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BILE FLOW IN RESPONSE TO PHARMACOLOGIC AGENTS

HEPATIC DNA AS A REFERENCE STANDARD*

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Abstract—Bile flow was measured in controls and in rats treated with phenobarbital, ethinyl estradiol, 3-methylcholanthrene and 6-methylprednisolone, using hepatic DNA as a reference index. Bile flow was increased after phenobarbital, decreased after ethinyl estradiol, and unchanged after 3-methylcholanthrene, consistent with the accepted effects of these drugs in studies using either body weight or liver weight as a reference index. In addition, the controversial effect of glucocorticoids (6-methylprednisolone) on bile flow was evaluated. The reported increase in bile flow when related to body weight and the failure of bile flow to increase when evaluated by liver weight were both confirmed. When measured as a function of hepatocyte number, bile flow was increased by glucocorticoids. The values for DNA demonstrate that drug-induced hepatomegaly by phenobarbital and 6-methylprednisolone is due to cellular hypertrophy rather than to an increase in cell number, suggesting that the increases in bile flow are due to enhanced hepatocyte function. These findings emphasize the benefit of relating physiologic and pharmacologic effects to a cell specific reference when studying subcellular functions.

In recent years, a number of reviews have emphasized the importance of subcellular events in the physiology of bile flow [1, 2, 3]. Although researchers stress these subcellular events, bile flow continues to be related to gross measurements such as body or liver weight. An ideal experimental protocol would study the function of individual hepatocytes which would eliminate problems of relating function to a non-specific, non-hepatocyte parameter. With some limitations, hepatic DNA is a close approximation of hepatocyte number [4]. By utilizing hepatic DNA, physiologic and subcellular events may be related more closely to hepatocyte function.

The purpose of this study was to evaluate the validity of quantitating bile flow as μl/min/mg of hepatic DNA in rats treated with three well-studied agents (phenobarbital, 3-methylcholanthrene and ethinyl estradiol) in which there is general agreement as to their effects on bile flow, using the standard methods of comparing bile flow to liver or body weight. In addition, the effect of a glucocorticoid (6-methylprednisolone) on bile flow was studied as an example of an agent whose effect on bile flow is controversial.

MATERIALS AND METHODS

Animals and operative procedures. Nonfasted male Sprague–Dawley rats (Charles River Labs), weighing 140–180 g, were maintained on standard laboratory chow, in drop-through cages, on a 12 hr light/dark cycle. Treatment groups were given intraperitoneal injections of one of the following: phenobarbital (80 mg/kg/day × 3 days), 6-methylprednisolone acetate (20 mg/kg/day × 5 days), ethinyl estradiol (5 mg/kg/day in corn oil × 5 days), or 3-methylcholanthrene (4 mg/kg in corn oil × 4 days).

Bile flow was measured by cannulating the bile duct with polyethylene tubing (PE-10, Clay Adams) while the rat was anesthesized with pentobarbital. Body temperature was maintained at 37 ± 0.5° by a heat lamp. After discarding the bile flow of the first 10 min, two 15-min bile samples were collected in tared microcentrifuge tubes. The tubes were weighed immediately after collection. The volume of bile, expressed as μl, was calculated from the weight, assuming the density of bile was identical with water. When the bile collection was complete, the liver was rapidly removed, rinsed in ice-cold saline, blotted dry, and weighed. Liver samples of 200 mg (for DNA concentration determination) were weighed and frozen in saline at −20°.

DNA assay. Preweighed, resected liver samples were blotted dry and homogenized in 4 ml of 6% ice-cold tricholoroacetic acid (TCA), using a glass homogenizing tube with a motorized Teflon pestle drill. The homogenate plus a 3-ml TCA rinse of the homogenizing tube were added to a centrifuge tube and spun at 5° for 30 min at 20,000 rev/min, using a Ti50 rotor (Beckman) in an L-2 ultracentrifuge (Beckman). After discarding the supernatant fraction, the pellet was resuspended in 1 N perchloric acid to extract the DNA. The remainder of the assay followed the diphenylamine reaction outlined by Burton [5], using twice recrystallized diphenylamine.

Statistics. Duncan's Multi-comparison test [6] was used to determine significant changes between the control group and the groups treated with phenobarbital, 6-methylprednisolone and ethinyl estradiol. Since the group treated with 3-methylcholanthrene was not included in the original protocol, results were compared with a separate set of control rats and the *t*-test for non-paired data was used to determine statistical differences [7]. Significance was defined as $P < 0.05$ in both sets of experiments.

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Table 1. Effects of phenobarbital, ethinyl estradiol and 6-methylprednisolone on basal bile flow compared with body weight, liver weight and total hepatic DNA*

	(μl Bile/min/100 g body wt)	(μl Bile/min/g liver wt)	(μl Bile/min/mg hepatic DNA)
Control	8.60 ± 0.22 (6)	2.21 ± 0.06 (6)	0.713 ± 0.025 (5)
Phenobarbital	10.95 ± 0.29† (8)	2.34 ± 0.08 (NS)‡ (8)	0.865 ± 0.029† (7)
Ethinyl estradiol	6.78 ± 0.47§ (6)	1.63 ± 0.14† (6)	0.459 ± 0.024† (6)
6-Methylprednisolone	10.64 ± 0.56† (8)	2.16 ± 0.12 (NS) (8)	0.860 + 0.040† (8)

* Rats were given intraperitoneal doses of phenobarbital (8 mg/100 g body weight/day for 3 days), ethinyl estradiol (0.5 mg/100 mg body weight/day in oil for 5 days) and 6-methylprednisolone (2 mg/100 g body weight/day for 5 days). Values indicate means ± one S. E. Numbers in parentheses indicate the number of experimental animals in each group. Experimental values were compared to control value, by Duncan's Multi-comparison test to determine significance.

† $P < 0.01$.

‡ NS means not significant with $P > 0.05$.

§ $P < 0.05$.

RESULTS

Bile flow. Alterations of bile flow using the different reference indices of body weight, liver weight and liver DNA are shown in Table 1. The reference did not affect the decrease in bile induced by ethinyl estradiol, as each determination of bile flow was significantly lower than control values. Treatment with phenobarbital and 6-methylprednisolone increased bile flow significantly when flow was measured against body weight or hepatic DNA. A significant difference in bile flow could not be determined using liver weight with either of these two drugs.

Changes in body weight and in the liver weight to body weight ratios. The effects of the three drugs on the change in body weight with time and on the liver weight/body weight ratio are shown in Table 2. The final weights of rats treated with ethinyl estradiol and 6-methylprednisolone were virtually identical to the initial body weights. Phenobarbital-treated rats were not compared with control rats in this set of experiments, but their weight increased significantly during the 3 days of treatment. In fact, in a separate study, three rats treated for 5 days with the same daily dose of phenobarbital actually increased their body weight by 21.4 ± 0.8 per cent (mean ± S.E.), an increase greater than the weight change in the control group. There was a significant increase in the ratio of liver weight to body weight after phenobarbital and 6-methylprednisolone, while no change was observed with ethinyl estradiol.

Hepatic DNA. Hepatic DNA, expressed as mg DNA/g of liver, was significantly lower after 6-methylprednisolone treatment (Table 2). Hepatic DNA compared with body weight showed a slight increase after ethinyl estradiol and no change with phenobarbital or 6-methylprednisolone treatment (Table 2).

3-Methylcholanthrene. An additional group of animals was treated with 3-methylcholanthrene, a known carcinogen and inducer of the microsomal enzyme system, and compared with a set of control rats. Bile flow in the treated animals was not different from the control animals, regardless of the denominator used

Table 2. Effects of phenobarbital, ethinyl estradiol and 6-methylprednisolone on the change in body weight with time, on the ratio of liver weight to body weight, on mg of hepatic DNA/g liver, and on mg of hepatic DNA/100 g body weight*

	Change in body wt† (%)	Liver wt/body wt × 100	Hepatic DNA (mg/g liver)	Hepatic DNA (mg/100 g body wt)
Control	+ 15.1 ± 0.8 (8)	3.90 ± 0.05 (8)	3.15 ± 0.09 (7)	12.20 ± 0.37 (7)
Phenobarbital	+ 10.5 ± 0.9 (8)	4.71 ± 0.12‡ (8)	2.86 ± 0.16 (NS)§ (7)	13.42 ± 0.75 (NS) (7)
Ethinyl estradiol	−1.0 ± 2.8‡ (6)	4.19 ± 0.11 (NS) (6)	3.55 ± 0.23 (NS) (6)	14.9 ± 0.75‖ (6)
6-Methylprednisolone	−2.5 ± 1.6‡ (6)	5.00 ± 0.22‡ (6)	2.40 ± 0.14‡ (6)	12.47 ± 0.58 (NS) (6)

* Rats were given intraperitoneal doses of phenobarbital (8 mg/100 g body weight/day for 3 days), ethinyl estradiol (0.5 mg/100 g body weight/day in oil for 5 days) and 6-methylprednisolone (2 mg/100 g body weight/day for 5 days). Values indicate means ± one S. E. Numbers in parentheses indicate the number of experimental animals in each group. Experimental values were compared to control values by Duncan's Multi-comparison test to determine significance.

† Change in weight is over 5 days for control group and for the time of treatment (3 or 5 days) in the treated groups. Because of the different treatment period, statistical significance was not tested for the weight change in the phenobarbital-treated group.

‡ $P < 0.01$.

§ NS means not significant with $P > 0.05$.

‖ $P < 0.05$.

Table 3. Effect of 3-methylcholanthrene on bile flow and hepatic DNA*

	Bile flow			Hepatic DNA	
	(μl/min/100 g body wt)	(μl/min/g liver wt)	(μl/min/mg hepatic DNA)	(mg/g liver wt)	(mg/100 g body wt)
Control (5)	8.76 ± 0.18	2.03 ± 0.10	0.714 ± 0.023	2.852 ± 0.140	12.30 ± 0.30
3-Methyl-cholanthrene (4)	9.01 ± 0.58	1.95 ± 0.14	0.661 ± 0.061	2.970 ± 0.064	13.73 ± 0.39
Significance	NS	NS	NS	NS	P = 0.021

* Treated rats were given intraperitoneal doses of 3-methylcholanthrene (0.4 mg/100 g body weight/day) for 4 days. Values indicate means ± S. E. Numbers in parentheses indicate the number of animals in each group. Student's *t*-test was used to determine significance. NS means not significant, P > 0.05.

(Table 3). The hepatic DNA measured per g of liver was not different from control, but total hepatic DNA was increased after 3-methylcholanthrene.

DISCUSSION

Bile flow is a measure of hepatic function which is dependent on blood flow to the liver, hepatocyte function and biliary ductular function. Although the importance of the hepatocyte membrane in biliary excretion has been stressed [1–3, 8], there has been no effort to relate bile flow to the individual hepatocyte. Bile flow has usually been measured as μl/unit time/100 g body weight or g liver weight. Neither of these denominators seems appropriate for the measure of hepatocytic function, since neither relates bile flow directly to the individual hepatocyte. The use of these different denominators by authors studying the same drug may explain their divergent conclusions. For example, some investigators have reported induction of bile flow by glucocorticoids [9], whereas others have refuted this [10]. The former study, indicating an increase in bile flow, used body weight as a denominator, while the latter, showing no change in bile flow, used liver weight. A possible explanation of these contradictory results is that, while cortisone increases liver weight, body weight is virtually unchanged (Table 2). If glucocorticoids do not affect the liver, and the increased weight of the liver reflects normal liver growth while body growth is halted, then liver weight would be the appropriate denominator for evaluating bile flow. With this assumption, the actual liver weight exceeds the predicted liver weight, suggesting there is a glucocorticoid-induced increase in liver size. This increase could be due to an increase in the number of hepatocytes (hyperplasia) or an increase in the size of each hepatocyte (hypertrophy). This distinction seems important for the evaluation of the effect of glucocorticoids on bile flow. Body weight or liver weight as reference indices do not allow this discrimination. Since hepatic DNA is an accurate representation of hepatocyte number [4], comparing function to hepatic DNA should elucidate the subcellular mechanism of action of glucocorticoids.

Phenobarbital induction of bile flow presents similar difficulties. Berthelot *et al.* [11] reported a 45 per cent increase in bile flow, using body weight as a denominator. The mean increase in liver weight was 38 per cent, suggesting that there probably would not have been an increase in bile flow if bile flow had been related to liver weight instead of body weight.

The values for bile flow in Table 1 confirm the data reported previously concerning phenobarbital and glucocorticoids, i.e. bile flow increases when compared with body weight and is not changed when related to liver weight. Using hepatic DNA as a measure of hepatocyte number, both pharmacologic agents increase bile flow. This increase in bile flow, using hepatic DNA as a reference, suggests that hepatocyte function is induced by phenobarbital and 6-methylprednisolone. These findings also explain the apparent contradictory results of previously published studies. The known cholestatic agent, ethinyl estradiol, decreases bile flow no matter which parameter is used as a denominator (Table 1).

Additional support for the use of hepatic DNA as a basis of comparison for hepatocyte function was obtained by studying the effect of 3-methylcholanthrene, an agent which induces the hepatic microsomal enzyme system while not changing bile flow [12]. In agreement with these observations, we found that bile flow did not change when comapred with body weight, liver weight or hepatic DNA (Table 3). Although there is no change in the concentration of DNA per g of liver, the increase in DNA per 100 g body weight (11.6 per cent) suggests that the increased hepatic weight (6.5 per cent) is due to hepatocyte proliferation and may be due to the known carcinogenic properties of 3-methylcholanthrene.

In addition to demonstrating the benefit of using hepatic DNA as a reference index for cellular function, these data also emphasize the important effects of drugs on cell replication and subcellular metabolism. Shenoy and Peraino [13] found that phenobarbital induced hepatomegaly by cellular hypertrophy rather than by an increase in cell number, as hepatic DNA per g of liver was significantly lower after phenobarbital with no change in the total hepatic DNA. Although the values shown in Table 2 do not confirm these observations, the changes in hepatic DNA per g of liver and in hepatic DNA per 100 g body weight parallel the published findings. The increase in liver weight after 6-methylprednisolone is due to hypertrophy, as the DNA per g of liver decreases significantly with the increase in liver weight while the DNA per 100 g body weight is unchanged from control values. In addition, since body weight after 6-methylprednisolone does not increase during the 5 days of treatment and the amount of DNA per 100 g body weight is the same as in the control animals, the dose of glucocorticoid used in these experiments probably inhibits cell replication. This is consisent with earlier observations on the subcellular effects of glucocorticoids [14].

The increase in DNA per 100 g body weight with ethinyl estradiol could be explained by the failure of this group of rats to gain weight while normal hepatocyte proliferation continues. Direct support for this hypothesis is not provided by these studies, but the 22 per cent increase in hepatic DNA per 100 g body weight is fairly close to the 15 per cent increase in body weight expected in the untreated control.

As the emphasis in hepatic physiology has shifted toward subcellular events, the drug effects on cell replication and cell hypertrophy shown in this study demonstrate the need to relate changes in hepatocyte function to a cell specific, rather than an organ specific, parameter. In studying hepatocyte function, DNA measurements should be particularly useful for interpreting subcellular events in the newer models of partial biliary obstruction, selective hepatic vascular occlusion and partial hepatic resection where important information regarding the adaptation of the liver by increasing cell proliferation or altering subcellular structure can be obtained by comparing function to hepatic DNA rather than to liver weight or to body weight.

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