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Mixed Function Oxygenases in Cultured Fish Cells: Contributions of *in vitro* Studies to the Understanding of MFO Induction

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(With 7 Figures)

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The expression of toxicological responses in biological systems can be studied by various approaches and methodologies. In comparison to *in vivo* approaches, *in vitro* systems provide several distinct advantages (BAKSI & FRAZIER 1990, BRAUNBECK 1993, BRAUNBECK & STORCH 1992, SEGNER & LENZ 1993, ZAHN et al. 1993):

(1) *Technical reasons*. Improved standardization of experimental conditions allows for better control of influences on the test systems by biological, chemical an physical factors and, thus, decreases variability of results; repeated sampling over time within the same preparation is possible; miniaturization of assay volumes drastically reduces health risks for personnel and amounts of hazardous wastes in toxicological experiments; requirements for space and equipment are usually reduced; in most cases, *in vitro* tests are quicker and cheaper to perform than *in vivo* studies.

(2) *Ethical considerations*. The number of animals spent for toxicological experiments can be significantly lowered.

(3) *Scientific reasons*. Elimination of systemic effects allows for the analysis of molecular and cellular mechanisms of toxic action; since biological variation is greatly reduced in cell systems, they provide a good experimental basis to study the effects of chemical variation, i.e. to establish structure-activity relationships.

The last 20 years have seen an immense increase in characterization of xenobiotic metabolism in fish by mixed function oxygenase (MFO) systems (e.g., ADDISON et al. 1991, ANDERSSON & FÖRLIN 1992, ANDERSSON et al. 1985, BEND & JAMES 1979, FÖRLIN & HAUX 1990, GOKSØYR & LARSEN 1991, GOKSØYR et al. 1987, 1991a, 1992, JAMES & BEND 1980, KLEINOW et al. 1987, LARSEN et al. 1992, MONOD et al. 1987, 1988, PESONEN et al. 1987, STEGEMAN 1989, STEGEMAN & JAMES 1989, STEGEMAN & KLOEPPER-SAMS 1987, STEGEMAN et al. 1981, 1991a, 1992). Such progress has resulted in the suggestion to use MFO systems activities as monitor systems in field studies on environmental pollution (FÖRLIN & CELANDER 1993, GOKSØYR & FÖRLIN 1992, GOKSØYR & HUSOY 1992, GOKSØYR & SOLBERG 1987, GOKSØYR et al. 1991b, 1992, HAASCH et al. 1989, MONOSSON et al. 1991, STAGG et al. 1992, STEGEMAN et al. 1981, 1988, 1991b, 1992, VAN DER WEIDEN et al. 1993, VAN VELD et al. 1992). In addition, increasing efforts have been devoted to the study of xenobiotic metabolizing enzymes in piscine *in vitro* systems (ANDERSSON & FÖRLIN 1985, ANDERSSON et al. 1983, DEVAUX et al. 1991, 1992, FÖRLIN & ANDERSSON 1981, MASFARAUD et al. 1992, MILLER et al. 1993a, b, PESONEN & ANDERSSON 1991, PESONEN et al. 1988, VAILLANT et al. 1989, VINDIMIAN & GARRIC 1989).

Piscine systems with permanent cell cultures as well as primary cultures of hepatocytes have already been applied in biomonitoring (AHNE & HALDER 1991, CASTANO et al. 1994, KOCAN et al. 1985, RUSCHE & KOHLPOTH 1993, ZAHN et al. 1995); however, in some cases difficulties have emerged with regard to bioactivation of certain classes of chemicals. Therefore, the present communication was designed to focus on the potential use of fish cell cultures for MFO research and their contribution to still existing problems in MFO application for biomonitoring. Since primary cultures of hepatocytes significantly differ from permanent fish cell cultures with respect to their biotransformation capacities (HAUCK & BRAUNBECK 1994a, b, HAUCK et al. 1993, SCHOLZ et al. 1994), a distinction between these two *in vitro* systems is necessary.

Cytochrome P-450 in Cultured Hepatocytes of Rainbow Trout *(Oncorhynchus mykiss)*

Although cytochrome P-450-dependent biotransformation enzymes are not restricted to the liver (MILLER et al. 1988, STEGEMAN et al. 1987), but have also been located in fish gills (MILLER et al. 1989), heart (STEGEMAN et al. 1990) and kidney (LORENZANA et al. 1988, MILLER et al. 1988, PESONEN & ANDERSSON 1987, STEGEMAN et al. 1987), hepatic cytochrome P-450 activities account for the largest portion of total MFO activities in fish (LESTER et al. 1992, 1993, LORENZANA et al. 1989). Within teleost hepato-

cytes, monooxygenases are associated with cisternae of the endoplasmic reticulum (Fig. 1; LESTER et al. 1992, 1993), with the major portion – in contrast to mammalian hepatocytes – being localized on the rough endoplasmic reticulum (LESTER et al. 1993). A typical zonal accentuation of cytochrome P-450 distribution similar to that in mammalian liver parenchyma (BARS & ELCOMBE 1991, BÜHLER et al. 1992) could not be found in fish liver (Fig. 2; LESTER et al. 1993, LORENZANA et al. 1989).

For most *in vitro* studies on biotransformation of fish, freshly isolated hepatocytes have been used (for review, see BAKSI & FRAZIER 1990). Only recently, techniques for primary cultures of teleost hepatocytes have been developed (BLAIR et al. 1990, BRAUNBECK & STORCH 1992, DEVAUX et al. 1991, 1992, KLAUNIG 1984, KLAUNIG et al. 1985, KOCAL et al. 1988, LIPSKY et al. 1986, MASFARAUD et al. 1992, PESONEN & ANDERSSON 1991,

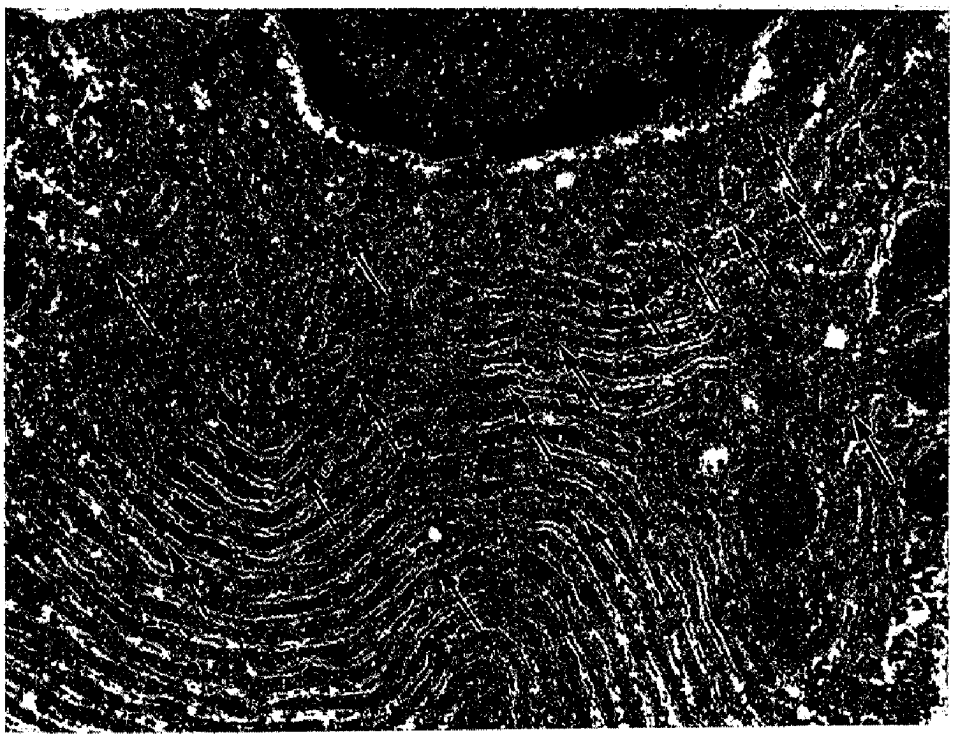

Fig. 1. Intracellular localization of cytochrome P-450 IA1 (CYP 1A) in hepatocytes from juvenile rainbow trout *(Oncorhynchus mykiss;* mean weight 5 g) stimulated by a single i.p. injection of β-naphthoflavone suspended in cod liver oil (50 μg/g; total volume of injectate, 500 μL) 5 days before sacrifice. Immunogold particles are abundant over cisternae of the rough endoplasmic reticulum. Additional labeling can be found on cisternae of the smooth endoplasmic reticulum, nuclear membranes and heterochromatin, basal as well as apical microvilli of hepatocytes (LESTER et al. 1993; data not shown). Section was treated with a monoclonal antibody (Mab 1-12-3 raised against cytochrome P-450 CYP 1A in scup, *Stenotomus versicolor;* KLOEPPER-SAMS et al. 1986, 1987), rabbit anti-mouse IgG, and protein G-colloidal gold (10 nm).

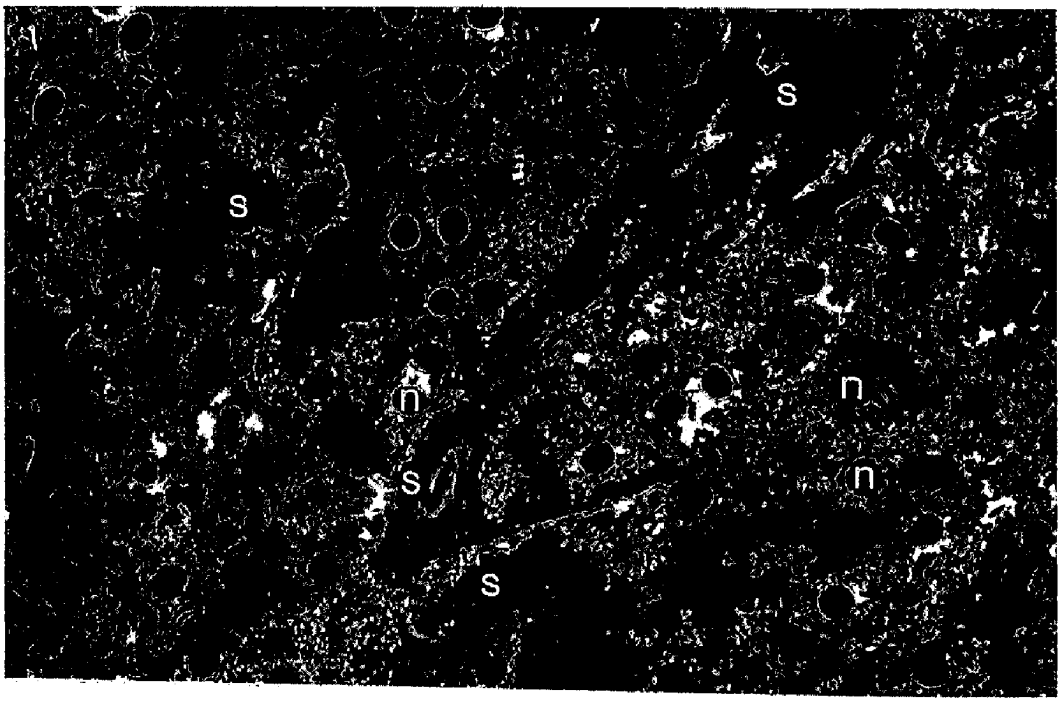

Fig. 2. Light micrograph of glycol methacrylate-embedded rainbow trout *(Oncorhynchus mykiss)* liver section (1 μm). The fish was given a single i.p. injection of β-naphthoflavone suspended in cod liver oil (50 μg/g; total volume of injectate, 500 μL) 5 days prior to sampling. Section was incubated with a monoclonal antibody (Mab 1-12-3; cf. Fig. 1) and a fluorescent secondary antibody. Labeled structures are nuclear envelopes and perinuclear regions of hepatocytes seen as light regions surrounding nuclei (n). Also note strongly positive reaction of endothelial cells lining sinusoids (s). Although there is some heterogeneity in labeling intensity, no typical zonal accentuation of cytochrome P-450 distribution is apparent.

SEGNER et al. 1993, 1994, 1995). Although in most cases this approach has only been used for short-term studies, it should also be useful for longer-term studies on MFO induction.

A central problem in the use of *in vitro* preparations of hepatocytes is the question as to what extent the cells retain basic characteristics of the more complex *in vivo* condition. For mammals, it is well documented that isolated hepatocytes in monolayer culture suffer a serious decline of MFO levels with ongoing incubation period (e.g., GUGUEN-GUILLOUZOU & CORLU 1993, ROGIERS et al. 1990, SKETT 1994). In contrast, rainbow trout *(Oncorhynchus mykiss)* hepatocytes in culture display different characteristics: apparently, they are able to maintain *in vivo*-like MFO activities over a 5 - 8 day-culture period (HAUCK et al. 1993, MILLER et al. 1993b, PESONEN & ANDERSSON 1991, PESONEN et al. 1988, SCHOLZ & SEGNER 1994, Table 1). However, this finding may not be generalized for teleosts, since in carp *(Cyprinus carpio)* hepatocytes, a drastic decrease of EROD activities during culture can be observed (Table 1).

Table 1
Activities of ethoxycoumarin-O-deethylase in cultured hepatocytes of carp *(Cyprinus carpio)* and rainbow trout *(Oncorhynchus mykiss)*

Days of culture	Carp	Rainbow trout
0	44 ± 2	7 ± 1
2	13 ± 8	27 ± 3
4	7 ± 3	217 ± 7
6	n.d.	361 ± 67
8	n.d.	685 ± 138

Data are given as activities in pmol resorufin/minute/mg protein.

In addition, *in vitro* induction of MFO activities in cultured trout hepatocytes is possible: Exposure of liver cells to a single dose of 1 µL/ml 3.6 mM β-naphthoflavone resulted in a significant increase of ethoxycoumarin-O-deethylase (ECOD) activities (Fig. 3). However, induction only lasts for one day and decreases again thereafter, if no new stimulus is given.

Responsiveness of MFO systems in isolated rainbow trout hepatocytes to inducing agents can be easily manipulated by appropriate pretreatment of donor fish: exposure of intact fish to MFO inducers prior to hepatocyte isolation enhances the response of monooxygenases to toxicants in cultured cells (HAUCK & BRAUNBECK 1994a, b). Moreover, cultured hepatocytes are an ideal system to investigate the interaction of anthropogenic and natural factors (temperature, sex hormones, adrenocorticoid stress hormones, etc.) on biotransformation enzymes. Thus, numerous incertainties which still obscure results of *in vivo* monitoring programs based on MFO activities could probably be solved by appropriate *in vitro* experiments with isolated hepatocytes.

Cytochrome P-450 in Permanent Fish Cell Lines

RACHLIN and PERLMUTTER (1968) were the first to suggest the use of permanent fish cell lines in cytotoxicity assays for screening purposes. During the last years, the application of fish cell lines for ecotoxicity testing has drastically increased (for review on acute and subacute toxicity tests with permanent fish cell lines, see BABICH & BORENFREUND 1991 as well as ZAHN et al. 1993). Among the cell lines presently available, for toxicological studies most attention has been given to RTG-2 cells

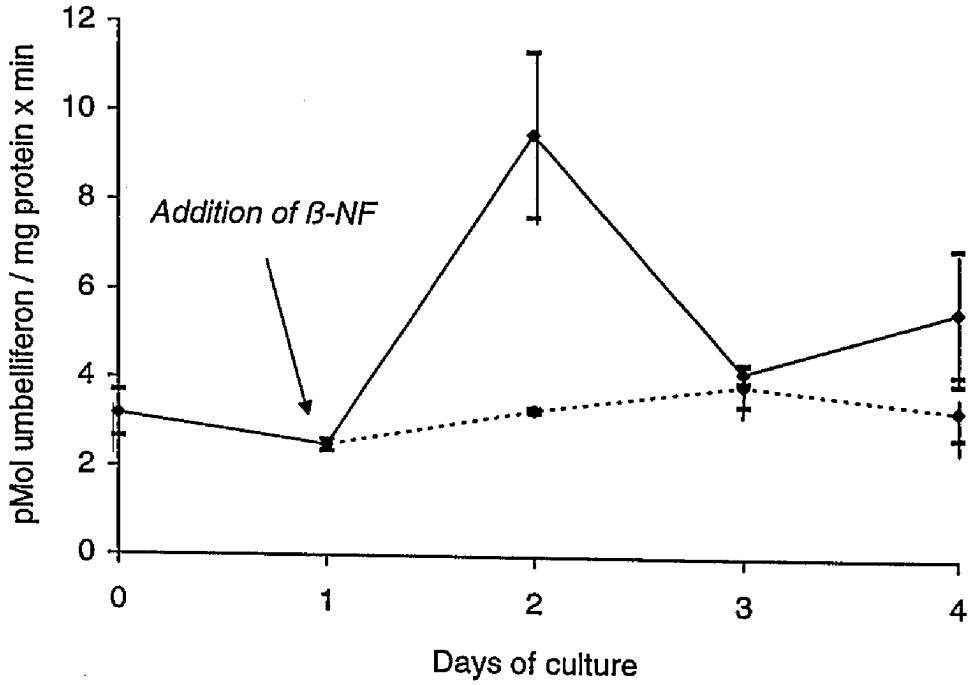

Fig. 3. Induction of specific activities of ethoxycoumarin-O-deethylase by a single *in vitro* application of β-naphthoflavone (1 μL/ml, 3.6 mM) in hepatocytes isolated from rainbow trout *(Oncorhynchus mykiss)* not previously stimulated by *in vivo* exposure to inducers (. . . = unstimulated controls).

derived from gonadal tissues of rainbow trout (WOLF & QUIMBY 1962), STE generated from steelhead trout embryos (KOCAN et al. 1979), BF-2 originating from the caudal trunk of bluegill sunfish *(Lepomis macrochirus,* WOLF & QUIMBY 1966), BG/F developed from bluegill sunfish (BABICH & BORENFREUND 1990), FHM from fathead minnow *(Pimephales promelas,* WALTON et al. 1983) and R1 originating from primary cultures of rainbow trout liver (AHNE 1985, AHNE & HALDER 1991, HALDER & AHNE 1990). Since cultures of permanent fish cells do not require living fish as donors any more and are substantially easier to handle, they appear to be promising candidates for routine toxicity testing and environmental monitoring.

The capacity for biotransformation is an important trait of cultured cells in order to be able to discriminate between directly acting chemical reagents and compounds requiring bioactivation. In human toxicology, several permanent cell lines are in use that preserved significant biotransformation capacities (DONATO et al. 1990, 1991, 1993). With regard to permanent fish cells, however, biotransformation capacities have not been adequately characterized. Fish cell lines such as RTG-2 or R1 display only very low or even lack detectable levels of phase I and phase II biotransformation enzymes (Fig. 4). Even 7-day-exposure to 0.15, 1.5 and 15 μM of the classical cytochrome P-450 inducer β-naphthoflavone,

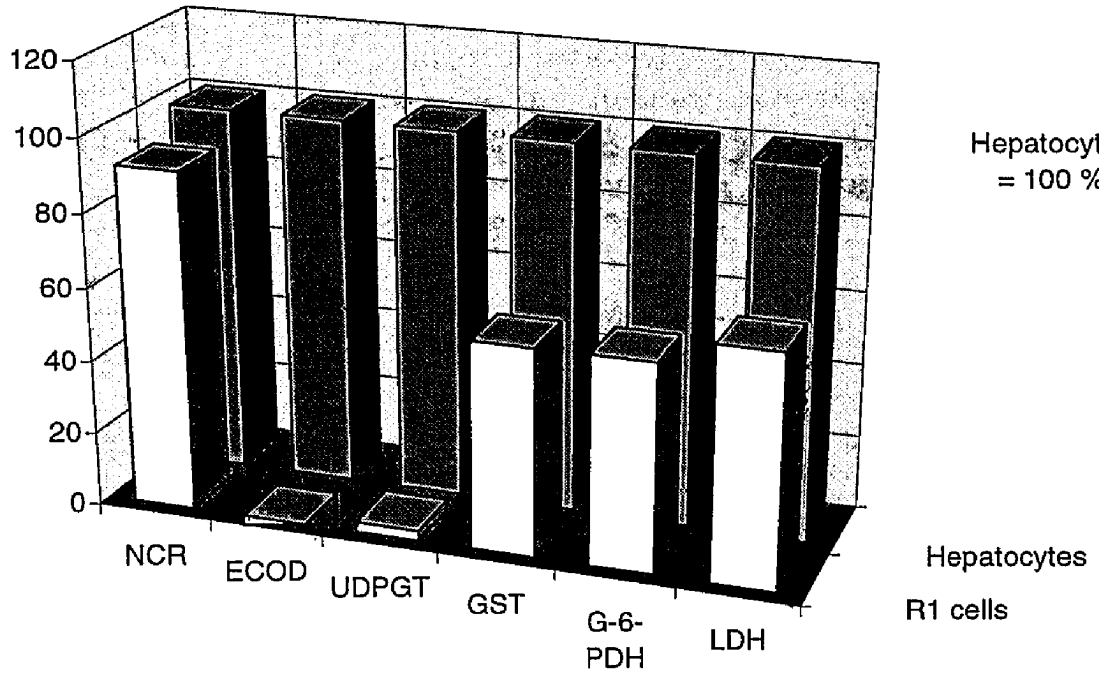

Fig. 4. Comparison of specific activities of selected biotransformation and metabolic key enzymes in isolated hepatocytes from rainbow trout *(Oncorhynchus mykiss;* 100%) and R1 cells derived from rainbow trout liver (NCR – NADPH-cytochrome P-450 reductase; ECOD – ethoxycoumarin-O-deethylase, UDPGT – UDP-glucuronyl transferase; GST – glutathione S-transferase; G-6PDH – glucose-6-phosphate dehydrogenase; LDH – lactate dehydrogenase).

is not able to induce measurable activities of ethoxycoumarin-O-deethylase (EROD). This is in line with the fact that RTG-2 and R1 display no or only fairly low sensitivities to indirectly acting toxicants such as benzo[a]pyrene (Fig. 5). On the other hand, experiments with radioactive benzo[a]pyrene have revealed that RTG-2 cells are able to transform this lipophilic compound at least partly into more water-soluble metabolites (DIAMOND & CLARK 1970, THORNTON et al. 1982). Apparently, monooxygenase activities of RTG-2 cells are too low for detection in enzymatic analysis, but seem to be sufficient for a measurable turnover of radioactively labelled benzo[a]pyrene. The production of metabolites, however, is not correlated with a dose-dependent toxic response of the cells (Fig. 5), which ist probably due to a high capacity of RTG-2 cells for glutathion conjugation (cf. SMOLAREK et al. 1987). In contrast to phase I metabolism, RTG-2 cells show distinct activities of conjugating enzymes such as glutathione-S-transferase (Fig. 6). Accordingly, prolonged exposure of RTG-2 cells to MFO inducers such as β-naphtoflavone results in elevation of glutathione-S-transferase levels.

Insufficient biotransformation capacities in many permanent fish cell lines can be compensated by complementation of the test assay with S-9

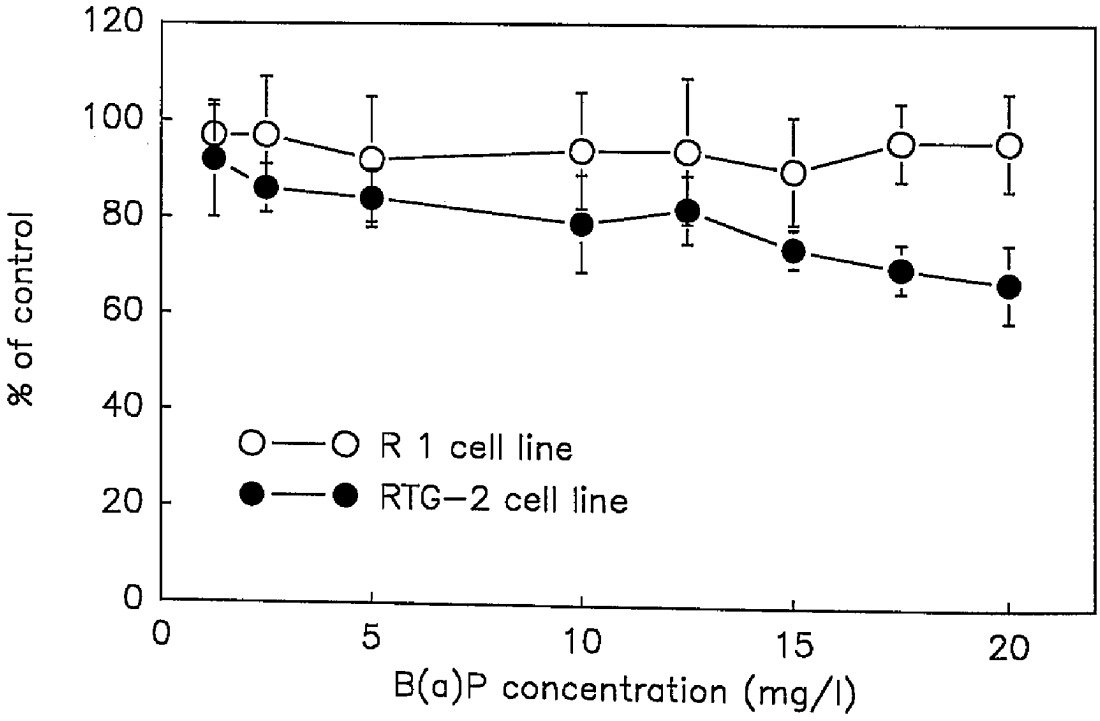

Fig. 5. Comparative cytotoxicity of benzo[a]pyrene in permanent R1 and RTG-2 cells after exposure for 6 days, as evidenced by crystal violet staining. Data given as % of controls (100% viability).

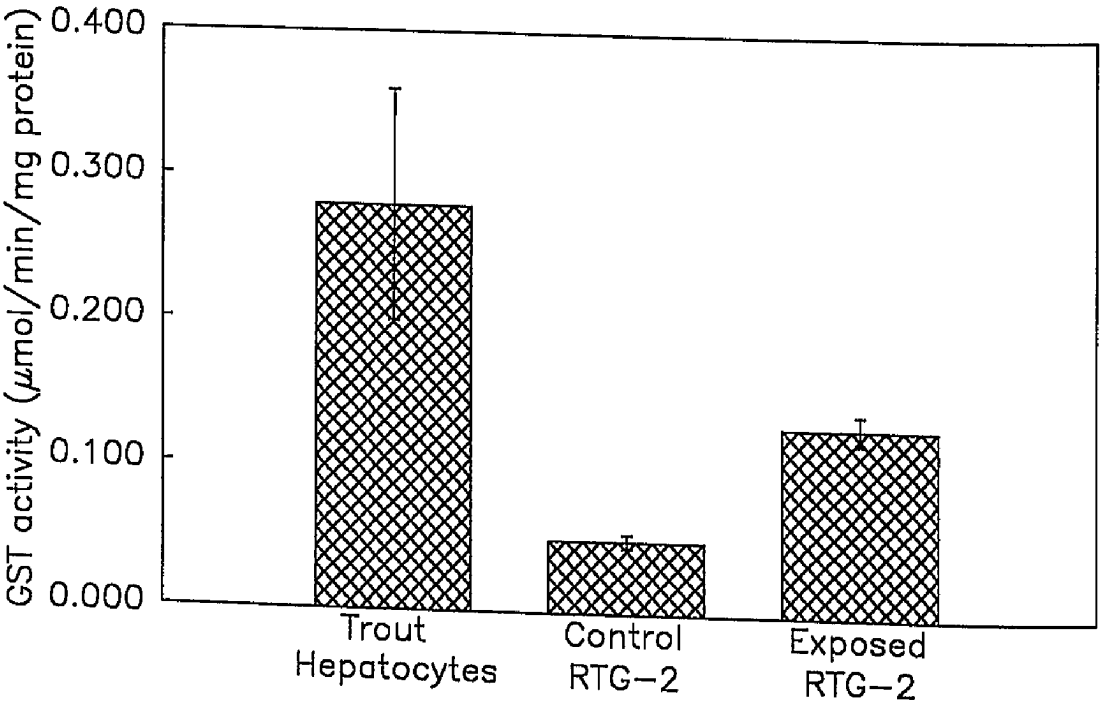

Fig. 6. Activity of glutathione S-transferase (GST) in primary rainbow trout hepatocytes, in confluent RTG-2 cells and GST levels in confluent RTG-2 cells exposed for 6 days to 1.5 μM β-naphthoflavone.

preparations from rat, which are highly competent of bioactivation by phase I monooxygenase reactions. The toxicity of cyclophosphamid, a model substance which requires bioactivation by cytochrome P-450 IA1 to exert its cytotoxic effects, is dramatically enhanced by addition of S-9 preparations from rat stimulated with β-naphthoflavone and phenobarbital (Fig. 7).

Conclusions

Results indicate that isolated hepatocytes represent a promising tool for the elucidation of basic characteristics of fish MFO. In contrast, permanent fish cell lines need to be further studied with respect to their biotransformation capacities prior to use in routine survey and monitoring of environmental pollution. More recent investigations have led to the introduction of novel fish cell lines, which are apparently well capable of expressing stable and inducible MFO activities (HAHN et al. 1993, GHOSH et al. 1994, LEE et al. 1993). Comparison of established

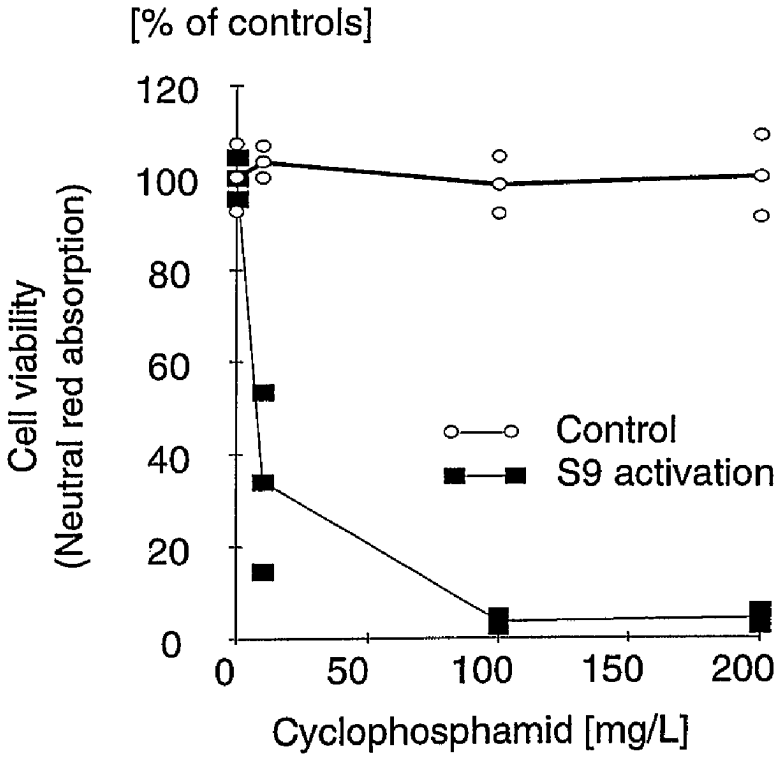

Fig. 7. Influence of bioactivation of the reference toxicant cyclophosphamid by S-9 preparations from rat stimulated by injection with β-naphthoflavone plus phenobarbital in the permanent fish cell line D11 derived from R1 cells from rainbow trout *(Oncorhynchus mykiss)*. Data are presented as means ± S.E. from 16 culture wells.

fish cell lines with these new cells competent of biotransformation is urgently required.

Summary

Mixed function oxygenase systems (MFO, cytochrome P-450) were studied in primary cultures of hepatocytes isolated from rainbow trout *(Oncorhynchus mykiss)* and carp *(Cyprinus carpio)* as well as in permanent piscine fibrocyte cultures (R1, D-11, RTG-2). In fish hepatocytes, inducible forms of cytochrome P-450 IA1 could be localized on membranes of both rough and smooth endoplasmic reticulum as well as on the outer face of the nuclear envelope. In culture, only rainbow trout hepatocytes maintain cytochrome P-450 activities at a certain, albeit low, level, whereas activity rapidly declines in carp hepatocytes. Moreover, rainbow trout hepatocytes preserve inducibility of cytochrom P-450 IA1 (CYP IA) by β-naphthoflavone (1 μL/mL, 3.6 mM), the efficiency of which can be significantly enhanced by isolation of cells from fish stimulated by a previous single injection of β-naphthoflavone (50 μg/g i.p.). In contrast, whereas significant activities of NADPH cytochrome P-450 reductase and glutathione-S-reductase can be measured, activities of, e.g., ethoxycoumarin-O-deethylase and UDP-glucuronyl transferase could not be demonstrated in permanent R1 cells. As a consequence, RTG-2 and R1 cells show only minor, if at all, sensitivity to indirectly acting toxicants such as benzo[a]pyrene. Since lacking biotransformation capacities can be compensated by addition of S9-mixes or liver microsomes isolated from the livers of fish stimulated by, e.g., β-naphthoflavone, toxicity tests with permanent fish cell lines should be complemented by such additions to guarantee for adequate bioactivation competence.

Zusammenfassung

Gemischtfunktionelle Oxygenasen (MFO, Cytochrom P-450) wurden in Primärkulturen von Hepatocyten aus Regenbogenforelle *(Oncorhynchus mykiss)* und Karpfen *(Cyprinus carpio)* sowie in permanenten Fibrocytenkulturen aus Fischen (R1, D-11, RTG-2) untersucht. In Fischhepatocyten konnte die induzierbare Form von Cytochrom P-450 IA1 (CYP IA) v.a. auf den Membranen des rauhen und glatten endoplasmatischen Retikulums sowie auf der äußeren Membran der Kernhülle lokalisiert werden. Unter Kulturbedingungen bleibt die Cytochrom P-450-Aktivität in isolierten Hepatocyten der Regenbogenforelle auf einem bestimmten, wenn auch niedrigen Niveau erhalten, während die Aktivität in isolierten Hepatocyten des Karpfens rasch stark absinken. Hepatocyten aus der Regenbogenforelle bewahren überdies die Induzierbarkeit von Cytochrom P-450 IA1 durch β-Naphthoflavon (1 μL/mL, 3.6 mM), deren Effizienz erheblich gesteigert werden kann, wenn die Hepatocyten aus Fischen isoliert werden, die zuvor mit einer einmaligen Injektion von β-Naphthoflavon (50 μg/kg i.p.) stimuliert wurden. Im Gegensatz dazu zeigen permanente Zellen wie die Zellinie R1 zwar beträchtliche Aktivitäten von NADPH Cytochrom P-450-Reduktase und Glutathion-S-Reduktase, jedoch konnten z.B. Ethoxycumarin-O-Deethylase oder UDP-Glucuronyltransferase nicht nachgewiesen werden. Infolgedessen zeigen RTG-2- und R1-Zellen, wenn überhaupt, nur eine sehr geringe Reaktion auf indirekt wirkende Schadstoffe wie z.B. Benzo[a]pyren. Da dieser Mangel an Biotransformationskapazität durch die Zugabe eines S9-Mix bzw. von Mikrosomen aus der Leber von Fischen, die zuvor z.B.

durch β-Naphthoflavon induziert wurden, kompensiert werden kann, empfehlen sich derartige Zusätze für Toxizitätstests mit permanenten Fischzellinien, um auf diese Weise ein adäquates Bioaktivierungspotential sicherzustellen.

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