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Inhibition Studies on the Involvement of Flavoprotein Reductases in Menadione- and Nitrofurantoin-Stimulated Oxyradical Production by Hepatic Microsomes of Flounder (*Platichthys flesus*)

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ABSTRACT: Inhibitors of mammalian cytochrome P450 and P450 reductase were used to investigate the enzymes in flounder (*Platichthys flesus*) hepatic microsomes involved in the stimulation of NAD(P)H-dependent iron/EDTA-mediated 2-keto-4-methiolbutyric acid (KMBA) oxidation (hydroxyl radical production) by the redox cycling compounds menadione and nitrofurantoin. Inhibitors were first tested for their effects on flounder microsomal P450 and flavoprotein reductase activities. Ellipticine gave type II difference binding spectra (app. K_S 5.36 μM; ΔA max 0.16 nmol^{-1} P450) and markedly inhibited NADPH-cytochrome c reductase, NADPH-cytochrome P450 reductase, and monooxygenase (benzo[a]pyrene metabolism) activities. 3-aminopyridine adenine dinucleotide phosphate (AADP; competitive inhibitor of P450 reductase) inhibited NADPH-cytochrome c but not NADH-cytochrome c or NADH-ferricyanide reductase activities. Alkaline phosphatase (inhibitor of rabbit P450 reductase) stimulated NADPH-cytochrome c reductase activity seven fold but had less effect on NADH-reductase activities. AADP inhibited nitrofurantoin- and menadione-stimulated KMBA oxidation by 45 and 17%, respectively, indicating the involvement of P450 reductase at least in the former. In contrast, ellipticine had relatively little effect, possibly because, unlike cytochrome c, the smaller xenobiotic molecules can access the hydrophilic binding site of P450 reductase. Alkaline phosphatase stimulated NAD(P)H-dependent basal and xenobiotic-stimulated KMBA oxidation, showing general consistency with the results for reductase activities. Overall, the studies indicate both similarities (ellipticine, AADP) and differences (alkaline phosphatase) between the flounder and rat hepatic microsomal enzyme systems.

KEY WORDS: **Flounder, *Platichthys flesus*, Ellipticine, Menadione, Nitrofurantoin, Cytochrome P450, Cytochrome P450 Reductase, Hydroxyl Radical, Redox Cycling.**

INTRODUCTION

Redox cycling of compounds such as quinones and nitroaromatics is a potential mechanism of chemical-caused carcinogenesis and other pathologies in mammals (1,2) and fish (3). Univalent reduction of the xenobiotic and reoxidation of the resultant anion radical by reaction with O_2 to complete the redox cycle result in the concomitant depletion of NAD(P)H and production of the superoxide anion radical (O_2^-). Conversion of O_2^- to hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and other oxidants can give rise to a spreading web of oxidative damage, resulting in cytotoxicity and cell death (4–6).

A wide variety of organic pollutant xenobiotics enter aquatic ecosystems, including polynuclear aromatic hydrocarbons (PAHs), nitroaromatics, quinones (7–10), and pulp mill effluents (11,12). Such lipophilic compounds are readily taken up into the tissues of fish, particularly the liver (10,13), and have been correlated with the onset or occurrence of hepatic neoplasms and other pathological lesions (14,15), including oxidative damage to DNA (16). Redox cycling by hepatic microsomes of fish has been demonstrated for nitroaromatics and quinones, including both model compounds, viz. N-(5-nitro-2-furfurylidene)-1-aminohydantoin (nitro-

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furantoin) and 2-methyl-1,4-naphthoquinone (menadione), and pollutant xenobiotics, viz. *p*-nitrobenzoic acid, *m*-dinitrobenzene, benzo[a]pyrene diones, and 1,4-tetramethylbenzoquinone (17–19).

Hepatic microsomal redox cycling of xenobiotics in mammals is catalyzed by flavoprotein reductases such as cytochrome P450 reductase (EC 1.6.99.3) (20,21), but little is known of the situation in fish. The present study was carried out to obtain evidence of flavoprotein reductase involvement in redox cycling by hepatic microsomes of flounder (*Platichthys flesus*), using menadione and nitrofurantoin as oxyradical generators and several inhibitors of mammalian microsomal enzymes, viz. clotrimazole and piperonyl butoxide (inhibitors of cytochrome P450s) (22,23), ellipticine (inhibitor of P450 and cytochrome P450 reductase) (24,25), alkaline phosphatase (inhibitor of P450 reductase) (26), and 3-aminopyridine adenine dinucleotide phosphate (AADP; competitive inhibitor of P450 reductase) and 3-aminopyridine adenine dinucleotide (AAD; competitive inhibitor of microsomal and other NADH dehydrogenases) (27,28). Given that some of these inhibitors have not been used before in fish studies, experiments were first carried out to characterize their effects on flounder hepatic microsomal NAD(P)H-dependent enzyme activities before applying them to the redox cycling experiments. In the latter studies, oxyradical generation was detected by oxidation of the scavenging agent 2-keto-4-methiolbutyric acid (KMBA) by ·OH, using iron/EDTA as a catalyst for the Haber–Weiss reaction (see the Materials and Methods Section).

MATERIALS AND METHODS

Chemicals, Animals, and Preparation of Hepatic Microsomes

Sodium azide, horse heart cytochrome c, NADH (β-nicotinamide adenine dinucleotide reduced form), NADPH (β-nicotinamide adenine dinucleotide phosphate reduced form), AADP, AAD, KMBA, menadione, nitrofurantoin, clotrimazole (1-[*o*-chloro-α,α-diphenyl-benzyl]imidazole), piperonyl butoxide, ellipticine (5,11-dimethyl-[6H]-pyridol carbazole), and alkaline phosphatase (EC 3.1.3.1) were obtained from Sigma Chemical Co., UK. All other chemicals were of AnalaR grade and were from Merck, UK.

Flounder (about 35 cm length and 200–300 g wet weight) were caught in local waters off Plymouth, UK. Fish were sacrificed by severing their spinal cords. The livers (about 1 g) were immediately removed and microsomes prepared at 4°C. Livers were homogenized in a 1:4 tissue weight:buffer volume ratio in 0.15 M KCl pH 7.5 containing 1 mM ethylenediaminetetraacetic acid (EDTA), using an electrically driven Potter–Elvehjem glass/teflon homogenizer. The homogenate was centrifuged at 9000 g for 20 minutes and the resulting supernatant at 100,000 g for 60 minutes. The microsomes were resuspended in a reduced volume of homogenization buffer containing 20% w/v glycerol to a protein concentration of about 10 mg per ml. Microsomal suspensions were stored in liquid nitrogen (without loss of reductase or oxyradical-generating activities) until required for assay.

Enzyme and Protein Assays

Spectrophotometric enzyme assays were carried out at 25°C on a Varian Cary 1 dual-beam spectrophotometer. Ellipticine binding spectra were determined by difference spectroscopy, as described in Lesca *et al.* (24) using split cuvettes to eliminate absorbance due to ellipticine itself. Thus, the following additions were made: ellipticine in dimethylformamide (DMF) into (1) the front section of a sample cuvette containing microsomes and (2) the back section of a reference cuvette lacking microsomes; and DMF alone into (1) the back section of a sample cuvette lacking microsomes and (2) the front section of a reference cuvette containing microsomes. Total cytochrome P450 and b_5 contents were measured, respectively, by the carbon monoxide (CO) difference spectra of sodium dithionite-reduced microsomes (Ext. Coeff. 91 $mM^{-1} \cdot cm^{-1}$) and the NADH-difference spectrum (Ext. Coeff. 185 $mM^{-1} \cdot cm^{-1}$), essentially according to Estabrook and Werringloer and Omura and Sato (29,30) using 100 mM Tris-HCl pH 7.4 as a buffer. Cytochrome P450 reductase activity was measured under anaerobic conditions using anaerobic cuvettes and argon flushing: all reagents except NADPH were added to the cuvettes, and, following addition of 0.3 mM NADPH, cytochrome P450 content was measured by CO-difference spectra as described earlier. Reductase activities were assayed as described in Livingstone and Farrar (31). NAD(P)H-cytochrome c reductase activities were determined by the increase in absorbance at 550 nm due to cytochrome c reduction (Ext. Coeff. 19.6 $mM^{-1} \cdot cm^{-1}$). Contained in a final volume of 1 ml were 100 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.3 mM NADH or NADPH, 60 μM cytochrome c, and 1 mM KCN. NADH-ferricyanide re-

ductase activity was determined by the decrease in absorbance at 420 nm due to ferricyanide reduction (*Ext. Coeff.* 1.02 $mM^{-1} \cdot cm^{-1}$). Contained in a final volume of 1 mL were 100 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.3 mM NADH, 0.5 mM potassium ferricyanide, and 1 mM KCN.

Benzo[a]pyrene (BaP) metabolism was determined radiometrically at 25°C under yellow light essentially as described in Lemaire *et al.* (32). Contained in an assay volume of 1.5 mL were 60 μM ^{3}H-BaP (185 μCi per μmol), 0.2 mM NADPH, 50 mM Tris-HCl pH 7.6, 3 mM $MgCl_2$, and about 4 mg microsomal protein. Incubation was terminated after 0 and 15 minutes by 1.5 mL cold acetone. Metabolites were extracted under argon with ethyl acetate/0.1 mM butylated hydroxytoluene, dried over anhydrous sodium sulfate, taken up in methanol, and separated by reverse phase HPLC (Waters C_{18} Bondapak 3.9 mm × 30 cm column, 60% methanol/40% water to 100% methanol [v/v] gradient over 40 minutes, flow rate 1 mL per min, 40°C). Metabolite quantification and identification were by on-line radioactivity monitoring (Reeve, UK) and retention time relative to authentic standards (254 nm detection).

Protein was measured by the method of Lowry *et al.* (33).

Hydroxyl Radical Production

Hydroxyl radical production (KMBA oxidation) in the absence or presence of menadione or nitrofurantoin was measured by oxidation of KMBA to ethylene, as described in Winston *et al.* (34). Ethylene was determined by direct measurement of an aliquot from the head space of rubber septum-sealed 25 mL conical flasks by gas chromatography. Assays were carried out at 25°C and contained in a 3 or 1 mL reaction volume in 25 mL conical flasks with 100 mM KH_2PO_4/K_2HPO_4 pH 7.4, 10 mM $MgCl_2$, 1 mM sodium azide (to inhibit endogenous catalase), 0.3 mM NADH or NADPH, 75 μM $FeCl_3$ in 150 μM neutralized EDTA, about 1 mg of microsomal protein, and with or without 500 μM menadione or nitrofurantoin. The $FeCl_3$/EDTA was included as a redox cycling catalyst for the production of ·OH by the Haber–Weiss reaction ($O_{2^-} + H_2O_2 = \cdot OH + OH^- + O_2$) via the sum of reactions:

$$Fe^{3+} + O_{2^-} = Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 = Fe^{3+} + \cdot OH + OH^-$$

The concentrations of $FeCl_3$ were approaching saturating for both basal and xenobiotic-stimulated NAD(P)H-dependent KMBA oxidation, as was the concentration of menadione and nitrofurantoin (19). The KMBA assay also detects other oxidants such as alkoxyl radicals, but studies have shown that approximately 90% of the oxidant formed in iron-catalyzed Haber–Weiss reactions is ·OH (35). The septa were applied to the flasks immediately after initiation of the reaction by addition of NADH or NADPH. Samples of head space were taken at variable times over the course of the incubation with a gas-tight plastic syringe. Samples of the gas phase were injected directly into a Varian model 2700 gas chromatograph equipped with a Poropack Q-column (80/100 mesh) and a flame ionization detector. Operating conditions were column 50°C, detector 180°C, and nitrogen (carrier gas) flow 30 mL per minute. Retention time for ethylene was 1.8 minutes, and quantification was by reference to ethylene standards prepared under the same conditions of incubation.

Inhibition Studies

Assays were carried out in duplicate or triplicate on multiple biological samples (see text for details). Inhibitors were added dissolved in water or dimethyl formamide (DMF), and details of concentrations used are given in the text. Controls contained water or DMF only. Inhibitors were preincubated with assay mixtures for 5 minutes prior to initiation of the reaction or added just before initiation.

Statistical and Kinetic Analysis

Values are quoted as mean ± SEM. Groups of values were compared by Student's paired *t*-test and changes over time by ANOVA with Fisher's F-test (36). Levels of confidence ≤ 0.05 were considered statistically significant. Michaelis–Menten parameters for ellipticine binding were determined by weighted Lineweaver–Burk regressions (37).

RESULTS

Ellipticine showed typical type II binding spectra, with a λ_{max} at 440 nm and a λ_{min} at 390 nm (Figure 1A). Maximal absorbance change (440–390 nm) occurred at 10 μM ellipticine (Figure 1B) and was 0.164 ± 0.015 absorbance units per nmol cytochrome P450. The apparent spectral dissociation constant (K_S) was 5.36 ± 0.43 μM. Total microsomal cytochrome P450 content was 210 ± 15 pmol per mg microsomal protein. Using anaerobic con-

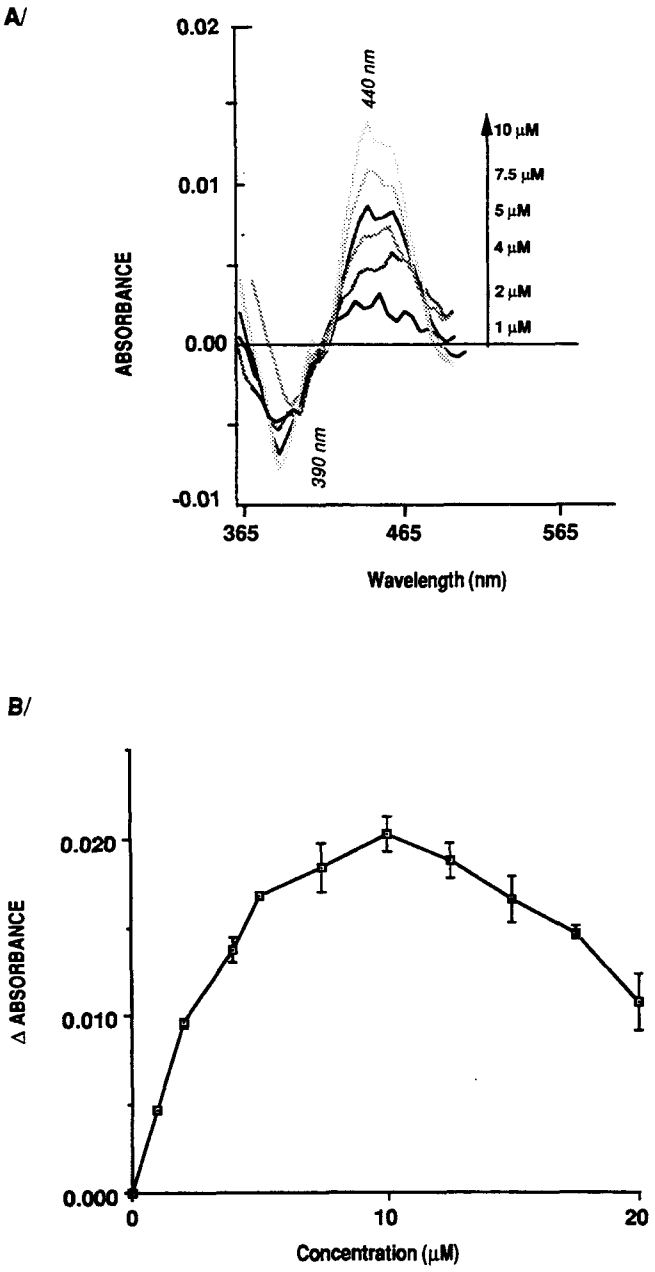

FIGURE 1. (A) Difference binding spectra and maximal absorbance change (440–390 nm) with (B) ligand concentration for ellipticine binding to hepatic microsomal cytochrome P450 of flounder (*Platichthys flesus*). Values are mean ± SEM; 195 pmol total P450 per assay.

ditions and NADPH instead of sodium dithionite, a clear CO-difference peak was discernable at 450 nm (approximating 30% of the dithionite-determined P450 levels) which was not observed in the presence of 200 μM ellipticine, indicating inhibition of NADPH-cytochrome P450 reductase activity (data not shown). BaP was mainly metabolized to 3-hydroxy-BaP (~90% of free polar metabolites) at a rate of 81.9 ± 4.4 pmol min^{-1} mg^{-1} protein. Metabolism to phenols and dihydrodiols was completely inhibited by 200 μM ellipticine, confirming the binding of ellipticine to cytochrome *P450* reductase and/or cytochrome P450 so as to inhibit monooxygenase activity.

The effects of inhibitors of mammalian flavoprotein reductases on flounder hepatic microsomal reductase activities (with artificial electron acceptors) are given in Table 1. Inhibition of NADPH-cytochrome c reductase activity was indicated at 10 μM ellipticine but was only statistically significant at 100 μM ellipticine (66% inhibition). A similar level of inhibition was found for NADH-cytochrome c reductase activity (73% inhibition at 100 μM ellipticine), but no effect was observed for NADH-ferricyanide reductase activity. Somewhat surprisingly, incubation with alkaline phosphatase markedly stimulated NADPH-cytochrome c reductase activity by up to 600% and also slightly increased NADH-reductase activities by 9 to 22%. At 100 μM reduced coenzyme, 300 μM AADP (competitive inhibitor with NADPH) inhibited NADPH-cytochrome c reductase activity by 67% but had no effect on either NADH-reductase activity. Inhibition of NADPH-cytochrome c reductase activity by AADP was similar at 100 and 300 μM NADPH and was not increased at 1 mM AADP (not shown). Three-hundred micromolar AAD (competitive inhibitor with NADH) had no effect on NADPH-cytochrome c reductase activity but reduced both NADH-reductase activities by 13–14%.

The effects of the inhibitors on ·OH production (KMBA oxidation to ethylene) are given in Table 2. Basal rates of KMBA oxidation (absence of menadione or nitrofurantoin) were greater for NADH than NADPH. Both coenzyme-dependent rates were stimulated by addition of xenobiotic, moreso for menadione than nitrofurantoin. Clotrimazole and piperonyl butoxide had no effect on basal or xenobiotic-stimulated NADPH-dependent KMBA oxidation but variously stimulated some of the equivalent NADH-dependent rates by up to 46%. Ellipticine had no effect on NADH-dependent KMBA oxidation but reduced significantly basal and menadione- but not nitrofurantoin-stimulated NADPH-dependent rates by 23 and 8%, respectively. AADP stimulated basal and xenobiotic-stimulated NADH-dependent KMBA oxidation by 31–61% but inhibited significantly menadione- and nitrofurantoin-stimulated NADPH-dependent rates by 17 and 45%, respectively. Alkaline phosphatase variously increased some of the basal and xenobiotic-stimulated rates for both NADH and NADPH by 16–47%.

TABLE 1. Effects of Inhibitors of Mammalian Flavoprotein Reductases on NAD(P)H-Cytochrome c and NADH-Ferricyanide Reductase Activities of Hepatic Microsomes of Flounder (*Platichthys flesus*)[a]

Conditions	*NADPH Cytochrome c Reductase*	*NADH Cytochrome c Reductase*	*NADH Ferricyanide Reductase*
Control (300 μM NAD(P)H)	22.4 ± 0.9	119.1 ± 5.1	668.7 ± 42.7
Ellipticine 10 μM	18.4 ± 3.0	—	—
100 μM	7.6 ± 0.9 (66% ↓)*	31.6 ± 3.2 (73% ↓)*	616.8 ± 66.5
Alkaline phosphatase 0.32U	155.7 ± 3.7 (600% ↑)*	145.1 ± 7.0 (22% ↑)*	727.9 ± 29.7 (9% ↑)*
0.16U	132.3 ± 7.5 (500% ↑)*	—	—
Control (100 μM NAD(P)H)	14.6 ± 1.5	114.1 ± 4.9	568.6 ± 12.4
3-Aminopyridine adenosine dinucleotide diphosphate (AADP, 300 μM)	4.8 ± 0.8 (67% ↓)*	114.7 ± 4.1	537.7 ± 22.9
3-Aminopyridine adenosine phosphate (AAD, 300 μM)	15.5 ± 0.64	99.6 ± 6.5 (13% ↓)*	486.7 ± 6.9 (14% ↓)*

[a]Activities in nmol · min^{-1} · mg microsomal protein^{-1}. Values are mean ± SEM (n = 3). Where there is a statistically significant difference, this is given in parentheses as percentage inhibition ↓ or stimulation ↑ of the control.—Not determined. *$p < 0.05$.

TABLE 2. Effect of Different Inhibitors of Mammalian Cytochrome P450 and Cytochrome P450 Reductase on Hydroxyl Radical Production (KMBA Oxidation to Ethylene) by Hepatic Microsomes of Flounder (*Platichthys flesus*)[a]

Conditions	*Control*	*Menadione*	*Nitrofurantoin*
NADH (300 μM)			
Control	0.68± 0.05	2.20 ± 0.12	1.01 ± 0.03
Clotrimazole (500 μM)	0.72 ± 0.07	2.50 ± 0.12	1.16 ± 0.03 (15% ↑)*
Piperonyl butoxide (1 mM)	0.75 ± 0.05 (10% ↑)*	3.20 ± 0.21 (46% ↑)*	1.28 ± 0.01 (27% ↑)*
Ellipticine (100 μM)	0.69 ± 0.06	2.01 ± 0.09	1.04 ± 0.03
3-Aminopyridine adenosine dinucleotide diphosphate (AADP, 300 μM)	0.89 ± 0.09 (31% ↑)*	3.55 ± 0.18 (61% ↑)*	1.40 ± 0.05 (39% ↑)*
Akaline phosphatase (0.16 unit)	0.69 ± 0.02	3.19 ± 0.14 (45% ↑)*	1.17 ± 0.09 (16% ↑)*
NADPH (300 μM)			
Control	0.30 ± 0.01	2.26 ± 0.15	1.08 ± 0.10
Clotrimazole (500 μM)	0.31 ± 0.01	2.29 ± 0.15	0.98 ± 0.05
Piperonyl butoxide (1 mM)	0.30 ± 0.03	2.45 ± 0.14	1.01 ± 0.07
Ellipticine (100 μM)	0.23 ± 0.01 (23% ↓)*	2.08 ± 0.14 (8% ↓)*	1.05 ± 0.06
3-Aminopyridine adenosine dinucleotide diphosphate (AADP, 300 μM)	0.30 ± 0.01	1.88 ± 0.13 (17% ↓)*	0.59 ± 0.03 (45% ↓)*
Alkaline phosphatase (0.16 unit)	0.44 ± 0.01 (47% ↑)*	2.35 ± 0.08	1.35 ± 0.08 (25% ↑)*

[a]Values are the mean ± SEM (n = 3). Activities are expressed in nmol · min^{-1} · mg microsomal protein^{-1}. Where there is a statistically significant difference, this is given in parentheses as percentage inhibition ↓ or stimulation ↑ of the control. *$p < 0.05$.

DISCUSSION

Properties of flavoprotein reductases and electron flow within the hepatic microsomal mixed-function oxygenase (MFO) system have been extensively characterized in mammals (38,39). NADPH-cytochrome c reductase activity is catalyzed predominantly or exclusively by cytochrome P450 reductase, NADH-ferricyanide reductase activity by predominantly cytochrome b_5 reductase, and NADH-cytochrome c reductase activity by cytochrome b_5 reductase and cytochrome b_5. Fewer studies have been made of flavoprotein reductases in fish hepatic MFO systems, but a similar situation is indicated (40–44). Experiments were thus first carried out to characterize the effects of the inhibitors on hepatic microsomal reductase activities and cytochrome P450 of *P. flesus* before their application to the redox cycling experiments.

Ellipticine inhibits rat hepatic MFO activity by binding to the heme moiety of cytochrome P450, producing a typical type II difference spectrum through binding of the ellipticine pyridinic nitrogen to the sixth coordination site of ferric-P450 (24). The same type of spectrum and binding was observed for flounder. Using the same assay conditions and oxidized microsomes, flounder gave a λ_{max} of 440 nm compared to 428 nm for (unin-

duced) rat and a λ_{min} of 390 nm compared to 390 and 408 nm (two troughs) for rat. The maximal absorbance and apparent spectral dissociation constant were both greater than those for uninduced or induced (phenobarbital, Arochlor 1254, or benzo[a]pyrene) rat, viz. 0.16 compared to up to 0.07 per nmol P450 and 5.4 compared to up to 0.9 μM, respectively. The latter indicates less tight binding of ellipticine to flounder cytochrome P450s compared to rat. While bound to the heme of cytochrome P450 in rat hepatic microsomes, ellipticine also interacts in such a way as to impede the transfer of electrons from the hydrophobic binding site of cytochrome P450 reductase (i.e., inhibits P450 reductase activity) but not from the hydrophilic binding site of the reductase to soluble electron acceptors (i.e., does not inhibit NADPH-cytochrome c reductase activity) (25). By comparison, in flounder hepatic microsomes, ellipticine was indicated to inhibit transfer of electrons from both the hydrophobic and hydrophilic bindings sites of cytochrome P450 reductase, although in the latter case, an ellipticine concentration of 100 μM was used (this inhibited NADPH-cytochrome c reductase activity by 66%) compared to a maximum concentration of only 40 μM (inhibited NADPH-cytochrome c reductase activity by ~8%) in the rat study (25). The binding of ellipticine so as to inhibit flounder hepatic microsomal cytochrome P450 reductase or cytochrome P450 function was confirmed by inhibition of MFO (BaP metabolism) activity, consistent with the results for rat (24). Ellipticine also inhibited flounder hepatic microsomal NADH-cytochrome c reductase activity by 73% but had no effect on NADH-ferricyanide reductase activity, possibly indicating that its binding to cytochrome P450 also interfered with the transfer of electrons through cytochrome b_5 to cytochrome c (38). Interactions between hepatic cytochromes P450 and b_5 have been indicated from reconstitution studies with purified enzymes from scup (*Stenotomus chrysops*) (43).

Alkaline phosphatase inhibits NADPH-cytochrome c reductase activity but not NADH-cytochrome c or NADH-ferricyanide reductase activities in rabbit liver hepatic microsomes by digestion of the flavoprotein mononucleotide (FMN) prosthetic group necessary for electron transfer to cytochrome c (26). Totally unexpectedly, similar units and incubation conditions of alkaline phosphatase stimulated flounder NADPH-cytochrome c reductase activity by up to sevenfold and had relatively little effect on the NADH-reductase activities. Although the structure of hepatic cytochrome P450 reductase is indicated to be similar for mammals and fish, including the presence of FAD and FMN and up to 79% overall homology in amino acid sequence (41,42,44), differences are also evident in regions concerned with function, e.g., in trout (*Salmo trutta*) compared to pig, only 33% amino acid sequence homology in the hydrophilic domain (residues 56–70) which is connected to the membrane segment (residues 1–55) (44). Such differences may account for the different effects of alkaline phosphatase on the rabbit and flounder hepatic microsomal NADPH-cytochrome c reductase activities. Regulation by dephosphorylation/phosphorylation mechanisms is also a possibility to explain the effect of alkaline phosphatase but was considered unlikely for rabbit, since P450 reductase is a poor substrate for protein kinases (26).

AADP is a specific and potent competitive inhibitor of rat NADPH-cytochrome P450 reductase (28). In contrast, AAD is much less effective toward rat hepatic microsomal NADH-cytochrome c reductase activity. Taking into account the higher levels of reduced coenzymes used in the current studies (needed for KMBA oxidation), comparable levels of inhibition of reductase activities were observed for these inhibitors with flounder hepatic microsomes. Thus, AADP inhibited NADPH-cytochrome c reductase activity 67% but had no effect on NADH-reductase activities, and AAD showed only minimal inhibition (13–14%) of NADH-reductase activities. Because of the latter, only AADP was used in the redox cycling studies. Overall, the reductase inhibition studies indicate that ellipticine and AADP are inhibitors of aspects of cytochrome P450 reductase function.

Univalent reduction of quinones and nitroaromatics to the anion radical, the first part of the redox cycle that produces O_2- from NAD(P)H and O_2, is catalyzed by flavoprotein reductases in mammalian hepatic microsomal systems (20). Binding of menadione to cytochrome P450 reductase has been demonstrated for cell culture in mammalian system (21). Cytochrome P450 can give rise to O_2- and H_2O_2 from NADPH (45,46) but plays no role in redox cycling, although xenobiotics could affect P450-mediated oxyradical generation via disturbance of the MFO system and P450 reductase/P450 coupling (34). The characteristics and evidence for redox cycling and involvement of both O_2- and H_2O_2 in menadione- and nitrofurantoin-stimulated ·OH production (KMBA oxidation) by flounder hepatic microsomes are reported elsewhere (19). The results are consistent with other studies on redox cycling of nitroaromatics by fish (17,18).

AADP inhibited both menadione- and nitrofurantoin-stimulated NADPH-dependent oxyradical production by 17 and 45%, respectively, indicating an involvement of cytochrome P450 reductase in redox cycling, particularly in the latter case. The lower inhibition of menadione cannot be related to differences in the tendency of the two compounds to accept electrons from P450 reductase, menadione, and nitrofurantoin having similar oxidation-reduction potentials (−0.22 and −0.25, respectively) (47) but must be related to other factors such as substrate binding. In contrast to the inhibition by AADP, the effect of ellipticine on xenobiotic-stimulated NADPH-dependent oxyradical production was minimal, possibly because cytochrome c is a much larger molecule than either of the redox cycling compounds and ellipticine does not therefore prevent access of the xenobiotics to the hydrophilic binding site of P450 reductase.

Cytochrome P450s were indicated not to have a role in basal or xenobiotic-stimulated NADPH-dependent oxyradical production, consistent with observations for nitrofurantoin-stimulated O_2^- production by hepatic microsomes of channel catfish (17). Alkaline phosphatase showed some consistency with the NADPH-cytochrome c reductase activity results, increasing both basal and nitrofurantoin-stimulated NADPH-dependent oxyradical production. Significantly, the reverse consistency was seen for rat hepatic microsomes, the inhibition of NADPH-cytochrome c reductase activity by alkaline phosphatase (see earlier) being correlated with an inhibition of NADPH-dependent oxyradical generation (48). This lends further support to the idea of alkaline phosphatase treatment producing a different functional effect in flounder compared to mammalian microsomal cytochrome P450 reductase. Somewhat surprisingly, AADP and cytochrome P450 inhibitors clotrimazole and piperonyl butoxide, which interact with NADPH-utilizing enzymes, stimulated to varying degrees basal and xenobiotic-stimulated NADH-dependent oxyradical production. Presumably, this must have occurred through some redirection of NADH electron flow in the microsomal MFO system. This or other mechanisms may also have been involved in the increase in xenobiotic-stimulated NADH-dependent oxyradical production by alkaline phosphatase.

In conclusion, the studies indicate both similarities (ellipticine binding, AADP and AAD inhibition) and differences (alkaline phosphatase) between fish and rat hepatic microsomal enzyme systems. Involvement of cytochrome P450 reductase in the stimulation of microsomal NADPH-dependent oxyradical production by nitrofurantoin, and possibly also by menadione, is indicated. Further interpretation, however, is limited by lack of information on the properties of purified reductases and the restricted specificity or effects of the inhibitors.

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