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Induction of propranolol metabolism in the Hep G2 human hepatoma cell line

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Abstract—Metabolism of propranolol by the human hepatoma cell line Hep G2 was studied. Although metabolism qualitatively was similar to that in-vivo, the P450-mediated *N*-desisopropylation clearly predominated. Pretreatment of cells with 3-methylcholanthrene increased the activity of this pathway 14-fold, whereas phenobarbitone had no effect. This is similar to the pathway-selective inductive response observed for cigarette smoking in-vivo. As in-vivo, secondary metabolism of *N*-desisopropylpropranolol was extensive. This could, however, be completely blocked by 0·1 μM clorgyline, a potent MAO type A inhibitor. As in human liver microsomes, the stereochemistry of propranolol metabolism demonstrated a preference for the *R*(+)-enantiomer. These observations emphasize the usefulness of the Hep G2 cell line as a model of man.

Propranolol is commonly used as a model compound in in-vivo and in-vitro human drug metabolism studies. A wide range of environmental and genetic factors is known to affect propranolol's metabolic clearance (Lennard et al 1984; Raghuram et al 1984; Walle et al 1985a; Ward et al 1989). Two such factors of importance for drug disposition in general are sex differences and cigarette smoking, both of which have selective effects on the cytochrome P450-mediated metabolism of propranolol. Thus, the metabolic clearance of propranolol is higher in men than in women (Walle et al 1989) and also higher in cigarette smokers compared with nonsmokers (Walle et al 1987), mainly due to higher clearance through side-chain oxidation (Fig. 1).

The objective of the present investigation was to examine the human Hep G2 cell line (Dawson et al 1985; Sassa et al 1987; Grant et al 1988; Doostdar et al 1990; Fischer & Wiebel 1990) as a potential in-vitro hepatic model for the human metabolism of propranolol, in particular side-chain oxidation, and its regulation by enzyme inducers and other factors.

Materials and methods

Materials. Hep G2 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and Williams' medium E was from Gibco BRL (Grand Island, NY, USA). Foetal calf serum, propranolol HCl, phenobarbitone sodium salt, clorgyline and pargyline were purchased from Sigma Chemical Co (St Louis, MO, USA). (±)-[4-^{3}H]Propranolol HCl (sp. ac., 25 Ci mmol^{-1}) was from Amersham Corp. (Arlington Heights, IL, USA), 3-methylcholanthrene from Eastman (Rochester, NY, USA) and (−)-menthyl chloroformate (MCF) from Aldrich Chemical Co (Milwaukee, WI, USA). 4-Hydroxypropranolol (4HOP)(Fig. 1), 5-hydroxypropranolol (5HOP), *N*-desisopropylpropranolol (DIP), propranolol glycol (Glycol) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) were synthesized in our laboratories (Nimura et al 1980; Oatis et al 1981; Bargar et al 1983). α-Naphthoxylactic acid (NLA) was a gift from ICI Ltd (Wilmslow, UK). All solvents were of HPLC grade from Burdick & Jackson (Muskegon, MI, USA).

Culture of Hep G2 cells. Cells were grown in a humidified atmosphere of 5% CO_2 in air in Williams' medium E supplemented with 10% (v/v) foetal calf serum, 100 int. units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Doostdar et al 1990). The cells were split 1:3 every 7 days and used for experiments on day 11 after passage (Doostdar et al 1990). The medium was renewed routinely 3, 6 and 10 days after passage. In the induction studies, 2 μM 3-methylcholanthrene (in dimethyl sulphoxide) or 2 mM phenobarbitone was added to the cells 24 h before experiments. These concentrations are inductive in the Hep G2 cells (Grant et al 1988; Labruzzo et al 1989). The dimethylsulphoxide, final concentration 0·2% in the medium, had no effect on metabolism.

Incubations. Washed cells were scraped off the culture into tubes with 100 mM phosphate buffer, pH 7·6 (1 mL 100 mm dish), and pressurized with nitrogen in a cell disruption bomb (1000 psi for 10 min). To 0·5 mL of disrupted cell homogenate was added an NADPH-generating system (0·5 mM NADP, 10 mM glucose-6-phosphate, 5·2 mM magnesium chloride and 2 int. units mL^{-1} glucose-6-phosphate dehydrogenase) in buffer for a total volume of 1·0 mL. Incubations were carried out for 30 min at 37°C in a

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shaking water bath with either radioactively labelled propranolol (10 μM, 0·5 μCi) or 1 μM DIP as the substrate, and were stopped by adding ice-cold 0·4% sodium bisulphite solution. The propranolol and DIP concentrations used were estimates of what may be expected to be present in the liver after a clinical oral dose and have been commonly used in microsomal studies (von Bahr et al 1982; Otton et al 1990). Preincubations with monuamine oxidase (MAO) inhibitors, clorgyline and pargyline, were carried out for 15 min at 37°C (Tipton et al 1983). Cell homogenate protein was measured by the method of Lowry et al (1951).

Analytical chemistry. All incubates were mixed with 10 μg of unlabelled propranolol metabolites (4HOP, 5HOP, DIP, Glycol, NLA). The pH 9·6 ethyl acetate extracts of the samples were taken to dryness and analysed by reversed-phase HPLC (Bargar et al 1983). The equipment was a Model 6000A pump, a Model U6K injector and a Model 440 UV detector with a 280 nm filter from Waters-Millipore (Milford, MA, USA). The metabolites were separated on a Spherisorb ODS-1, 5 μm column, 25 cm × 4·6 mm (Phenomenex, Torrance, CA, USA). The mobile phases were acetonitrile-methanol-water (30:5:65, v/v/v) in 0·05 M ammonium acetate, pH 4, from 0 to 14 min, and acetonitrile-water (60:40) in 0·05 M ammonium acetate, pH 4, from 14 to 26 min. The flow-rate was 1·0 mL min^{-1}. The separation pattern for propranolol and metabolites was similar to previous studies (Bargar et al 1983). Metabolite and unchanged propranolol fractions were collected and their radioactivity counted. NLA was determined similarly after pH 2 benzene extraction and HPLC using a Spherisorb ODS-2 column with acetonitrile-methanol-water (40:5:55) in 1 M acetic acid as the mobile phase.

HPLC fractions containing DIP, 4HOP and 5HOP (see above) were freeze-dried. The enantiomers were then separated by HPLC on a Spherisorb ODS-1 column after chiral derivatization of DIP with GITC (Walle et al 1985b) and of 4HOP and 5HOP with MCF (Mehvar 1989). The mobile phases used were acetonitrile-methanol-water (38:5:57) in pH 4, 0·05 M ammonium acetate for the DIP diastereoisomers and acetonitrile-water (80:20) in the same buffer for the 4HOP and 5HOP diastereoisomers.

For all radioactive metabolite measurements, the between-culture coefficient of variation was 10–15%, whereas the coefficient of variation for repeated injections was less than 5%. The minimum detectable concentration was about 10 ng mL^{-1}.

Following incubations with DIP and extraction at pH 7 with hexane-butanol (99:1, v/v), the peak height of the glycol metabolite formed was measured by HPLC/fluorometry using 290 nm excitation with a 320 nm emission cut-off filter. The HPLC equipment was the same as described above, with a Model FD 200 fluorescence detector (Spectrovision, Chelmsford, MA, USA). The mobile phase was acetonitrile-methanol-water (37:5:58) in 0·05 M ammonium acetate, pH 4, and the flow-rate was 1·0 mL min^{-1}. The coefficient of variation was 3–8% with a minimum detectable concentration of 5 ng mL^{-1}.

Results

The metabolic scheme for propranolol by the Hep G2 cells (Fig. 1) was established based on the use of radioactive propranolol and the addition of synthetic reference metabolites as carriers in the HPLC analysis. The base line metabolism was about 2 pmol min^{-1} (mg cell protein)$^{-1}$. This corresponded to about 1% metabolism in a 30 min incubate. Total side-chain oxidation clearly predominated over total ring oxidation (Fig. 2), with DIP accounting for 29 ± 5% (mean ± s.e.) and Glycol 42 ± 6% of the metabolism. NLA, surprisingly, was only a trace metabolite.

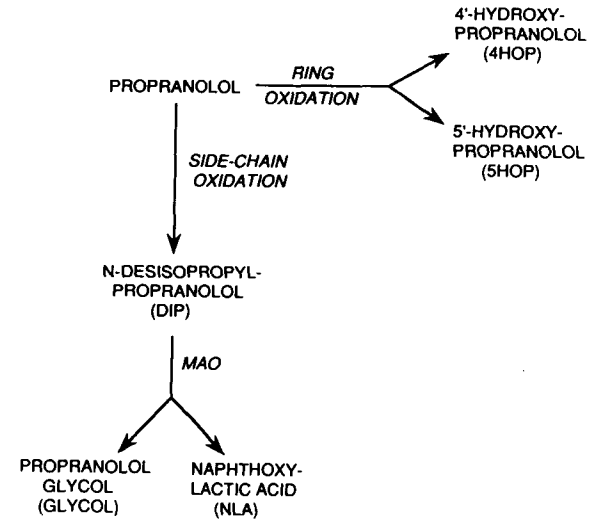

FIG. 1. Summary of propranolol metabolism in Hep G2 cell homogenates.

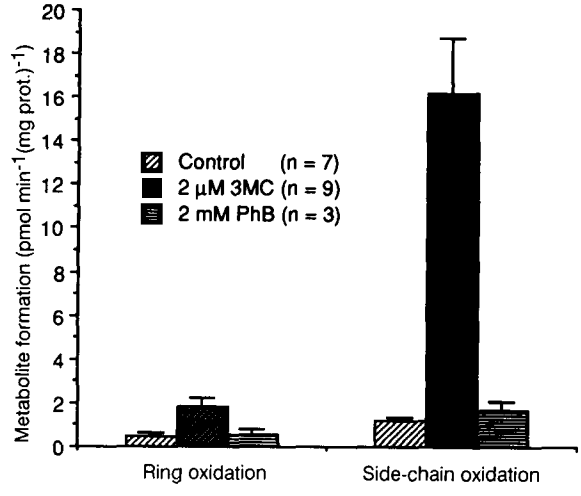

FIG. 2. Effects of 3-methylcholanthene (3MC) and phenobarbitone (PhB) on the two primary pathways of propranolol metabolism in Hep G2 cells.

Ring oxidation includes 4HOP and 5HOP; side-chain oxidation includes DIP, Glycol and NLA (see Fig. 1). Mean values ± s.e. are shown. The figures in brackets refer to number of separate cell culture preparations used.

Total ring oxidation accounted for 29 ± 5% of metabolism, equally divided between 4HOP and 5HOP.

Pretreatment of the hepatic cells with 2 μM 3-methylcholanthrene for 24 h and then washing the cells before determining the metabolism of propranolol produced a large induction of metabolism (Fig. 2). Total side-chain oxidation was increased 13·5 times ($P < 0·001$) with similar increases in the individual metabolites, i.e. DIP and Glycol. Total ring oxidation, including both 4HOP and 5HOP, increased to a lesser extent, i.e. 3·8 times ($P < 0·001$), as a result of 3-methylcholanthrene pretreatment. After induction, DIP accounted for 43 ± 6% and Glycol 47 ± 7% of the metabolism, whereas 4HOP and 5HOP each accounted for only 5 ± 1%. In similar experiments, the effect of 2 mM phenobarbitone on propranolol metabolism was also investigated. In contrast to 3-methylcholanthrene, pretreatment of the Hep G2 cells with this drug for 24 h produced no alteration in any of the metabolites of propranolol (Fig. 2).

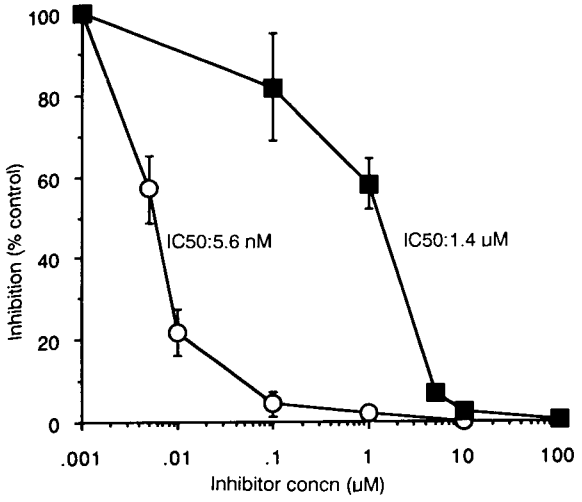

FIG. 3. Effect of MAO inhibitors on Glycol formation from DIP in Hep G2 cells. Mean values ± s.e. of 4 experiments with separate cell culture preparations are shown. The inhibitors used were clorgyline (○) and pargyline (■).

As the metabolism secondary to the P450-mediated formation of DIP (Fig. 1) was extensive in the Hep G2 cells, which also is true in-vivo (Walle et al 1985c), this was examined initially with 3-methylcholanthrene-induced cells. As MAO previously has been shown to deaminate DIP (Goldszer et al 1981), the effect of the MAO inhibitor clorgyline was determined. These data demonstrated complete inhibition of deamination of DIP derived from propranolol by 0·1 μM clorgyline in the Hep G2 cells, i.e. no Glycol was formed. In further experiments using non-induced cells, we determined the effect of the selective MAO inhibitors clorgyline and pargyline on deamination of DIP. As can be seen from the concentration-response relationships in Fig. 3, the MAO A inhibitor clorgyline produced a single-sigmoidal inhibition curve for DIP deamination with an IC50 of 5·6 ± 0·8 nM, which is similar to the inhibition of the deamination of the pure MAO A substrate 5-HT in human liver mitochondria (Tipton et al 1983). The less potent inhibition exerted by pargyline, IC50 1·4 ± 0·3 μM, is also consistent with the MAO A rather than the MAO B form,

The stereochemistry of propranolol metabolism was investigated in the 3-methylcholanthrene-induced Hep G2 cells, using established stereospecific HPLC methodology. This was done in the presence of 0·1 μM clorgyline to limit side-chain oxidation to production of DIP only. The enantiomer ratios all favoured the (+)-enantiomer with a (+)/(−) ratio for DIP of 1·87 ± 0·09 ($P < 0·001$ compared with a racemic value), for 4HOP, 1·36 ± 0·15 ($P < 0·05$) and for 5HOP, 1·39 ± 0·09 ($P < 0·01$).

Discussion

Our study with propranolol using the Hep G2 cell line had the advantage that comparisons could be made with substantial in-vivo (Walle et al 1985c) and liver microsomal (von Bahr et al 1982; Nelson & Shetty 1986; Otton et al 1990) human data. As our cell study used a cell homogenate, the data, however, showed more secondary metabolism than would be expected from a microsomal system. On the other hand, as appropriate cofactors were not included, conjugative metabolism such as glucuronidation and sulphation, important in in-vivo propranolol metabolism (Walle et al 1985c), did not occur.

Taking the difference in experimental conditions into consideration, the qualitative metabolic fate in the Hep G2 cells (Fig. 1) was identical to that demonstrated in-vivo (Walle et al 1985c). Quantitatively, the rather slow metabolism of propranolol in the uninduced cells, estimated to be about 10% of that in fresh human liver, would seem to be the major deficiency. Similar findings have also been reported for several other substrates undergoing cytochrome P450-mediated oxidations, although for other drug metabolism reactions the activity in the Hep G2 cells is comparable with fresh tissue (Grant et al 1988). In order to enhance the base line activity of propranolol metabolism, we tried the modified Earle's medium, previously shown to increase certain drug metabolizing enzyme activities (Doostdar et al 1988). However, with propranolol as the substrate, no significant improvement over Williams' medium E was observed. Also, whereas P450-mediated side-chain oxidation and ring oxidation contribute about equally to the metabolism of propranolol in-vivo in man (Walle et al 1985c), side-chain oxidation clearly predominates in the Hep G2 cells, accounting for 71% of total metabolism compared with 29% by ring oxidation in the uninduced cells. These differences may point to a selective loss in the Hep G2 cells compared with normal liver of the particular P450 responsible for ring oxidation, which could involve debrisoquin hydroxylase, or P450IID6, known to contribute to ring oxidation of propranolol in man (Raghuram et al 1984; Lennard et al 1984; Ward et al 1989; Otton et al 1990). Another large difference compared with in-vivo metabolism is that Glycol is a more important metabolite than NLA in Hep G2 cells following deamination of DIP. This suggests that NADH predominates over NAD^+ in the Hep G2 cells as the determinant of whether the intermediate aldehyde in this reaction is reduced to Glycol or oxidized to NLA (Goldszer et al 1981). However, of greater importance was the observation that this cell line expresses all the characteristics of in-vivo oxidative deamination of propranolol, i.e. P450-mediated *N*-desisopropylation to DIP and subsequent MAO-mediated deamination.

The most interesting finding was the induction by 3-methylcholanthrene. Thus, side-chain oxidation increased about 14-fold (Fig. 2). In contrast, there was no induction by phenobarbitone, emphasizing the specificity of the 3-methylcholanthrene induction. These observations are very similar to the aromatic hydrocarbon induction of resorufin *O*-de-ethylase in Hep G2 cells, i.e. a 15-fold induction by benz(a)anthracene but no effect of phenobarbitone (Grant et al 1988). Other enzyme activities are, however, inducible by phenobarbitone in the Hep G2 cells, including P450s as well as glucuronosyl transferases (Grant et al 1988). Most interestingly, the side-chain oxidation activity of propranolol after 3-methylcholanthrene (16 pmol min^{-1} (mg protein)$^{-1}$) was very similar both to the induced resorufin *O*-de-ethylase activity of 36 pmol min^{-1} (mg protein)$^{-1}$ (Grant et al 1988) and to the 3-methylcholanthrene-induced benzo(a)pyrene activity of 12 or 28 pmol min^{-1} (mg protein)$^{-1}$ (Labruzzo et al 1989; Roberts et al 1990), also in the Hep G2 cells. These observations suggest that the induced side-chain oxidation of propranolol in Hep G2 cells is mediated by arylhydrocarbon hydroxylase, i.e. P450IA1, or by P450IA2. This may explain the prominent effect of cigarette smoking on propranolol clearance in-vivo, an effect which is selective for side-chain oxidation (Walle et al 1987). Clinically, it appears clear that an additional P450 enzyme, i.e. mephenytoin hydroxylase (P450IIC9), is a contributor to propranolol side-chain oxidation in man (Ward et al 1989). This isoenzyme may also be responsible for side-chain oxidation in the uninduced Hep G2 cells. Thus, multiple P450s are most likely involved in this oxidation pathway in man.

The comparatively small induction of ring oxidation of propranolol by 3-methylcholanthrene in the Hep G2 cells (Fig. 2), suggests that the inducible P450 form can oxidize propranolol both in the ring and in the side-chain, although at different

rates. This finding is similar to the observation in cigarette smokers (Walle et al 1987).

MAO is a major contributor to the intermediary metabolism of propranolol (Goldszer et al 1981). Side-chain oxidation resulting in DIP formation is thus rapidly followed by deamination to Glycol and NLA (Fig. 1). The single-sigmoidal inhibition curve and very potent effect of clorgyline on this reaction compared with pargyline, about a 250-fold difference (Fig. 3), supports the view that DIP is deaminated by an MAO type A enzyme. This is based on the selectivity of these inhibitors for different MAO forms (Tipton et al 1983). MAO activity, at least of the A type, is thus well expressed in the Hep G2 cells.

Finally, as previously seen in human liver microsomes (von Bahr et al 1982; Nelson & Shetty 1986; Otton et al 1990), the formation of DIP as well as 4HOP and 5HOP in the Hep G2 cells, all favoured the (+)-enantiomer. Although this may be taken as a sign of equivalent properties among the enzymes in the Hep G2 cells and human liver, it should be noted that the stereochemistry of propranol metabolism in-vitro deviates from that in-vivo (Walle et al 1988).

In conclusion, the Hep G2 cell line has previously been shown to be a useful in-vitro model in drug metabolism studies (Dawson et al 1985; Sassa et al 1987; Grant et al 1988; Doostdar et al 1990; Fischer & Wiebel 1990). It is the only well-developed human hepatic cell line with a clear expression of a number of drug metabolizing enzyme activities. Its greatest advantage is the potential for studying human enzyme induction, which requires an intact cell system. The present investigation substantiates these conclusions.

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