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INTERCONVERSIONS OF HALOPERIDOL AND REDUCED HALOPERIDOL IN GUINEA PIG AND RAT LIVER MICROSOMES

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Abstract—An alcohol metabolite of haloperidol, reduced haloperidol, is present in the tissues of haloperidol-treated patients. We have studied whether rat and guinea pig liver microsomes have the capability to reduce haloperidol and thus serve as models for human haloperidol metabolism. Interestingly, the rat microsomes did not reduce haloperidol, but possessed an NADPH-dependent, carbon monoxide-inhibited mechanism to oxidize the reduced haloperidol back to haloperidol. Guinea pig microsomes efficiently reduced haloperidol molecules in a fashion not dependent on nicotinamide cofactors and not inhibited by carbon monoxide. Both of these activities were confined to the microsomal fraction. In guinea pigs, reduction of haloperidol was observed also in kidney slices, whereas brain slices proved inactive. Reduced haloperidol was also oxidized to haloperidol to a small extent in guinea pig microsomes. These *in vitro* experiments confirm our findings *in vivo*, which showed that in rats haloperidol is not reduced, while guinea pigs have a very active mechanism for reducing haloperidol. Thus, guinea pigs constitute a model for human haloperidol metabolism, and they should be used for further characterization of the reductive drug-metabolizing system.

Reduced haloperidol (RHAL) is a major metabolite of haloperidol (HAL) in human beings ([1–3]; Fig. 1). The mechanisms for reducing HAL to the alcohol metabolite are unknown. Characterization of the mechanisms appears meaningful for several reasons. First, reductive drug-metabolizing systems are much more poorly known than the oxidative processes. Second, RHAL may have antipsychotic potency [3]. Third, establishment of a specific enzymatic reducing mechanism would have clinical relevance, if variation of its activity among schizophrenic patients were found, especially with low activity linked to better response than high activity.

We have found recently that *in vivo* HAL is greatly reduced in guinea pigs, but not in rats [4, 5]. We have now studied liver microsomal preparations from these two animal species and observed that HAL metabolism *in vivo* can be qualitatively mimicked by incubation experiments *in vitro*.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (200 g) (Zivic Miller, Allison Park, PA) and adult male guinea pigs (300–350 g) (NIH, Bethesda, MD) were used in this study.

Preparation and incubation of microsomes. Livers from nonperfused animals were quickly excised, homogenized in 3 vol. (w/v) of 1.15% (w/v) KCl in 20 mM Tris–HCl (pH 7.4). The homogenate was centrifuged for 20 min at 9000 *g* at the rotor temperature of +4°. The resulting supernatant fraction was further centrifuged for 60 min at 88,000 *g* at +8°, and the pellet was resuspended in KCl solution (1 ml/1 g initial liver) and centrifuged again for 30 min at 88,000 *g*. The final pellet was either resuspended (0.8 to 1.2 g fresh weight/ml) in ice-cold 0.2 M potassium phosphate buffer (pH 7.5) and used within 1 hr, or it was stored for later use as a pellet in a freezer at −70°. The frozen pellet was then resuspended in 0.2 M phosphate buffer just prior to the incubations that took place within 7 days of the preparation.

Six rats and six guinea pigs were anesthetized with ether and perfused with saline (pH 7.4 with sodium phosphate) through the left ventricle and out the right atrium. When the livers were cleared of visible blood, microsomes were prepared from them as

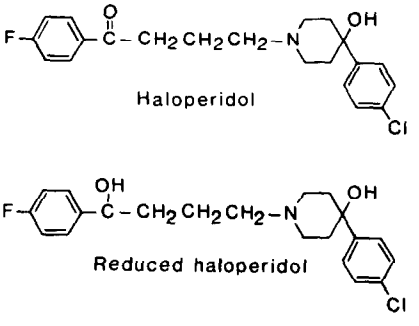

Fig. 1. Structural formulae of haloperidol (HAL; 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone) and reduced haloperidol (RHAL; 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanol).

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described above except that the supernatant fractions were saved and only one high-speed centrifugation was needed. These microsomes and supernatant fractions were used on the day of preparation.

The microsomal preparation of the rats was incubated in borosilicate tissue culture tubes with Teflon-lined screw caps in a final volume of 2 ml, containing the equivalent of 1.0 g of fresh liver (about 16 mg protein) and 1 mM $NADP^+$, 1 unit of glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate, 10 mM $MgCl_2$ and various concentrations (0.0265 to 26.5 μM) of RHAL or HAL. Incubations took place at 37° under an atmosphere of O_2–CO_2 (95:5, v/v) for several periods of time, usually for 30 min. Incubations were terminated by placing the tubes in an ice-bath and by adding 250 μl of 2 M NaOH. Preliminary experiments indicated that the microsomes that were stored at −70° and thawed within 1 week of the preparation fully retained their activity. In experiments with microsomes from perfused rats, part of the incubation volume was replaced by the supernatant, and in the corresponding control incubations the same volumes of KCl solution were added.

Experiments with guinea pig liver microsomes were carried out in the same way as those with rat microsomes with the exception that phosphate buffer replaced the NADPH-regenerating system unless otherwise stated. Microsomes were used with a final dilution of 0.5 g of liver/ml, but in the kinetic analyses only 0.3 g/ml was used to slow down the reaction rate. Guinea pig microsomes could also be stored frozen for at least 1 week without any loss of activity (longer periods were not tested).

Tissue slice incubations. Slices 0.4 mm thick were prepared with a non-wetted McIlwain-type tissue chopper from liver, kidney and two brain regions (striatum, dopaminergic area; cerebellum, non-dopaminergic area) of rats and guinea pigs immediately after decapitation. The slices were preincubated for 15 min in prewarmed and gassed Krebs–Ringer solution [6] at 37°. The gassing was with the O_2–CO_2 mixture. Then either 0.265 μM HAL or RHAL was added and the incubation continued for 30 or 60 min. The reactions were stopped by 2 M NaOH, the internal standard was added, and the tissue was carefully sonicated with a small probe. Then the butyrophenones were measured as described below.

Analysis of HAL and RHAL. Procedures used for the extraction and quantitation of the butyrophenones have been described earlier [2, 7]. Briefly, chlorohaloperidol as an internal standard was added into the incubation tubes after the microsomal solutions were alkalinized. Similarly, various standard amounts of HAL, RHAL and chlorohaloperidol were added to unincubated standard samples already alkalinized with NaOH. The butyrophenones were extracted with an isoamyl alcohol/hexane mixture 1.5:98.5 (v/v), then back-extracted into aqueous dilute hydrochloric acid, and finally re-extracted into the organic phase. After evaporation, the residues were solubilized in the mobile phase [40 mM potassium phosphate (pH 6.8)/acetonitrile, 55:45 (v/v)] and injected into the liquid chromatograph. It consisted of a Waters solvent delivery system (model 6000A, Waters Associates, Milford, MA), a Waters automatic sample injector 710B, either a μBondapak CN (30 × 3.9 cm, 10 μm, Waters Associates) or an Altex Ultrasphere Cyano column (15 × 4.6 cm, 5 μm, Rainin Instrument Co., Inc., Woburn, MA), an LC-4A electrochemical detector equipped with a glassy carbon TL-5 flow cell (Bioanalytical Systems, Inc., West Lafayette, IN), and a Beckman two-channel chart recorder. The flow rate was 1.2 ml/min, the injection volume 100 μl, the electrochemical potential 0.90 V, and the sensitivity of the method about 2 pmoles per injection. The concentrations of HAL and RHAL were determined by comparison with the standard samples.

The final results are expressed as percent recovery of HAL and RHAL after the incubations. This presentation was considered appropriate for the present experiments, since it could not be excluded that the initial substrate or the conversion product was metabolized by other reactions [8].

Microsomal protein was measured by the assay of Lowry *et al.* [9]. Haloperidol, reduced haloperidol and chlorohaloperidol were donated by Janssen Pharmaceutica, Belgium.

RESULTS

Oxidation of RHAL to HAL in rat liver. The formation of HAL during incubation of rat liver microsomes in the presence of RHAL was easily

Table 1. Oxidation of RHAL by rat liver microsomes*

Incubation conditions		Recovery (%) as HAL	RHAL
(1) No cofactors		0	92 ± 3
+ NADPH	(1 mM)	41 ± 1	21 ± 1
+ NADP	(1 mM)	26 ± 1	49 ± 1
+ NADH	(1 mM)	42 ± 1	52 ± 2
+ NAD	(1 mM)	30 ± 2	58 ± 1
+ FAD	(1 mM)	0	91 ± 2
+ FMN	(1 mM)	0	102 ± 5
+ Riboflavin	(1 mM)	0	96 ± 4
(2) Control		69 ± 9	29 ± 4
CO gassing		9 ± 1	88 ± 10
N_2 gassing		40 ± 3	41 ± 1
0°, control		26 ± 2	73 ± 5
0°, CO gassing		8 ± 1	83 ± 5
(3) Control (perfused rats)		40 ± 1	11 ± 2
+ Supernatant 200 μl		40 ± 3	11 ± 2
+ Supernatant 390 μl		42 ± 1	13 ± 4
(4) 15 min		41 ± 1	25 ± 2
60 min		45 ± 1	26 ± 1
120 min		45 ± 1	25 ± 2
(5) Control		38 ± 2	38 ± 2
Boiled microsomes		0	101 ± 3

* Unless otherwise stated, the concentration of RHAL (0.265 μM) and the amount of microsomal protein (15–20 mg) were in a final incubation volume of 2 ml; incubation was under an oxygen–carbon dioxide (95:5, v/v) atmosphere at 37°. Results are given as percent recovered of the initially added RHAL as HAL or RHAL after 30-min incubations. Experiments 2–5 were carried out in the presence of the NADPH-regenerating enzyme system. The values are means ± S.E.M. of three to seven determinations.

demonstrated (Table 1). It was dependent on the presence of an NADPH-regenerating system (or NADPH). Low temperatures inhibited the reaction. Under the present conditions, equilibrium was reached after about 15 min of incubation. The oxidation was inhibited by an atmosphere of carbon monoxide; a slight inhibition was also evident in an atmosphere of nitrogen. The supernatant fraction of the microsomal pellet did not have the ability to oxidize RHAL, nor did it affect the reaction produced by the microsomes. In the absence of glucose-6-phosphate dehydrogenase, of the various cofactors tested to facilitate the oxidation of RHAL, the reduced adenine nucleotide (NADPH) was the best, giving the highest recovery of HAL and the lowest of RHAL. NAD, for example, gave about 10% lower recovery of HAL and about 50% higher recovery of RHAL than NADPH. The flavin nucleotides were totally ineffective. Boiling of the microsomes destroyed the oxidizing capacity.

The oxidation of RHAL was dependent upon the RHAL concentration in a complex fashion (Fig. 2). After a 30-min incubation, the ratio of HAL to RHAL increased from about 1 (26.5 nM initial RHAL concentration) to about 6 (2.65 μM), but the percentage recovery of the drugs after incubation decreased when the concentration was increased.

Striatum and cerebellum slices of the rat were ineffective in oxidizing RHAL, whereas liver slices produced HAL from RHAL (Table 2). The recovery of HAL decreased during incubation of liver slices with added HAL, thus indicating the metabolism of HAL. No RHAL could be recovered in these incubations (data not shown). Metabolism of HAL was also demonstrated in rat liver microsomes (Fig. 2), but no RHAL was detected. RHAL also was not formed from HAL during incubation of rat microsomes in the absence of NADPH (data not shown).

Reduction of HAL by guinea pig liver microsomes. Like the oxidation of RHAL by rat microsomes, the reduction of HAL by guinea pig microsomes also was dependent upon the incubation temperature, totally inhibited by boiling of the microsomes before incubation, and not affected by the presence of liver supernatant (Table 3). But, in contrast to the rat liver oxidizing system, the reduction was not affected by prior gassing with carbon monoxide or nitrogen.

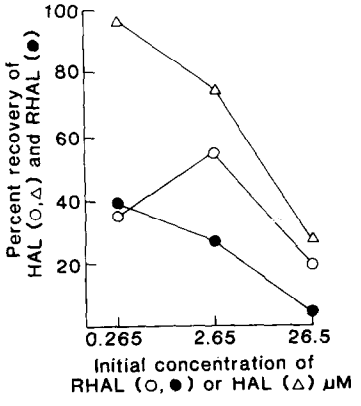

Fig. 2. Concentration dependence of RHAL oxidation by rat liver microsomes.

Table 2. Oxidation of RHAL by rat liver slices*

Tissue slices from	Recovery (%) as HAL	RHAL
Liver	24 ± 6	16 ± 2
Striatum	0	86 ± 11
Cerebellum	0	87 ± 5

* Slices of striatum (80 mg wet weight), cerebellum (200 mg) and liver (200 mg) were preincubated for 15 min at 37° under O_2–CO_2 atmosphere in 1 ml of Krebs–Ringer solution; then RHAL was added to make the initial concentration of 0.265 μM and incubation was continued for 60 min. Results describe the recovery of the butyrophenones as unaltered RHAL or oxidized HAL. They are given as means ± S.E.M. of five experiments.

Furthermore, when the NADPH-regenerating system was added to the incubation mixture, the actual recovery of RHAL was lower than without NADPH.

The reaction exhibited a complex dependence on the initial concentration of HAL during 30-min incubations, with intermediate HAL concentrations (0.265 and 2.65 μM) yielding the greatest percent RHAL recoveries (Table 4). The reduction rate was quick (Fig. 3), with the formation of RHAL apparently stopping after 15 min, when the concentration of HAL also had decreased.

We characterized the HAL reduction by incubating microsomes in the presence of various initial HAL concentrations for 2-min periods to give initial reduction rates, since the reduction was not dependent on NADPH and probably no other form of HAL catabolism occurred in the absence of NADPH. It is at least clear that RHAL was not converted back to HAL without the added cofactors. Figure 4 describes the dependence of the reduction rate upon the initial HAL concentrations. It should

Table 3. Reduction of HAL by guinea pig liver microsomes*

Incubation conditions	Recovery (%) as HAL	RHAL
(1) Control	18 ± 1	72 ± 3
+ NADPH	27 ± 1	61 ± 1
(2) Control	35 ± 1	50 ± 1
CO gassing	34 ± 1	49 ± 1
N_2 gassing	34 ± 2	59 ± 2
0°, control	80 ± 4	0
0°, CO gassing	85 ± 3	0
0°, N_2 gassing	76 ± 3	0
(3) Control (perfused guinea pigs)	17 ± 1	72 ± 2
+ Supernatant 800 μl	16 ± 1	82 ± 1
Supernatant (1 ml) w/o microsomes	80 ± 2	0
(4) Control	21 ± 2	66 ± 3
Boiled microsomes	105 ± 2	0

* Unless otherwise stated, the concentration of HAL initially was 0.265 μM and the amount of microsomal protein 12–18 mg with a 2-ml incubation volume without the NADPH-regenerating system; incubations took 30 min at 37° under O_2–CO_2 atmosphere. Results (mean ± S.E.M. from three to eight incubations) are expressed as percent recoveries of HAL and RHAL after incubations.

Table 4. Concentration dependence of HAL reduction*

Initial concn of HAL (μM)	Recovery (%) as HAL	RHAL
0.0265	103 ± 13	0
0.265	35 ± 2	55 ± 2
2.65	40 ± 2	58 ± 2
26.5	80 ± 2	11 ± 2

* Microsomes (about 10 mg protein) from nonperfused guinea pig livers were incubated in a final volume of 2 ml for 30 min at O_2–CO_2 atmosphere in the presence of different initial HAL concentrations. Results are given means ± S.E.M. for four experiments.

be noted that even during such a short incubation time the substrate concentrations were reduced significantly (by 10–20%), thus obviously reducing the value of the kinetic analysis. Nevertheless, the reaction rates apparently gave a straight line against the HAL concentration in the double-reciprocal plot, giving the estimates of 5 μM and 800 nmoles/(kg protein × sec) for K_m and V_{max} respectively.

The slices of guinea pig striatum and cerebellum were not able to reduce HAL (Table 5), whereas liver and kidney slices had this ability. RHAL was not converted to HAL by guinea pig microsomes without the NADPH-regenerating system.

DISCUSSION

The efficient reduction of HAL by guinea pig liver and kidney and the inability of rat liver to carry out this reaction indicate an important species difference in drug metabolism. The HAL reducing reaction apparently was saturable and temperature-dependent, both characteristics of enzyme-mediated reactions. Its independence of NADP and NADPH clearly distinguished it from the oxidation of RHAL that has features (preference for NADPH; microsomal localization; nearly complete inhibition by carbon monoxide, but not by anoxia) suggesting it to be a part of the microsomal oxidative drug-detoxicating mechanism.

Experimental data on reductases, especially on ketone reductases, have been reviewed recently [10]. These enzymes obviously are designed to convert hydrophobic carbonyl compounds (e.g. daunorubicin, naloxone, and metyrapone) to more hydrophilic alcohols. The main properties of these enzymes are ubiquitous tissue distribution, cytosolic localization, preference for NADPH as a cofactor and low molecular weight. Our results indicate that the HAL reducing system of guinea pig liver has little in common with these ketone reductases (cf. microsomal localization and nonpreference for NADPH), although we do not have any knowledge about the molecular weight and we have not carried out detailed tissue distribution studies. In the present experiment, there was no indication that HAL was reduced in brain tissue (Table 5), whereas the ketone reductase activity is present in the brain [10]. It

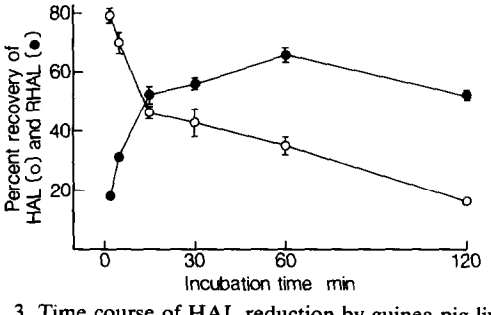

Fig. 3. Time course of HAL reduction by guinea pig liver microsomes.

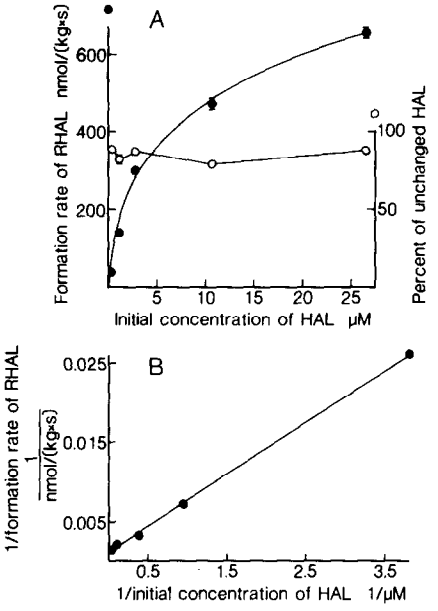

Fig. 4. (A) Dependence of RHAL formation on HAL concentration. Points are means ± S.E.M. or only means, when S.E.M. was smaller than the symbol. Number of incubations = 3–5. The rate of RHAL formation is expressed as nmoles per kg protein in a second. (B) Lineweaver–Burk plot of the initial rate of HAL reduction by guinea pig liver microsomes. Data points are calculated from the results of panel A. The linear regression line was $y = 0.00122 + 0.00651x$ ($r^2 = 0.9998$), yielding an estimate for K_m of 5 μM and for V_{max} of 800 nmoles/(kg protein × sec).

Table 5. Reduction of HAL by guinea pig liver and kidney slices*

Tissue slices from	Recovery (%) as HAL	RHAL
Liver	14 ± 1	77 ± 4
Kidney	31 ± 4	70 ± 9
Striatum	100 ± 3	0
Cerebellum	98 ± 4	0

* Slices of liver (200 mg), kidney (200 mg), striatum (100 mg) and cerebellum (200 mg) were preincubated for 15 min in a volume of 1 ml. Then 0.265 μM (initial concentration) HAL was added. The incubation continued for 30 min, after which the amounts of remaining HAL and RHAL were determined and given below as means ± S.E.M. for three to six experiments.

is not known whether our assay method was too insensitive to monitor the reduction by brain slices, since Felsted and Bachur [10] state that "the absence of a specific reductase activity in a tissue merely reflects the lack of sufficient assay sensitivity". Thus, it may be premature to conclude that we are dealing with a new, previously undescribed ketone reducing mechanism. Further work is needed to determine the exact tissue distribution, but it would require a more sensitive assay method, probably using radioactively-labeled substrates. Improved sensitivity would also be needed for ascertaining the complete inability of rat tissues to reduce haloperidol.

The independence of the reduction of nicotinamide cofactors indicates that, in the guinea pig tissues, some other type of substance is supplying the reducing equivalents. The identity of the substrate is unknown to us, but apparently either it has a large capacity or else there is an endogenous "recycling" mechanism to keep it in the reduced state.

Our preliminary characterization of the kinetics of the HAL reduction in guinea pig microsomes may be biased by two experimental reasons. First, the rate measured during 2 min may already be starting to taper off from the linear part because of falling substrate concentration, which would decrease the apparent V_{max}. Second, lipophilic drugs like butyrophenones are readily absorbed into cell membrane lipid bilayers and thus escape any action of the enzymes studied [11], which would increase the apparent K_m of the reduction.

In a cautious extrapolation to man, we may suggest that interconversions of HAL and RHAL have clinical significance and an important modulating role in the pharmacokinetics of HAL administration. During a chronic HAL treatment with a steady dose, RHAL may be accumulating in all tissues, thus possibly reducing the effectiveness of high HAL doses. On the other hand, when decreasing the dosage, RHAL could be converted back to HAL, thus preserving the concentrations of HAL for prolonged periods. It is not known whether RHAL is broken down by mechanisms other than the oxidation back to the parent molecule, and it is still to be demonstrated that RHAL can be oxidized to HAL in man. If RHAL is not greatly metabolized otherwise, then the RHAL "storage" pool would artificially lengthen the half-times of haloperidol elimination after a single dose and after discontinuation of prolonged therapy.

Further investigation on the reduction of HAL seems important, and efforts should be directed to the characterization and eventual isolation of the HAL reducing enzyme.

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