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INFLUENCE OF PHENOBARBITAL ON FACTORS RESPONSIBLE FOR HEPATIC CLEARANCE OF INDOCYANINE GREEN IN THE RAT: RELATIVE CONTRIBUTIONS OF INDUCTION AND ALTERED LIVER BLOOD FLOW

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Abstract—The influence of phenobarbital, 3,4-benzpyrene and 3-methylcholanthrene on the clearance of indocyanine green (ICG) in the rat was investigated. ICG clearance following rapid i.v. administration of 1, 10 and 50 mg/kg, body wt was dose-dependent and pretreatment for 10 days with the inducing agents produced an increase in clearance only in the case of phenobarbital. All three inducers when given in this manner have been reported to produce equivalent increases in ligandin, consequently, this protein cannot be a major rate-limiting factor for hepatic ICG removal in the rat.

Of the three inducing agents studied, only phenobarbital increases liver blood flow. The relative contribution of this change versus any increase in the liver's intrinsic ability to remove ICG was estimated using a perfusion-limited model of hepatic elimination. Although the influence of the altered flow decreased with increasing ICG dose, the changes in both flow and intrinsic clearance were directly proportional to the increase in liver mass produced by phenobarbital. It may be concluded, therefore, that the influence of phenobarbital on ICG clearance in the rat is due to a larger liver with a proportionate increase in blood flow and not a consequence of the induction of any specific uptake protein.

The liver plays an important role in the removal from the body of a wide variety of substances. A number of processes are involved in this hepatic drug clearance including delivery to the liver, uptake, storage and biotransformation or excretion into the bile. Little quantitative information is available, however, concerning the relative importance of each of these steps in the overall *in vivo* removal process. Hence, the mechanisms by which factors such as ontogeny, drug interactions and disease-states produce changes in hepatic clearance is not always clear. This is particularly true of the increase in clearance of many drugs caused by pretreatment with phenobarbital. An increase in activity of the drug-metabolizing enzymes is probably involved in many cases [1] but other factors must be contributory, particularly for those compounds which are eliminated unchanged by the liver. The anionic dye, indocyanine green (ICG) used extensively in man and experimental animals for the determination of cardiac output [2] and hepatic function [3–6] is such a substance. The rate of elimination of ICG in the rat is significantly increased by phenobarbital pretreatment [7–9] and it has been proposed that this is a consequence of increased hepatic uptake secondary to the induction of the cytoplasmic anion binding protein ligandin (Y protein) [7]. The present study was designed to investigate this hypothesis and to assess the relative contributions of induction and altered liver blood flow to the increased clearance of ICG in the rat.

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METHODS

Groups of 6 male, 300–400 g body wt Sprague–Dawley rats (Harlan Industries, Indianapolis) with free access to food and water were injected i.p. once daily for 10 days. Phenobarbital (80 mg/kg, body wt.) dissolved in physiological saline was administered in a total vol. of 10 ml/kg, body wt; 3-methylcholanthrene (10 mg/kg, body wt) and 3,4-benzpyrene (10 mg/kg, body wt) were injected in 5 ml corn oil/kg, body wt., and control groups received equivalent vol. of either saline or corn oil, respectively. The animals were anesthetized with sodium pentobarbital (35–45 mg/kg, body wt, i.p.) 15–20 hr after the final pretreatment injection, and PE-50 catheters placed in a femoral artery and vein.

ICG (Hynson, Westcott and Dunning, Baltimore) was rapidly injected i.v. in 1 ml saline at three different doses, 1, 10 and 50 mg/kg, body wt. Heparinized arterial blood samples were obtained at suitable times after the injection to define the rate of elimination of the dye. The sample vol. varied according to the expected ICG concentration but the total vol. of blood removed from each rat was similar in all groups and approximated 2 ml. Withdrawn blood was replaced with an equal vol. of saline, infused through the arterial cannula after each sampling and, in addition, the hematocrit was determined on the first and last samples. The plasma concentration of ICG was determined promptly after sampling by

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Table 1. Influence of inducing agents on the pharmacokinetics of ICG

ICG Dose		Saline	Corn Oil	Phenobarbital	3,4-benzpyrene	3-methyl cholanthrene
1 mg/kg, body wt.	Half-life, min	2.39 ± 0.07	2.10 ± 0.07§	1.86 ± 0.13*†	1.95 ± 0.09	2.18 ± 0.09
	Volume of distribution, ml/100 g, body wt	6.52 ± 0.60	5.76 ± 0.21	7.17 ± 0.80†	4.72 ± 0.40	4.82 ± 0.40
	Blood clearance (ml/min/100 g, body wt)	3.07 ± 0.29	2.95 ± 0.13	4.30 ± 0.26*†	2.87 ± 0.29	2.51 ± 0.28
10 mg/kg, body wt	Half-life, min	4.03 ± 0.38	4.25 ± 0.36	2.68 ± 0.11*†	3.38 ± 0.18	4.27 ± 0.63
	Volume of distribution, ml/100 g, body wt	4.21 ± 0.29	4.34 ± 0.46	3.93 ± 0.48	3.42 ± 0.10	3.74 ± 0.25
	Blood clearance (ml/min/100 g, body wt	1.21 ± 0.09	1.18 ± 0.12	1.78 ± 0.20*†	1.23 ± 0.05	1.05 ± 0.09
50 mg/kg, body wt	Half-life, min	29.75 ± 1.68	28.30 ± 2.33	19.02 ± 1.62*‡	25.96 ± 2.03	25.65 ± 2.64
	Volume of distribution, ml/100 g, body wt	5.10 ± 0.12	5.29 ± 0.16	4.32 ± 0.20*	4.52 ± 0.20*	4.04 ± 0.22*
	Blood clearance (ml/min/100 g, body wt	0.19 ± 0.01	0.23 ± 0.02	0.27 ± 0.02*†	0.21 ± 0.02	0.20 ± 0.02

Values are mean of six rats ± S.E.M.
* control v active agent, $P < 0.05$.
† phenobarbital v 3,4-benzpyrene and 3-methylcholanthrene, $P < 0.05$.
‡ phenobarbital v 3,4-benzpyrene, $P < 0.05$.
§ saline v corn oil, $P < 0.05$.

measuring its absorption at 800 nm in a Gilford 2400-S spectrophotometer [3, 5] using 0.1% crystalline bovine albumin in 0.9% saline to dilute the samples [10].

Calculations. The plasma half-life for ICG elimination was obtained by linear regression of the logarithm of the plasma concentrations and time. Other individual pharmacokinetic parameters were calculated from the following relationships:

$$\text{Vol. of distribution} = \frac{\text{Dose}}{\text{Plasma concentration at zero time}} \quad \text{ml/100 g, body wt}$$

$$\text{Blood clearance} = \frac{0{\cdot}693 \text{ Volume of distribution}}{\text{Plasma half-life } (1 - \text{Hematocrit})} \quad \text{ml/min/100 g, body wt}$$

Statistical analysis of the mean data was performed by the unpaired Student t-test with $P = 0.05$ as the minimal level of significance.

RESULTS

Not unexpectedly [6, 11] the elimination of ICG from the plasma in the rat was dose-dependent, the half-life at 50 mg/kg, body wt being approximately 10-fold greater than at 1 mg/kg, body wt, irrespective of the pretreatment (Table 1). However, at each dose level the elimination appeared to be mono-exponential over the time period studied. There were no differences in the half-lives of the two control groups and pretreatment with either 3-methylcholanthrene or 3,4-benzpyrene produced no changes in the rate of ICG disappearance at any dose level. However, phenobarbital pretreatment caused a significant shortening of the plasma half-life after all three ICG doses (Fig. 1) relative to both the saline control

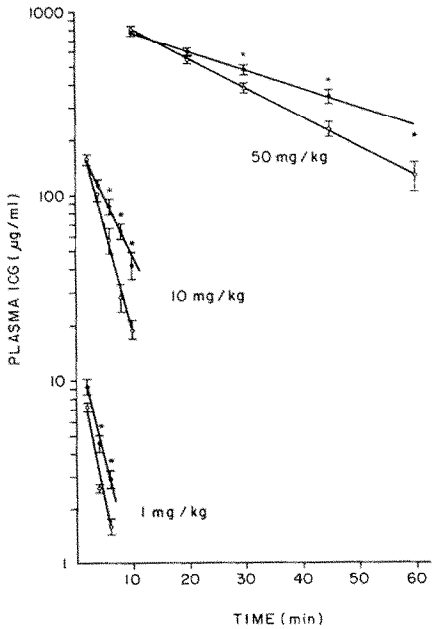

Fig. 1. Plasma elimination of ICG after rapid i.v. administration in saline control (●) and phenobarbital pretreated (○) rats. Each point is the mean ± standard error of six determinations and a statistical difference ($P < 0.05$) between the two groups is indicated by an asterisk.

groups and the other treatment groups, except after 50 mg/kg, body wt where the half-life did not differ statistically from that of the 3-methylcholanthrene group (Table 1).

The vol. of distribution of ICG showed some variability with respect to dose and pretreatment agent, and this may reflect the inherent error in its estimation due to the very rapid disappearance of ICG from the plasma [12]. However, there were no consistent trends suggestive that the dose-dependent changes or the effect of phenobarbital on the elimination half-life could be caused by alterations in this parameter (Table 1). On the other hand, ICG blood clearance decreased significantly with increasing dose in both the saline and corn oil control animals, and there was no difference between these two groups. Neither 3,4-benzpyrene nor 3-methylcholanthrene pretreatment altered ICG blood clearance at any dose level, but phenobarbital significantly increased this process at all doses studied compared to the control or the 3,4-benzpyrene and 3-methylcholanthrene groups. Relative to the saline group the mean increases were 40.1%, 47.1% and 42.1% at the 1, 10 and 50 mg/kg, body wt, dose levels respectively.

DISCUSSION

The present study confirms the dose-dependent elimination of ICG in the rat [6, 11], the mechanism of which is not clearly understood. The absence of any consistent distributional changes indicates that the phenomenon predominantly reflects a decrease in hepatic clearance, i.e. the efficiency with which the liver removes the dye. This would appear to be associated with saturable uptake of ICG into the liver which follows Michaelis–Menten kinetics [6]. The role of ligandin in this uptake process has not been definitively investigated. But, based primarily upon experiments with the model compound, sulfobromophthalein, it was postulated that this protein plays an important role in the transfer of organic anions from plasma to liver, and that its induction by a variety of agents was responsible for the enhancement in elimination of these anions after pretreatment with the agents [7]. The present findings would suggest, however, that this is not the case for ICG in the rat. Previous studies in the rat have shown that pretreatment with phenobarbital, 3,4-benzpyrene and 3-methylcholanthrene, according to the same protocol as used herein, causes an increase in hepatic ligandin of about 100 per cent for all three agents [7]. However, only phenobarbital pretreatment resulted in an alteration in ICG clearance. Accordingly, it must be concluded that ligandin is not the major rate-limiting factor in the hepatic removal of ICG in the rat, and another mechanism must be responsible for the observed increase in the rate of ICG elimination after phenobarbital pretreatment. Recent studies with other drugs have been similarly interpreted, and have suggested that ligandin plays a secondary role in the net hepatic transport of anions by functioning as an intracellular "storage" protein rather than a primary transport protein [13, 14].

It is well established that phenobarbital causes liver enlargement in the rat and the induction of certain hepatic proteins [1]. In addition, liver blood flow increases, 6.68 ± 0.23 to 8.86 ± 0.39 ml/min/100 g, body wt., in proportion to the increased mass of the liver, 12.0 ± 0.06 to 15.1 ± 0.2 g, and such changes are not seen after either 3,4-benzypyrene or 3-methylcholanthrene pretreatment [15]. The relative contributions of each of these factors in the enhancement of ICG clearance by phenobarbital may be assessed at each dose level by consideration of the perfusion-limited model of hepatic elimination [16–18]. According to this analysis, total hepatic clearance is a function of two independent physiological variables, liver blood flow (Q) and the total intrinsic clearance ($Cl_{intrinsic}$) of the liver. This latter term is a quantitative indication of the maximal overall ability of the liver to irreversibly remove ICG in the absence of any flow limitations and, therefore, reflects functional liver mass and specific intrinsic clearance ($Cl_{intrinsic}$/g liver). No indication of the location and/or identity of the several potential rate-limiting steps in the elimination process is provided by a drug's intrinsic clearance. Because there is no extrahepatic elimination of ICG [5], hepatic and blood clearance are synonymous, and therefore,

$$\text{Blood clearance} = Q\left[\frac{Cl_{intrinsic}}{Q + Cl_{intrinsic}}\right] = QE \text{ ml/min}$$

where the parenthetic term is equivalent to the steady-state hepatic extraction ratio, E. Thus, if ICG clearance and the appropriate liver blood flow are known, $Cl_{intrinsic}$ may be calculated, and from this, the expected change in clearance associated with any given change in blood flow or $Cl_{intrinsic}$ may be estimated. These calculations were made for the saline control and phenobarbital pretreatment groups using the mean data for clearance from the present investigation and the results for liver blood flow and mass from a separate study where the animals were treated in an identical fashion [15]. Phenobarbital pretreatment produced a large increase in effectiveness of the hepatic removal process as assessed by $Cl_{intrinsic}$ (Table 2), but the extraction ratio remained unchanged because of the equal and opposite influences of increased $Cl_{intrinsic}$ and liver blood flow. The changes in both of these parameters contributed to the overall increase in ICG clearance but their relative contributions varied with the ICG dose. This is consistent with earlier findings that the influence of liver blood flow upon drug clearance is dependent on the magnitude of the extraction ratio and, therefore, $Cl_{intrinsic}$ [17, 18]. Thus the 33 per cent increase in liver blood flow produced by phenobarbital was responsible for 47.8 per cent of the increase in ICG clearance after administration of 1 mg ICG/kg, body wt, 19.2 per cent after 10 mg/kg, body wt, and only 3.3 per cent after 50 mg/kg, body wt. Conversely, the 47.2, 50.7 and 42.3 per cent increases in $Cl_{intrinsic}$ contributed the balance of the changes, respectively. Importantly, when the absolute increases in $Cl_{intrinsic}$ were expressed per unit wt of liver, phenobarbital caused no change in the specific intrinsic clearance of ICG (Table 2). This would indicate that the pretreatment did not produce induction of any protein involved in the hepatic uptake and storage of the dye. Consequently, it may be concluded that the enhanced clearance of ICG at all dose levels after phenobarbital

Table 2. Changes in hepatic clearance, extraction ratio and intrinsic clearance ($Cl_{intrinsic}$) in saline and phenobarbital pretreated rats, and the calculated contributions of liver blood flow and $Cl_{intrinsic}$ to the changes in clearance

ICG dose mg/kg, body wt.	Blood clearance and (extraction ratio) ml/min/100 g, body wt.		Hepatic intrinsic clearance*				% Increase* in clearance due to	
			ml/min/100 g, body wt.		ml/min/g liver			
	Saline	Phenobarbital	Saline	Phenobarbital	Saline	Phenobarbital	Flow	Intrinsic clearance
1	3.07 (0.46)	4.30 (0.48)	5.68	8.36	1.60	1.59	47.8	52.2
10	1.21 (0.18)	1.78 (0.20)	1.48	2.23	0.42	0.44	19.2	80.8
50	0.19 (0.03)	0.27 (0.03)	0.196	0.279	0.055	0.055	3.3	96.7

* Based on mean liver mass and blood flow data from Ref. [15].

pretreatment is simply due to the presence of a larger liver and a proportional increase in the rate of delivery of ICG to its site of elimination, the exact contribution of each of these factors being dependent upon the dose of ICG; flow changes are less important as the administered dose increases.

The ability of phenobarbital to alter the disposition and elimination of many exogenous and endogenous compounds removed by the liver is well known. Such pretreatment produces perturbations in a number of physiological and biochemical factors which may or may not be involved in the disposition and elimination of the drug. The determination of a cause and effect relationship requires appreciation of the relative importance of each of the various physiological factors controlling these processes. With respect to hepatic elimination, the described perfusion-limited approach to hepatic elimination offers many advantages over more traditional pharmacokinetic analyses, and should permit a clearer understanding of the multiple mechanisms responsible for drug elimination and the alterations produced by disease and other drugs [18].

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