EFFECT OF HEAVY METALS ON HEPATIC CYTOCHROME P450-DEPENDENT ENZYME SYSTEM OF FRESHWATER FISH SPECIES

Thesis of Ph.D. dissertation

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2009

INTRODUCTION

Large amounts of substances harmful to the living world such as various heavy metals and derivatives are discharged into our living waters resulting in serious damages in the aquatic ecosystem. When released into the air, soil, groundwater and surface waters heavy metals and their compounds may damage the members of the ecosystem depending on concentration and duration of exposure and due to their accumulation these substances may cause toxic effects indirectly through the food chain. The rate of accumulation significantly depends on external environmental factors, the internal physiological condition of the body, the habits of living organism and its place in the food chain. The stress caused by the aquatic environment triggers complex cellular reactions in living organisms. The effect of foreign compounds entering into the cells primarily manifests in their reactions with macromolecules (proteins, lipids, nucleic acids) leading to changes in the molecular processes of the cells. Initially such processes can be reversible and are easily detectible based on the change in biological activist.

Biodegradation processes in fish are less known since the pharmacological and toxicological research is primarily limited to mammalians. In an aquatic environment increasingly contaminated with chemical pollutants fish are highly exposed directly and/or indirectly to the effects of chemical pollutants; thus, they are sensitive indicators of the condition of aquatic ecosystems. They are in constant and close contact with water and the toxic compounds in the water through their entire body surface and gill and thereby these foreign materials may be accumulated in their body directly or indirectly or through their feed.

Like with all living organism, the body of fish has a quite conserved protein systems providing molecular defence to ensure metabolism and elimination of foreign material. The main region of metabolism of pollutant entering into the fish body is the liver. Due to biotransformation processes these substances are

converted to other compounds that can be eliminated from the body thereby becoming less toxic either. The hepatic detoxifying enzymes, the cytochrome P450-dependent mixed function monooxygenases have important role in this process. P450 enzyme activity in the body is not constant, foreign substances, xenobiotics or some endogenous regulating molecules are able increase (inducers) or decrease (inhibitors) the activity of P450 enzymes.

Although the cytochrome P450 enzyme induction alone is not sufficient to identify the nature of pollutants, in addition to traditional analytical methods it is a suitable method to detect deterioration of water quality. Since continuous exposure with high concentrations often leads to desolation of individual animals or some more sensitive species, and changes in life communities for long term, it is very important to assess the ecological condition of our waters.

Numerous literature data are available on biodegradation processes occurring in the body of various seewater fish species, but the hepatic detoxifying cytochrome P450-dependent monooxygenase system of freshwater fish is much less known. We have little knowledge on the toxic effects of heavy metals contaminating freshwaters, the metabolism of such compounds, differences of biotransformation processes among species as well as the effects associated with other environmental factors.

AIMS

In our work we analyzed *in vivo* and *in vitro* the effect of heavy metal ions - cadmium, copper and lead – on the hepatic detoxifying cytochrome P450 enzyme system of fish species resident in Hungarian living waters i.e. silver carp (*Hypophthalmichthys molitrix* V.), wels (*Silurus glanis* L.) and the omnivorous common carp (*Cyprinus carpio* L.).

Our objectives are summarized as follows:

- We tested *in vivo* and *in vitro* the effects of treatment with the most toxic heavy metal, cadmium ion on the hepatic detoxifying enzymes of common carp, silver carp and wels.
- We studied the effects of Cu^{2+} ion administered at various concentrations on the hepatic cytochrome P450 enzyme system of silver carp and wels.
- We tested *in vivo* and *in vitro* the effect of lead ion on the hepatic cytochrome P4501A isoenzymes of common carp.
- We compared the sensitivity of hepatic detoxifying enzymes of fish species with different nutritional needs after *in vitro* treatment with heavy metal ions.
- We tried to find out it infrared resonance spectroscopy is suitable for detection of protein structure damaging effect of heavy metals in fish hepatic microsome.

METHODS

Care and treatment of fish

The experiments were performed with silver carp (*Hypophthalmichthys molitrix* V.), wels (*Silurus glanis* L.) and the omnivorous common carp (*Cyprinus carpio* L.) from both genders. Two fish were stored in a 100 l in temperature controlled aquarium, aerated for 24 hours in tap water maintained at 16 ± 2˚C and 10 ± 2˚C.

In vivo treatments

Intraperitoneal (i. p.) treatment of fish:

- β-naphthoflavone: the CYP1A specific inducer was used at a dose of 50 mg kg^{-1} calculated for body weight. We used fish treated only with corn oil as control.

- I. p. treatments with heavy metal ions:

Cadmium treatment: a stock solution 1 mg mL^{-1} Cd^{2+} ion concentration was prepared from cadmium acetate. Common carp was treated with i. p. doses of 2 and 10 mg Cd^{2+} kg^{-1}. For short term treatment 24 hours following treatment with 10 mg Cd^{2+} kg^{-1} we treated the fish with 50 mg kg^{-1} β-naphthoflavone. For long term treatment on day 6 following treatment with cadmium ion (2 mg Cd^{2+} kg^{-1}) 50 mg kg^{-1} β-naphthoflavone was injected into the fish.

- Lead ion treatment: Common carp was treated with lead at a dose of 2 mg kg^{-1} calculated for Pb^{2+} ion.

Treatment of fish with heavy metal ions in water:

Silver carp and wells were treated with cadmium and copper in water. Cadmium ion concentration in the aquarium was 10 mg L^{-1}, and the copper ion concentration for silver carp was 1 mg L^{-1} and 10 mg L^{-1}, while for wels 10 mg L^{-1}. Control animals were stored in heavy metal free water in the same conditions.

In vitro experiments

The *in vitro* experiments were performed with microsome prepared from liver of fish treated with β-naphthoflavone. *In vitro* effect of Cd^{2+}, Cu^{2+} and Pb^{2+} ions was tested in the following concentration ranges: Cd^{2+} 0-4.0 μg mL^{-1}; Cu^{2+} 0-2.0 μg mL^{-1}; Pb^{2+} 0-4.0 μg mL^{-1}.

Prior to measurement of values for 50% enzyme inhibition the microsomes were pre-incubated for 10 minutes at 30°C with heavy metal solutions of various concentrations (Cd^{2+} 0-0.120 μg mL^{-1}; Cu^{2+} 0-1.0 μg mL^{-1}; Pb^{2+}0-4.0 μg mL^{-1}).

Microsome preparation

After sedation of fish the liver was taken out and the gallbladder removed. Liver tissue was disintegrated with scissors, washed three times with 0.1M phosphate buffer (pH 7.4) containing 0.15M potassium chloride. It was pre-homogenized in Potter and centrifuged at 10,000 g. Supernatant was centrifuged twice at 105,000 g at 4 ˚C. Precipitate was suspended and homogenized in 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol to obtain a microsome with protein content of approximately 20 mg mL^{-1}.

Measurement of cytochrome P450 content

It was determined by the measurement of absorption on the differentiated spectrum of microsome reduces with dithionite and saturated with CO at 450 nm.

Enzyme activities

The **EROD** (ethoxyresofurine-O-deethylase) isoenzyme activity was measured by fluorimetric analysis of resofurine (E_{ex}/E_{em}=540/5909) generated in the catalyzed reaction.

The **ECOD** (ethoxycoumarine-O-deethylase) isoenzyme activity was measured by fluorimetric analysis of the quantiy of hydorxycoumarine (E_{ex}/E_{em}=390/465) generated in the reaction.

The **APND** (aminopyrene-N-desmethylase) activity was measured by photometry. The obtained formaldehyde was determined by measurement of extinction at 412 nm with Nash reagent.

Record of FTIR spectra

Fourier transformation IR measurements were performed with Varian (Digilab) FTS-175 and FTS-60A IR spectrometers. These instruments were equipped with dynamically controlled interferometer, liquid nitrogen-cooled MCT (Mercury-Cadmium-Telluride) and Peltier-cooled DTGS (Deutero-Triglycine Sulphate) detector. Spectra were made by averaging 256 individual spectra and with 4 cm^{-1} spectral resolution. Liquid samples were measured by using windows made of silicon or KRS-5 (Thallium Bromo-Iodide TlBr-TlI) with optical lengths of 6 and 12 mm.

To detect the structural changes of proteins fist we measured the spectra of microsomes not treated with heavy metals (reference spectrum) and then the spectra of samples treated with heavy metal ions were measured. For measurement and evaluation of spectra double subtraction was used.

RESULTS

1. We tested the *in vivo* effect of Cd^{2+}, Cu^{2+} és Pb^{2+} ions on the biotransformation enzymes of fish. We have demonstrated that heavy metals induce not only CYP1A biotransformation enzymes but CYP2B and CYP3A isoenzymes as well and at high ion concentrations enzyme inhibition was observed.

2. We tested *in vitro* effect of Cd^{2+} ion on the activity of hepatic microsomal EROD isoenzyme of common carp. Metal ion inhibited the enzyme. We analysed the inhibition kinetic based on the substrate and metal ion concentration

dependence of the catalyzed reaction. When the cadmium concentration increased V_{max} value decreased gradually compared to the control, and K_M value was unchanged. It has been concluded that cadmium ion caused non-competitive inhibition. The inhibitor cadmium ion is likely to bind to protein and changes the spatial structure of protein and its active centre or reduce the rate of reaction by binding to the catalytic site.

3. For *in vitro* treatment with heavy metals it was observed that Cd^{2+}, Cu^{2+} and Pb^{2+} ions reduced cytochrome P450 content and the absorption maximum were shifted toward higher wavelengths indicating enzyme destruction.

4. *In vitro* results for 50% enzyme inhibition have shown that Cd^{2+} ion exerted inhibitory effects at various extents on the activity of three selected isoenzymes (EROD, ECOD and APND). It was demonstrated that ECOD isoenzyme was the most sensitive to the treatment with heavy metal ions for silver carp while APND has shown the same sensitivity in all three fish species. The carnivorous wels has shown to be the most sensitive while the omnivorous common carp has shown to be the less sensitive.

5. In testing the *in vitro* effect of Cu^{2+} and Pb^{2+} ions the values for 50% enzyme inhibition were measured. ECOD isoenzyme reacted more sensitively to the effect of Cu^{2+} ion compared to EROD isoenzyme. The rate of inhibition by EROD isoenzyme based on the obtained result is as follows: wels < common carp < silver carp. Pb^{2+} ion has shown similar inhibitory effect at high concentration ranges. EROD isoenzyme changed in the most sensitive common carp, while the ECOD isoenzyme changed the least in silver carp.

6. Experimental results have also demonstrated that heavy metals exert various rate of inhibition on the activity of cytochrome P450-dependent monooxygenase enzymes. We compared the rate of inhibition and Cd^{2+} has shown to be the most toxic and Pb^{2+} the less toxic in the tested concentration range. The order of inhibitory effect of the tested ions is as follows: Cd^{2+}>Cu^{2+}>Pb^{2+}.

7. We tested the effect of heavy metals on the protein structures by measurement of FTIR spectra of hepatic microsomes. The spectral changes we detected confirmed the toxicity of heavy metals and indicated detectable harmful effect on the protein structure. Disappearance of α-helix structure and appearance of β-lamellar structure suggest modification of the native enzyme structure. We have confirmed that Fourier transformed IR resonance spectroscopy is suitable for detection of changes in protein structure induced by heavy metals.

In conclusion, the difference changes in enzyme activity and cytochrome P450 content induced by the various heavy metal treatments may also be explained by the living and feeding habits of individual fish species. It is well known that the greatest pollution in living waters can be measured at the bottom of the water in the mud. The carnivorous fish (wels) spends most of their life in this zone; they get their feed from this area thereby this fish species is exposed to the environmental pollution in the greatest extent. The omnivorous fish species (common carp) lives in a less exposed environment, while the herbivorous fish species (silver carp) is exposed to a relatively low hazardous substance concentration due to its habits. EROD isoenzyme specifically indicates only CYP1A induction while the increase in ECOD isoenzyme activity is an indicator of CYP1A and CYP2B induction, and APND enzyme activity is an indicator of CYP2B and CYP3A induction. Considering that cytochrome P450 enzymes are

located in the ER membrane, change in the micro-environment cannot be excluded as a possible reason for the decrease in enzyme activity meanwhile lipid-protein reaction protects proteins against further denaturation. Enzyme activity may be decreased for several reasons including oxidation processes, damage of the membrane structure, reaction of enzyme and inhibitor, protein destruction.

The results of our experiments has demonstrated that heavy metal ions react with cytochrome P450-dependent enzyme system of fish species leading to changes in conformation of protein structure. They result in inhibition of activity of enzymes involved in biotransformation, decrease in detoxifying enzymes. In conclusion, the difference changes in enzyme activity and cytochrome P450 content induced by the various heavy metal treatments may also be explained by the living and feeding habits of individual fish species.

PUBLICATIONS

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