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# Basal Level and Induction of Cytochrome P450, EROD, UDPGT, and GST Activities in Carp (*Cyprinus carpio*) Immune Organs (Spleen and Head Kidney)

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Many vertebrate cytochromes P450 have an essential role in the primary or phase I metabolism of lipophilic xenobiotics. Numerous studies have reported the induction of cytochrome P450 in fish by many pollutants such as 3-methylcholanthrene (Kleinow et al. 1987). The Polynuclear Aromatic Hydrocarbon (PAH) inducible fish cytochromes P450 are homologous to mammalian cytochrome CYP 1Al. Induction has been detected by measurement of catalytic activity : ethoxyresorufin 0-deethylase (EROD). Biotransformation of many xenobiotics involves conjugation reactions (phase II enzymes) catalysed by a variety of transferases, for example UDP-glucuronosyltransferases (UDPGT) and glutathione S-transferase (GST). Induction of phase II enzymes by PAH has also been demonstrated in numerous piscine species (Kleinow et al. 1987). As in mammmals, the major organ involved in xenobiotic metabolism in fish seems to be the liver. However, few studies have show presence of biotransformation activity in organs other than liver. P450 1A protein is induced in many fish organs: heart, gill, kidney, ovary, testis and brain (Stegeman at al. 1991); and phase II enzyme activity has been characterised in kidney for UDPGT (Pesonen and Andersson 1987; Pangrekar and Sikka 1992) and in kidney (Nimmo and Spalding 1985) and gills (Gallagher et al. 1992) for GST. However, no detailed study is available on the presence and activity of biotransformation enzymes in immune organs of fish species.

Keeping in view the paucity of information on this biotransformation system, it was thought worthwhile to investigate the detoxication capacity of two main immune organs, the spleen and head kidney, in comparison with liver, the major organ of detoxication in fish. Hence, we have measured cytochrome P450 levels and EROD, UDPGT, and GST activities in carp exposed to a prototypical CYP 1A family inducer, 3-methylcholanthrene.

## MATERIALS AND METHODS

Juvenile carp (Cyprinus carpio) were obtained from local commercial farms. During experimentation, carp were kept in 15 liter glass aquaria. Water temperature (20°C), oxygen concentration (80-90%), pH (7.95-8.05) and

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**Table 1.** Statistical analysis of mean of cytochrome P450 content, EROD, UDPGT and GST activities of carp administred 3-MC at dose of 40 mg/kg; in liver, in spleen and head kidney. Control received carrier alone.

| | EROD (pmoles/min/mg) | P450 (nmoles/mg) | UDPGT (nmoles/min/mg) | GST (nmoles/min/mg) |
|---|---|---|---|---|
| Control | 7.10±1.48 | 0.384±0.043 | 2.33±1.16 | 209±67 |
| **LIVER** (induction factor) | **(43.13±10.5)** | **(2.42±0.86)** | **(7.63±1.56)** | **(4.24±2.13)** |
| Test | 306.27±80.5*** | 0.93±0.07*** | 17.78±7.92* | 888±134*** |
| Control | 2.73±0.86 | 0.475±0.06 | 3.27±0.70 | 117±32 |
| **SPLEEN** (induction factor) | **(2.64±1.2)** | **(1.55±0.5)** | **(2.22±1)** | **(1.92±0.23)** |
| Test | 7.22±1.31* | 0.738±0.17* | 7.28±1.06* | 225±28** |
| Control | 1.71±0.23 | 0.349±0.056 | 1.89±1.68 | 264±41 |
| (induction factor) **HEAD KIDNEY** | **(9.41±3.2)** | **(1.58±0.4)** | **(5.85±1.1)** | **(1.54±0.25)** |
| Test | 16.10±6.65** | 0.554±0.22* | 11.07±3.71 | 407±29** |

Values are the mean of five experiments ± SEM. The statistical significance of data was evaluated using the non parametric test of Mann and Whitney. (*) marginally significant different ($p<0.05$) (**) significant difference ($p<0.01$) and (***) highly significant difference ($p<0.001$) from non treated sample. The values presented the induction factor ± SEM of cytochrome P450 content and enzymatic activities were obtained in comparaison to control animals.

photoperiod 12h-12h were maintained during the acclimation and exposure periods.

Fish received a single intraperitoneal injection of 3-MC dissolved in corn oil at 40 mg/kg body weight at day 0. Fish receiving corn oil alone were used as controls. At day 3, fish were killed by a blow to the head and the liver, spleen and head kidney were quickly removed and washed in ice-cold 0.15 mM KCl.

Liver, spleen and head kidney were homogenized in 250 mM sucrose, 10 mM HEPES, 1mM EDTA, 1 mM PMSF and DTT, pH 7.4 using a motor driven Ultra turax glass teflon homogenizer (Ika labartechnik). The homogenate was centrifuged at 10000 xg for 20 min at 4 0°C and the resulting supernatant was ultracentrifuged at 106000xg for 1 hr at 4°C. The resulting supernatant containing cytosol fractions was stored at -80 °C for further analysis of GST activity. The microsomal pellet was resuspended in buffer containing 20 % of glycerol (w/v).

Microsomal suspensions were examined for cytochrome P450, UDPGT andEROD activities daily.

Ethoxyresorufin O-deethylase (EROD) activity was determined according to Burke and Mayer (1974). Assays were carried out at 37°C in a final volume of 1 mL phosphate buffer (200mM $Na_2HPO_4$ 50 mM $KH_2PO_4$, pH 7.4) containig 2.5 mM G-6-P; 0.25 mM NADP ; 1U / mL G-6-P dehydrogenase. The substrate concentration (7-ethoxyresorufin) that allows an optimal enzymatic reaction under saturation conditions was 2.7µM. Reaction was stopped by addition of 2 ml acetone. Fluorescence (excitation: 537 nm, emission: 583 nm) values were recorded by Kontron spectrofluorimeter using rhodamine B as an external standard. Values were expressed as picomoles of resorufin produced per minute per mg protein( pmole/min/mg ).

Cytochrome P450 content was analysed according to the method of Matsubara et al. (1976). Microsomal fractions were diluted in phosphate buffer (50 mM $KH_2PO_4$, 50 mM $K_2HPO_4$, pH,7.4) containing 0.1 mM EDTA, 0.1 mM PMSF and DTT. Samples were bubbled with CO and the CO difference binding spectrum was scanned between 400 and 500 nm. For spleen and head kidney, contamination by reduced hemoglobin was taken into consideration by bubbling control and test samples with carbon monoxide and adding dithionite only to the assay sample. The cytochrome P450 concentration was determined from the absorbance differences (450-490 nm) using an extinction coefficient of 91 mM/cm and was expressed as mnoles of cytochrome P450 /mg protein.

UDP-glucuronosyltransferase (UDPGT) activity was determined using 4-nitrophenol. Before treatment microsome proteins were treated with 0.02% triton X-100 at 4°C during 30 min. Incubation was carried out in a mixture containing 550mM Tris-HCl buffer, pH 7.4; 1.8 mM 4-nitrophenol; 12.7 mM UDPGA. The incubation was started by adding microsomes (400µg protein) and lasted for 30 min. at 37°C. The reaction was stopped by adding trichloroacetic acid and samples were centrifuged 5 min at 2000xg. The pH of the supernatant was alkalinized by adding NaOH (0.5 M final concentration) and absorbance was measured at 400 nm. Values are expressed as nmoles/min/mg.

GST activity was measured according to Habig et al (1974) using 1-chloro -2,4 - dinitrobenzene as substrate. The assay was carried out in 2.8 ml of 0.1 M phosphate buffer, pH 6.9, 3 mM 1-chloro-2,4-dinitrobenzene and 3 mM glutathione and the reaction was started by the addition of 200 µg cytosolic protein. The increase in absorbance at 340 nm was recorded at 30°C for 1 min. An extinction coefficient of 9.6 mM/cm was used for calculations, and the activity was expressed in nmoles/min/mg.

Microsomal and cytosolic protein concentrations were determined by the colorometric method using bovine albumin as a standard. We calculated Km and Vmax values by the Wilkinson non graphic method using a Enzpack software (Biosoft) which also gives the standard error deviation of kinetic constants.

## RESULTS AND DISCUSSION

First, in our laboratory we have characterized cytochrome P450 1A activity by determining Michaelis-Menten constants (Km and Vmax) in liver, spleen and

**Table 2.** Michaelis-Menten constants of UDPGT determined by Wilkinson method.

| Organs | Km (mM) | Vmax (nmoles/min/mg) |
|---|---|---|
| Liver | 0.32 ± 0.12 | 11.1 ± 3.8 |
| Spleen | 0.305 ± 0.14 | 8.5 ± 1.35 |
| Head Kidney | 0.25 ± 0.08 | 7.7 ± 2.1 |

The saturation experiment used different concentrations of substrate . Each value is the mean of the three experiments ± SEM.

head kidney of carp (Marionnet et al. 1997). In our study, cytochrome P450 is present in immune organs i.e. spleen and head kidney (Table 1). Moreover, we measured cytochrome P450 dependent activity: EROD activity in all three organs. But this activity was lesser in the immune organs than in liver. UDPGT is a membrane-enzyme deeply embedded in membrane lipids.

This activity was measured in microsomal fraction using triton X-100 as a detergent. First, we calculated the Michaelis-Menten constants (Km and Vmax) in order to determine the affinity of UDPGT for the substrate 4-nitrophenol. Considering the standard error of Km and Vmax values for different organs, we did not observe a significant difference in enzyme activity (Table 2), demonstrating that for optimal assays we can use the substrate at concentrations five fold the Km values (1.8 mM). UDPGT activity was 1.89 nmoles/min/mg and 3.27 nmoles/min/mg for head kidney and spleen respectively (Table 1). We found a comparative activity in liver: 2.33 nmoles/min/mg . Some authors have reported extra hepatic UDPGT activity: in rainbow trout kidney (Pesonen et al., 1987); and gills and intestines of *Pleuronectes platena* (Clarke et al. 1988) but no data has been reported for immune organs in fish.

As for UDPGT we determined the Km and Vmax values of GST activity in cytosolic fraction (Table 3). The apparent Km was 0.61, 0.39 and 0.55 mM for CDNB in liver, spleen and head kidney respectively. One study used the same experimental conditions (substrate, temperature, time of incubation) for trout liver and described the presence of 6-7 isoforms of GST exibiting values in the range of 0.4-0.5mM for CDNB (Rammage and Nimmo 1984). For optimal assay conditions we used CDNB at concentrations five fold the Km values (3 mM) for all three organs.GST is present in spleen and head kidney with activity of 117 and 264 nmoles/min/mg respectively. It's identical that's found in liver: 209 nmoles/min/mg. This activity has been reported in extra hepatic organs at different levels as follows: in trout kidney, 350 and 385 nmoles/min/mg

**TABLE 3:** These values presented Michaelis constants of UDPGT determined by Wilkinson method.

| Organs | Km (mM) | Vmax (μmoles/min/mg) |
|---|---|---|
| Liver | 0.617 ± 0.111 | 0.30 ± 0.017 |
| Spleen | 0.392 ± 0.133 | 0.25 ± 0.015 |
| Head Kidney | 0.555 ± 0.159 | 0.23 ± 0.026 |

The saturation experiment using different concentrations of substrate . Each value is the mean of the three experiments ± SEM.

(Nimmo and Spalding 1985; Pangrekar et al. 1992) in rainbow trout olfactory mucosa, 477 nmoles/min/mg (Starcenic 1995).

We evaluated the biotransformation capacity of immune organs in comparison to liver after an exposition with a PAH: the 3-methylcholanthrene. 3-MC treatment induces EROD activity in liver. This is in accordance with our previous results for four injections of 3-MC at 30 mg/kg/day during 4 days (Marionnet et al. 1997). 3-MC induced EROD activity in immune organs with an lower induction factor than for liver. Moreover, we noticed an induction of cytochromes in liver and immune organs with an lower increasing than for hepatic organs. After 3-MC exposure induction of UDPGT activity was found in immune organs with an enhansement factor of 2.2 and 5.8 for spleen and head kidney respectively (Table 1). These values were lower than in liver; head kidney seems to be the most important immune organ implicated in this conjugating enzyme. Little attention has been directed towards the induction process of UDPGT in extrahepatic tissue.

Our results found induction of GST activity by factors of 4.24, 1.92 and 1.54 for liver, spleen and head kidney (Table 1). Induced levels between spleen and head kidney were comparable and lower in comparison to liver. GST induction has been reported in i.e. kidney (Pangrekar and Sikka 1992) but our results are the first characterizing induction of this enzyme activity in fish immune organs. Activities of conjugating enzymes have been poorly described for extrahepatic organs in the literature; our results establish that UDPGT and GST activities are inducible in spleen and head kidney.

The major organ involved in xenobiotic metabolism in fish is the liver. Spleen and head kidney are immune organs in fish implicated in the integrity of the organism. Our study suggests that these two organs can also contribute to the biotransformation process for the elimination of xenobiotics.

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## REFERENCES

Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inductible by 3-methylcholanthrene. Drug Metab Disp2: 583-588

Clarke DJ, Burchell B (1988) Characterisation and molecular analysis of hepatic microsomal UDP-glucuronosyltransferase in the marine fish, *Pleuronectes platessa.* Mar Environ Res 24: 105-109

Gallagher EP, Canada AT., Di Giulio RT (1992) The protective role of glutathione in chlorothalonil induced toxicity to channel catfish. Aquat Toxicol 23: 155-168

Habig WH, Pabst MJ, Jakoky WB (1974) Glutathione S-transferase the first step in mercapturic acid formation J Biol Chem 249: 7130-7139

Kleinow KM (1987) Biotransformation and induction: implication for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environ Health Perspect 71: 105-119

Marionnet D , Taysse L, Chambras C, Deschaux P (1997) 3-methylcholanthrene induced EROD activity and cytochrome P450 levels in liver, spleen and head kidney of a cyprinid fish (Cyprinus carpio). Comp Bioch Physiol 118C. In Press

Matsubara T, Koile M, Touchi, AY ,Tochini A, Sugeno K. (1976) Quantitative determination of cytochrome P450 in rat liver homogenate. Anal Biochem 75: 596-603

Nimmo IA, Spalding. CM (1985) The glutathione S-transferase activity in the kidney of rainbow trout (Salmo gairdeneri). Comp Biochem Physiol 82B: 91-94

Pangrekar J, Sikka HC (1992) Xenobiotic metabolizing enzyme activity in the liver and kidney of the brown bullhead (*Ictalurus nebulosus).* Marine Environ Research 34: 287-291

Pesonen M, Andersson T (1987) Subcellular localization and properties of cytochrome P450 and UDP glucuronosyltransferase in the rainbow trout kidney. Biochem Pharmacol 6: 823-829

Rammage PIN, Nimmo IA (1984) The substrate specificities and subnit compositions of the hepatic glutathione S-transferase of rainbow trout (*Salmo gairdneri*). Comp Biochem Physiol 78B: 189-194

Starcenic S L, Zienlinski BS (1995) Immunohistochemical localization of glutathione S-transferase pi in rainbow trout olfactory receptor neurons, NeurosLetter, 183: 175-180

Stegeman JJ,.Somolowitz RM,. Hahn ME (1991) Immunohistochemical localization of environmentally induced Cytochrome P450 1A1 in multiple organs of the marine *teleost Stenotomus chrysops* (scup). Toxicol and applied Pharmacol 110: 486-504.