotreatment with neuroleptics and antidepressants, these interactions may have a clinically significant effect on the ratio of RHAL/HAL. It is conceivable that drug interactions are important not only in the liver but also in the brain where P450IID6 (sparteine/debrisoquine monooxygenase) is also present (Fonne-Pfister *et al.*, 1987; Niznik *et al.*, 1990).

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