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Effects of butylated hydroxytoluene upon protein transport in the isolated perfused rat liver

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Plasma proteins constitute the major source of biliary protein in most species [1]. These proteins may be transferred from plasma to bile by both paracellular and transcellular routes. Paracellular transfer involves the non-specific movement of proteins across tight junctions between adjacent hepatocytes or bile ductule cells and is inversely proportional to the molecular weight of the protein, i.e. low molecular weight proteins show higher bile:plasma ratios than do larger proteins [2]. Transcellular transfer involves either non-specific endocytosis of plasma components or receptor mediated endocytosis of specific proteins (e.g. polymeric IgA) to which a receptor is expressed at the sinusoidal surface of the hepatocyte. The resultant vesicles are routed mainly to the lysosomes for degradation whilst small amounts are discharged into bile due to mis-sorting at the endosome [3]. In the case of polymeric IgA, vesicles bypass the lysosomes and are routed via the endosomal compartment direct to the canaliculus where they discharge their contents into bile [4, 5]. Horse radish peroxidase (HRP*) provides an easily measured marker of both paracellular and transcellular pathways. When given as a single, short pulse to the isolated perfused rat liver operating under single pass conditions, HRP output into bile shows two components; an initial peak at approx. 3–5 min corresponding to paracellular movement and a second peak at approx. 15–20 min corresponding to non-specific, transcellular movement [2, 6].

Butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene, BHT) is a phenolic antioxidant used as a preservative (E321) in the food industry. Ingestion of BHT at doses >75 mg/kg/day produces dose dependent liver hypertrophy which is reversible when BHT is removed [7]. However, BHT does appear to cause some liver damage since the serum activities of aspartate and alanine aminotransferases are elevated following BHT administration to rats [8] and sublethal doses of 1000 mg/kg/day cause centrilobular necrosis [9].

In order to further investigate the potential hepatoxocity of BHT, we have examined its effect upon the paracellular and transcellular transport of HRP from perfusate to bile in the isolated perfused rat liver. In addition, the biliary output of two other proteins, namely rat serum albumin (RSA) and bovine serum albumin (BSA) was also examined.

Materials and methods

Materials. Butylated hydroxytoluene, horse radish peroxidase (Type 11), bovine serum albumin (fraction V) and other fine chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.). Cannulation tubing PP10 was from Portex Ltd (Hythe, U.K.) and cannulae (17.5 and 16 gauge) were from Medical Assist (Colchester, U.K.). Nembutal (pentobarbitone, 60 mg/mL) was supplied by Ceva Ltd (Watford, U.K.) and heparin (5000 units/mL) by C.P. Pharmaceuticals (Wrexham, U.K.). Rabbit anti-bovine serum albumin and goat anti-rat serum albumin were from Nordic Immunologicals (Maidenhead, U.K.). Human blood was obtained through the courtesy of the Merseyside Blood Transfusion Service. All other chemicals were from BDH Ltd (Speke, U.K.) and were the highest grade available.

Animals. Male Wistar rats (220–350 g) fed a standard laboratory diet and maintained at 22° in a constant 12 hr light:dark cycle were used throughout. Butylated hydroxytoluene was dissolved in olive oil to a final concentration of 80 mg/mL and administered by stomach tube at a dose of 200 mg/kg body wt/day for up to 4 days. Control animals were administered similar volumes of olive oil.

Isolated perfused rat livers. Livers were perfused essentially as described by Gores *et al.* [10]. The perfusion medium was Krebs–Ringer bicarbonate, pH 7.4, containing 1 mM $CaCl_2$, 5 mM glucose, a physiological amino acid mixture [11], 1% (w/v) bovine serum albumin and 20% (v/v) washed human erythrocytes. The medium was continually gassed with O_2/CO_2 (19:1) and was warmed to 37° by means of a heat exchanger. All perfusions were carried out in a thermostatistically controlled cabinet.

Briefly, the common bile duct was cannulated with PP10 tubing while the rats were under Nembutal anaesthesia; bile was collected on ice throughout the experiment into tared tubes. Bile flow was determined gravimetrically. Approximately 10 min after cannulation of the common bile duct, heparin (2500 units in 0.5 mL) was administered via the inferior vena cava and after a further 1 min a 17.5-gauge Wallace cannula was inserted into the hepatic portal vein. To minimize anoxia (<15 sec), perfusion was started immediately at a flow rate of 17 mL/min. The thoracic vena cava was then cannulated with a 16-gauge cannula and recycling perfusion established.

The viability of the liver was assessed macroscopically by a marked colour change in the afferent and efferent perfusate, its homogeneity and a bile flow of at least 0.8 μL/min/g of liver. Leakage of aspartate aminotransferase (measured by the ultraviolet method using kits supplied by Boehringer Mannheim Ltd, Sussex, U.K.) into the perfusate was <0.5% of the total liver activity during 1.5 hr of recycling perfusion.

Livers were allowed to approach steady state for 30 min with recycling perfusion, after which they were converted to a one pass perfusion using fresh medium and maintained in this mode for 5 min. At the start of this 5 min period, 25 mg of HRP (dissolved in 1 mL of erythrocyte-free perfusion medium) was infused via a syringe pump over a 1 min period into the perfusion line just prior to the portal cannula. After a further 4 min, during which any HRP not taken up by the liver was removed, the liver was converted back to recycling perfusion. Bile was collected as 2 × 15 min samples prior to HRP infusion, then as 10 × 2 min samples followed by 8 × 5 min samples. Thus, the total time of liver perfusion was 90 min.

Assays. HRP in bile was assayed as the functional enzyme by the spectrophotometric method of Steinman and Cohn [12]. The final assay volume was 1 mL and up to 10 μL of bile was assayed. One unit of enzyme activity was taken to be equivalent to a change of one absorbance unit per minute. Results are expressed as units/min of bile/g of liver.

BSA and RSA in bile were determined by quantitative radial immunodiffusion [13]. The biliary output of BSA was quantified by comparison with authentic standards whilst that of RSA (for which no standard was available)

* Abbreviations: HRP, horse radish peroxidase; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; RSA, rat serum albumin.

Table 1. Liver:body weight ratios following oral administration of butylated hydroxytoluene to rats (200 mg/kg/day)

Days of BHT administration	0	1	2	3	4
Liver:body weight ratio	0.040 ± 0.001	0.049 ± 0.001	0.062 ± 0.004	0.056 ± 0.004	0.063 ± 0.006
% Increase	—	23	55	40	58
Number of animals	18	5	16	6	7
Significance (P)	—	0.010	0.001	0.011	0.006

Values are means ± SE. Statistical differences from controls were assessed using Student's *t*-test.

is expressed as arbitrary units relating to the diameter of the precipitation zone.

Results

Increases in liver:body weight ratios became apparent 1 day after BHT administration, and within a further day liver:body weight ratios had increased by over 50% when compared to controls (Table 1). No further increase in liver weight was seen after 4 days of BHT administration.

Immediately following liver isolation, bile flow rates were in the range 1.2 to 1.4 μL/min/g of liver; these declined steadily throughout the experiments to reach 0.8 to 1.0 μL/min/g of liver after 90 min of perfusion (results not shown). This is to be expected since the perfusion medium was not supplemented with exogenous bile salts and as biliary bile salt concentrations decline, the bile salt dependent fraction of bile flow decreases (see Ref. 2). Bile flow rates were unaffected by pretreatment of animals with BHT (results not shown).

HRP, when infused as a 1 min pulse into the perfusion medium of isolated perfused rat livers, appeared in bile as 2 peaks; a paracellular peak at 8–10 min and a transcellular peak at 18–20 min (Fig. 1). The volume of the biliary tree in rat liver has been reported as 2.3 μL/g of liver [14] and the dead volume of the cannula was 49 μL. Thus, there is a delay of approx. 5 min between HRP entering the bile canaliculi and reaching the collection tube. Therefore, peak paracellular secretion of HRP occurred at 3–5 min whilst peak transcellular secretion occurred at 13–15 min.

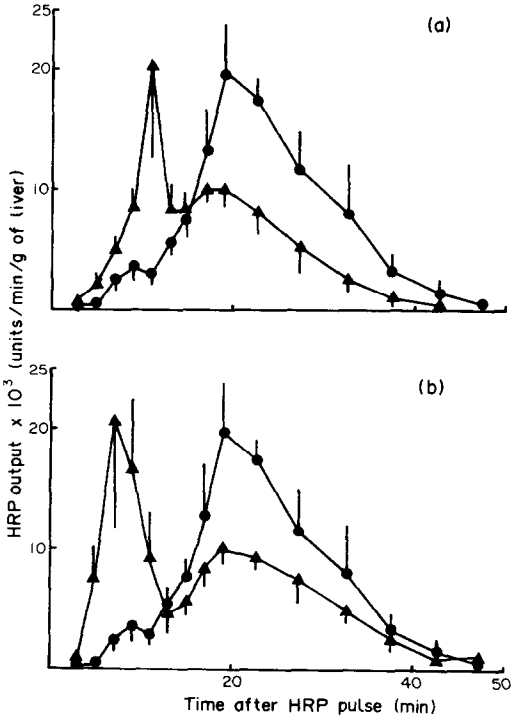

Fig. 1. Biliary output of horseradish peroxidase. HRP was administered as a 1 min pulse to isolated perfused rat livers. Symbols are: control (●), and rats administered BHT (▲) for either 2 days (a) or 4 days (b) prior to liver isolation. Results are means ± SE of 3–5 observations.

Pretreatment of rats with BHT increased the paracellular secretion of HRP into bile; this effect was evident 48 hr after BHT administration but was more apparent after 96 hr (Fig. 1). Conversely, the transcellular secretion of HRP into bile was decreased to a similar extent in livers from rats treated with BHT for either 48 or 96 hr (Fig. 1).

Biliary output of BSA in the 0–15 min sample from livers of BHT treated rats was significantly greater (by approx. two-fold) than that of controls (Fig. 2a). Thereafter, BSA output increased throughout the perfusion period and was essentially similar in livers from control and BHT treated rats (Fig. 2a). Biliary output of RSA declined throughout the perfusion period and was similar in livers from control and BHT treated rats (Fig. 2b).

Discussion

HRP, when given as a 1 min pulse to the isolated perfused rat liver operating under single pass conditions, appeared in bile as two peaks. After allowing for the volume of the biliary tree and the dead volume of the cannula, peak paracellular output of HRP occurred at 3–5 min after the pulse whilst peak transcellular output occurred at 13–15 min. These times are in approximate agreement to those reported by Lowe *et al.* [6, 15] although these authors report a slightly slower transcellular transit time of 17 min.

When livers from rats pretreated with BHT were pulsed with HRP two contrasting effects were apparent. Firstly, paracellular transport of HRP was dramatically increased (Fig. 1). This occurred after both 2 and 4 days of BHT administration but was more apparent at 4 days when the size of the paracellular peak was increased in excess of 10-fold. Clearly, BHT increases the permeability of hepatic tight junctions. A second effect of BHT was to decrease by approximately 50% the transcellular peak of HRP secretion into bile; this effect was similar after both 2 and 4 days of BHT administration.

A variety of other compounds have been shown to increase hepatic tight junction permeability. For example, α-naphthylisothiocyanate increases tight junctional permeability to small molecules such as sucrose, phosphate and inulin [16, 17] and to HRP [6] and even molecules as large as IgG (MW 160,000) [18]. Similarly, oestrogens increase hepatic tight junction permeability to sucrose, phosphate and inulin [19] and to HRP [20]. Bile duct ligation of rats also increases the permeability of hepatic tight junctions to HRP [21]. All of these treatments are marked by decreased bile flow; α-napthylisothiocyanate and oestrogens produce intrahepatic cholestasis whilst bile duct ligation produces total extrahepatic cholestasis. It is surprising, therefore, that the increase in paracellular permeability produced by BHT was not accompanied by a reduction in bile flow; indeed, bile flow was similar in livers from control and BHT-treated rats. However, Kan and Coleman [18] showed that the increase in permeability during early α-naphthylisothiocyanate treatment preceded

by several hours decreased bile flow rates, and Jaeschke *et al.* [19] showed that bile flow was decreased before junctional permeability was increased in rats treated with oestradiol valerate. Thus, permeability increases do not occur simultaneously with decreases in bile flow and junctional permeability and bile flow may be unrelated [20]. Indeed, tight junctions are not static permeability barriers and it has been shown recently that their permeability can be increased by vasopressin, adrenaline and angiotensin II (hormones which act via the intracellular mediators Ca^{2+}, diacylglycerol and the inositol phosphates) [15] and by the Ca^{2+} ionophore A23187 [22].

The endocytic transcellular movement of vesicles relies upon intact microtubules. Thus, microtubule inhibitors such as colchicine inhibit the blood to bile transfer of polymeric IgA, a ligand endocytosed by a specific receptor mediated process and subsequently transferred to bile by a direct vesicular pathway [23, 24]. Similarly, colchicine inhibits the transcellular secretion of HRP into bile [6]. Therefore, it is tempting to suggest that the decreased transcellular peak of HRP seen in BHT-treated rats is due to an inhibition of microtubule function. However, it must also be remembered that the hepatocyte internalizes the equivalent of its entire plasma membrane every 1–2 hr [25] and clearly any decreases in this endocytic/pinocytic activity would also decrease the transcellular transport of HRP.

The biliary output of BSA and RSA were also studied. BSA was present in the perfusion medium throughout the experiments and therefore was continuously presented to the liver whereas HRP was presented under single pass conditions for 1 min only. Thus, biliary output of BSA represents the continual summation of paracellular and transcellular components. The results presented in Fig. 2 show that the initial (0–15 min) bile sample from BHT-treated animals contained a significantly greater amount of BSA than corresponding controls. A large proportion of this BSA will be transported paracellularly and therefore probably reflects the increase in junctional permeability of livers from BHT-treated animals. Subsequent bile samples from BHT-treated animals contained similar amounts of BSA to controls and it is likely that at this time point increased paracellular permeability is balanced by decreased transcellular transport. Biliary output of BSA reached a maximum at 60–90 min after liver isolation and is in approximate agreement to that obtained by Barnwell *et al.* [11]. On the other hand, RSA can arise in bile by two possible routes: either transport from the perfusate of residual amounts present in rat plasma or misdirection of normal vesicular traffic such that RSA is discharged at the biliary pole of the cell rather than the sinusoidal membrane. The fact that the biliary output of RSA declines throughout the perfusion implies that most arises from residual amounts in serum and that in transit through the liver [11]. Since biliary RSA output is similar in livers from control and BHT-treated rats, it is likely that BHT does not cause significant misdirection of secretory vesicles to the canaliculus ('blundersomes'—see Ref. 2).

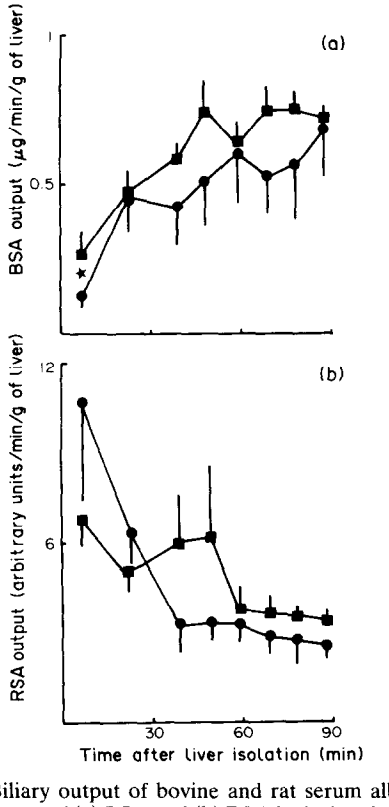

Fig. 2. Biliary output of bovine and rat serum albumins. Biliary output of (a) BSA and (b) RSA by isolated perfused livers from control rats (●) and rats administered BHT for 4 days (■). Results are means ± SE of four observations. Statistical differences from controls were assessed using Student's *t*-test and are indicated by ★ ($P < 0.05$).

In summary, the results presented here show that BHT administration to rats increases hepatic tight junction permeability to HRP whilst decreasing the endocytic transfer of HRP from perfusate to bile.

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In vitro hepatic, renal, and pulmonary N-dealkylation of amiodarone

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Amiodarone, an iodinated benzofuran derivative, is used clinically for treating arrhythmias refractory to other drug therapies. However, its use is often associated with serious adverse effects, including life-threatening pulmonary fibrosis and hepatitis [1]. In light of recent evidence indicating a role for the N-dealkylated amiodarone metabolite, desethylamiodarone, in amiodarone-induced toxicities [2, 3], it is of interest to characterize the biotransformation of amiodarone to desethylamiodarone.

Experiments performed with inhibitors and inducers of cytochrome P450 have confirmed the role of the polysubstrate monooxygenase system in the conversion of amiodarone to desethylamiodarone [4–6]. In examining the effects of inducing agents on *in vitro* desethylamiodarone formation, phenobarbital treatment was found to enhance greatly rat hepatic microsomal activity, and to result in measurable activity in lung and kidney, whereas none was found in lung or kidney microsomes from control animals [6]. Larrey and colleagues also reported that amiodarone can form an inactive complex with cytochrome P450, and that formation of this complex is increased greatly by prior treatment with dexamethasone [7], which induces products of the cytochrome P450III gene family, principally the isozyme referred to as P450PCNa [8] or P450p [9]. However, the effect of dexamethasone treatment on amiodarone biotransformation has not been explored.

The aim of the present study was to investigate the role of steroid-inducible cytochrome(s) P450 on the biotransformation of amiodarone to desethylamiodarone, by examining the effect of dexamethasone treatment on hepatic and extrahepatic *in vitro* desethylamiodarone formation. These activities also were compared to those found in tissues from animals treated with the polycyclic aromatic inducing agent, β-naphthoflavone. To confirm the inducing effects of dexamethasone and β-naphthoflavone, we employed the isozyme-selective marker enzyme assays. erythromycin *N*-demethylase and 7-ethoxyresorufin *O*-deethylase.

Methods

Animals and treatments. Male Sprague–Dawley rats (Charles River Canada, Inc., St. Constant, Quebec, Canada) weighing 250–300 g, were maintained on a 12-hr light/12-hr dark cycle and fed laboratory chow and water *ad lib.* They were treated with dexamethasone (50 mg/kg) or β-naphthoflavone (80 mg/kg) i.p. in corn oil (2.0 mL/kg). Controls for the above treatments received corn oil (2.0 mL/kg). Treatments were given once daily for three successive days, and rats were killed by cervical dislocation 48 hr after the final dose.

Chemicals. Chemicals were obtained from suppliers as follows: β-naphthoflavone, dexamethasone, erythromycin, and chemically reduced NADPH from the Sigma Chemical Co., St. Louis, MO, U.S.A.; resorufin and ethoxyresorufin from Molecular Probes Inc., Junction City, OR, U.S.A.; and amiodarone hydrochloride and desethylamiodarone hydrochloride from Ayerst Pharmaceuticals, Montréal, Quebec, Canada. All other chemicals were of reagent grade and were obtained from common commercial suppliers.

Preparation of microsomes. Kidneys, lungs, and livers were perfused *in situ* with ice-cold 1.15% KCl. Minced tissues were homogenized in 4 vol. of 1.15% KCl–0.1 M potassium phosphate buffer (pH 7.4) using a Potter–Elvehjem glass–teflon tissue homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°. The 10,000 *g* supernatant fraction was centrifuged at 104,000 *g* for 60 min at 4°. The microsomal pellets were resuspended in 0.25 M sucrose–0.1 M potassium phosphate buffer. Aliquots of resuspended pellet were frozen in liquid nitrogen and stored at −70°. Protein concentration was assessed by the method of Lowry *et al.* [10].

Assays. O-Deethylation of 7-ethoxyresorufin was