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Detection and Induction of Cytochrome *P*450IA (CYP 1A)-Like Proteins in *Julus scandinavius* (Diplopoda) and *Oniscus asellus* (Isopoda): A First Analysis

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Based on numerous studies on the cytochrome P450 system in vertebrates (e.g. Nebert & Jones 1989; Stegeman 1989; Nebert et al. 1991, Goksøyr & Förlin 1992), the induction of cytochrome P450 monooxygenases has been accepted as an established biomarker of sublethal xenobiotic stress (for reviews see Hahn & Stegeman 1994, Bucheli & Fent 1995). In this context, numerous polycyclic aromatic hydrocarbons (PAH), dioxins, furans, and a variety of other organics have been shown to be potent inducers of CYP 1A genes, depending on the substance-specific affinity to the intracellular Ah-receptor (Goksøyr & Förlin 1992; Stegeman & Hahn 1994). However, the degree of this response is not only controlled by binding affinities, but also by a wide spectrum of other abiotic and biotic factors such as temperature, sex, and moulting cycle which may result in divergent responses of individuals exposed to cytochrome P450I-inducing chemicals (e.g. Stegeman & Kloepper-Sams 1987; Goksøyr & Förlin 1992; Braunbeck et al. 1996). In contrast, the current knowledge on organisation and distribution of cytochrome P450 in invertebrates, especially in soil invertebrates, is very limited. Thus, the question whether the activity of this system may serve as a biomarker of soil contamination is still in debate.

For invertebrates, comprehensive data on CYP are only available for a few annelids (Berghout et al. 1991), molluscs (Kirchin et al. 1992), insects (Waters et al. 1992), crustaceans (James 1989), and some echinoderms (Payne 1977), which have been reviewed by Lee (1981) and Livingstone (1990). Studies focussing on cytochrome P450-related activities in soil invertebrates are particularly rare (Berghout et al. 1991; Triebskom 1991) and, to the best of our knowledge, the present investigation of the cytochrome P-450 system in the diplopod *Julus scandinavius* and the isopod *Oniscus asellus* represents the first analysis of CYP 1A induction in this important group of leaf litter decomposers. This study has been designed (1) to reveal the presence of the cytochrome P-450 system in diplopods and isopods, (2) to investigate the induction potential in comparison to vertebrates, and (3) to discuss further perspectives of this

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biomarker in ecotoxicological research with soil invertebrates.

MATERIALS AND METHODS

Adult *Julus scandinavius* Latzel, 1884, and *Oniscus asellus* Latreille, 1758, were collected from almost uncontaminated forest sites (near the cities of Hokenheim and Mauer (no artificial contamination with organics, soil metal content < 0.5 mg/kg Cd, <25 mg/kg Pb, < 50 mg/kg Zn)) near Heidelberg (Germany). For laboratory tests, the animals were kept at 9 °C (diplopods) or 15 °C (isopods) in plastic boxes (11.5 x 11.5 x 4 cm) on moistened gypsum. For exposure, 2-15 individuals per box were fed leaf litter material containing 7.5 g ß-naphthoflavone (ßNF)/kg dry wt. (2 g leaf litter were soaked with 15 mg ßNF in 10 ml acetone), a potent inducer of the cytochrome P450 system (Goksøyr & Förlin 1992). Control food was moistened with tap water. Exposure time was 5 days.

The low individual body mass of diplopods and isopods in combination with the restricted sensitivity of the analytical assays necessitated pooling of all exposed specimens into a single sample for immunological and enzymatical analysis. Thus, for each species and condition, 10 to 15 specimens formed one pool for enzymatical analysis, and the pooled samples analysed by immunoblotting consisted of 2 to 5 specimens (Table 1). Although no variance of the results could be given in this case, the results obtained for the respective pools represent the mean for all specimens within one pool and at least give a tendentcious overview.

According to their mass, pooled samples of 2 to 5 animals were manually homogenized in a buffer solution (potassium acetate 80 mM, magnesium acetate 4 mM, Hepes 20 mM, pH 7.5) in an ice bath and centrifuged at 20,000 g for 15 min at +3°C. Total protein content of the supernatants was determined according to Bradford (1976). Constant protein weights (100 µg per lane for diplopods, 125 µg for isopods) were separated by SDS-PAGE (12% acrylamide, 0.1% bisacrylamide (w/v), total 1.5 hr, 15 mA in the stacking gel, 25 mA in the separation gel), transferred to nitrocellulose (30 min at 100 mA) and the filters blocked for 2 hr at 20 °C in 0.15 M PBS (137 mM NaCl, 2,68 mM KC1, 1.66 mM $NaH_2PO_4{}^xH_2O$, 9.58 mM $Na_2HPO_4{}^xH_2O$, pH 7.4) in 10% NCS (newborn-calf-serum) and 0.05% Tween 20. Subsequently, the nitrocellulose filter was incubated for 2 hr at 20 °C in primary antibody solution (mouse anticod CYP1Al IgG MAb NP-7; Terra, Bergen, Norway; 1:1,000 in 0.15 M PBS buffer solution containing 1% NCS) and then washed in PBS. The antibody was detected with protein A gold *(Staphylococcus aureus)* (1:30 in 0.15 M PBS containing 1% NCS, pH 7.4) by overnight incubation at 20 °C. Subsequently, a silver staining procedure was conducted (Aurion R-gent developer, Aurion G-gent enhancer, dilution 1:1). Grey values of the Western blot protein bands were quantified by densitometric image analysis (Cybertech CS-1, version 1.20, Berlin, FRG) after background subtraction. Grey values of the control bands

were set to 1.0, as a standard reference for the respective data obtained for the exposed animals.

For enzymatic analysis pools of 10 diplopods (15 isopods) per test condition were homogenized manually in 4 mL (diplopods) or 2.5 mL (isopods) of homogenization buffer (50 mM Tris, 0.25 M saccharose, 2 mM EDTA, 150 mM KCl, 1 mM dithioerythritol (DTE), 0.25 mM phenyl-methanesulfonate (PMSF, pH 7.6). Homogenates of each pool were centrifuged at 8,000 g for 10 min, 20,000 g for 20 min and 105,000 g for 90 min at 4 °C. The microsome pellet was washed in homogenization buffer solution lacking saccharose for 45 min at 105,000 g at 4 °C, resolved in 125 µL 50 mM microsome buffer solution (50 mM K_2PO_4, 20% glycerin, 1 mM EDTA 0.1 mM DTE, pH 7.4), and aliquots of 30 µl were frozen in liquid nitrogen and stored at -80°C for at most 2 days. Protein concentrations were determined according to Bradford (1976).

For analysis of ethoxycoumarin-O-deethylase (ECOD, E.C. 1.14.14.1), 10, 20, 40 or 80 µL 0.2 mM 7-hydroxycoumarin in 0.1 M glycine buffer (pH 10.4) were topped up to 2 mL with glycine buffer solution and used as references. For sample analysis, 12.5 µg (diplopods) or 150 µg (isopods) of microsomal protein were diluted with 200 µL 0.1 M Tris buffer (pH 7.5) and 200 µL of a solution of 1 mM 7-ethoxycoumarin in two times destilled water. To start the catalytic reaction, 50 µL of 2 mM NADPH in a 0.1 M Tris buffer solution (pH 7.5) were added and incubated at 25 °C for 16 min. Blanks contained 50 µL Tris buffer solution lacking the microsome fraction. The catalytic activity was stopped by addition of 300 µL 10% trichloroacetic acid, and samples were centrifuged at 8,000 g at 4 °C. Subsequently, 100 µL of the supernatant were diluted with 2.9 mL of 0.1 M glycine buffer, pH 10.4, and the concentration of hydroxycoumarin was measured at 25 °C at a excitation wavelength of 375 nm and at an emission wavelength of 455 nm (Spectrometer LS 50, Perkin Elmer). The enzymatic activity was calculated in µU and related to the total microsomal protein.

RESULTS AND DISCUSSIONS

Protein A gold staining in the Western blot revealed a protein band of approximately 61 kDa (diplopods) or 62 kDa (isopods) in control and ßNF-treated animals. In diplopods, the intensity of this band, however, differed strikingly between exposed and control animals and, hence, image analysis revealed an increase to 780% of control intensity for the ßNF-exposed animals. The isopods exhibited an induction to 169% of controls in reponse to ßNF. The cross-reactivity of the anti-cod antibody with arthropod proteins in combination with the faint background of the blot and the size of these proteins indicated specifity of antibody binding and suggested structural similarities between the vertebrate CYP 1A group and the stained invertebrate proteins which, hence,

will be called CYP lA-like (Fig. 1). Likewise, catalytic ECOD activities of ßNF-exposed diplopods increased to 335% in comparison to controls. Mirroring the faint induction of the CYP lA-like proteins, isopods showed only a 1.23-fold ECOD induction in response to ßNF (Tab. 1).

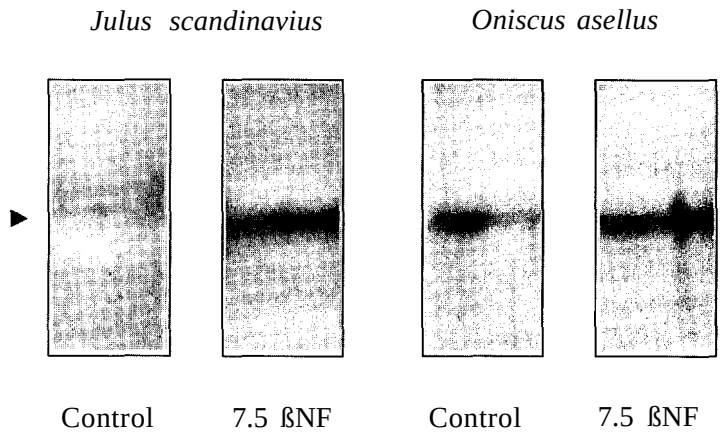

Figure 1. Immunoblots detecting CYP 1A-like proteins (arrow) in diplopods *(Julus Scandinavius)* and isopods *(Oniscus asellus)* under control conditions and in ßNF-treated animals. ßNF concentrations in g/kg dry wt.

Table 1. Induction of CYP lA-like gene products and ECOD activity in ßNF-treated *Julus scandinavius (J.s.)* and *Oniscus asellus (O.a.).*

	CYP lA[1]		ECOD[2]	
Condition	*J.s.*	*O. a.*	*J.s.*	*O.a.*
Control	1.00 (n=5)	1.00 (n=2)	0.59 (n=lO)	11.53 (n=15)
7.5 ßNF[3]	7.80 (n=5)	1.69 (n=3)	1.96 (n=lO)	14.11 (n=15)

[1] Relative grey value of protein bands in the Western blot,
[2] mU/mg microsomal protein,
[3] g/kg dry wt., n=number of pooled individuals.

The present study provides evidence of the presence and induction of the cytochrome P450 system in diplopods and isopods, both representing important groups of terrestrial soil invertebrates. As mentioned by numerous authors (e.g. Förlin & Celander 1993; Stegeman & Kloepper-Sams 1987) the polycyclic aromatic hydrocarbon, ß-naphthoflavone, represents one of the most efficient activating agents of the cytochrome P450 system in vertebrates. In the present study, the induction of a CYP lA-like protein and enzymatic activity of ECOD by ßNF indicated a similar mode of induction in both arthropods.

CYP lA-like proteins and ECOD were more strikingly induced in the diplopod *J. scandinavius than* in *O. asellus.* Although the presence of an Ah-receptor has not been proved for these soil organisms, it seems likely that the variable

potential of the different species to increase their CYP lA-like activity is due to interactions of the inducing agent and an Ah-analogous protein or differential uptake and/or excretion kinetics. The size of the CYP 1A-like gene products determined in diplopods and isopods corresponds to similar vertebrate proteins (Goksøyr and Förlin 1992; Förlin & Celander 1993) and the cross-reactivity with antibodies raised against cod CYP 1A1 also revealed structural similarities to vertebrate cytochromes.

With respect to ecotoxicological routine testing, the induction of the cytochrome lA-like subfamily in diplopods and isopods seems only conditionally suitable as a biomarker for xenobiotic soil contamination. In general, cytochrome P450-related enzyme activities in both investigated soil organisms are very low compared to vertebrate samples (e. g. Jedamski-Grymlas et al. 1992) and, accordingly, necessitates pooling of samples. For routine monitoring, therefore, very high numbers of test specimens would be required for adequate statistical analysis. As shown by the present study, however, even in pools of 15 specimens, stressor-induced catalytic activities of ECOD (which may have been further decreased by sample freezing) are hard to be distinguished from background values. Thus, the used catalytic assay for ECOD in diplopods and isopods cannot be recommended as routine biotests for xenobiotic stress, so far. In contrast, immunological assays detecting elevated levels of the CYP lA-like proteins appear more promising. Based on the present results, immunoblot studies revealed clear signals in pools of specimens; but should additionally render individual analyses of single specimens possible. Prior to the future use of the CYP lA-like proteins as markers in monitoring studies, however, their identity should be established by sequence analysis and the specifity for their induction should be further determined to reveal similarities and/or differences to the vertebrate cytochrome P450 system and its mode of induction.

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REFERENCES

Berghout AGRV, Wenzel E, Buld J, Netter KJ (1991) Isolation, partial-purification and characterization of the cytochrome P-450 dependent monooxygenase system from the midgut of the earthworm *(Lumbricus terrestris).* Comp Biochem Physiol C 100:389-369

Bucheli TD, Fent K (1995) Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit Rev Environ Sci

Technol 25:201-268
Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254
Braunbeck T, Hauck C, Scholz S, Segner H (1996) Mixed function oxygenates in cultured fish cells: contributions of *in vitro studies* to the understanding of MFO induction. Z angew Zool, in press
Förlin L, Celander M (1993) Induction of cytochrome P450IA in teleosts: environmental monitoring in swedish fresh, brackish and marine waters. Aquat Toxico1 36:41-56
Goksøyr A, Förlin L (1992) The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. Aquat Toxicol 22:287-312
Hahn ME, Stegeman JJ (1994) Regulation of cytochrome p450IA1 in teleosts: sustained induction of CYPIA1, mRNA, protein and catalytic activity by 2,3,7,8-Tetrachlorodibenzofuran in the marine fish *Stenotomus chrysops.* Toxicol Appl Pharmacol 127(2): 187-198
James MO (1989) Cytochrome p450 monooxygenases in crustaceans. Xenobiotica 19:1063-1076
Jedamski-Grymlas J, Siebers D, Krabe L (1992) Investigation on the cytochrome P-450 system of golden ide *(Leuciscus idus melanotus* L.). In: Braunbeck T, Hanke W, Segner H (eds) Fish in Ecotoxicology and Ecophysiology. VCH, Weinheim, FRG pp, 29-3
Kirchin MA, Wiseman A, Livingstone DR (1992) Seasonal and sex variation in the mixed-functioned oxygenase system of digestive gland microsomes of the common mussel, *Mytilus edulis* L. Comp Biochem Physiol 10 lC: 81-91
Lee RF (1981) Mixed function oxygenates (MFO) in marine invertebrates. Mar Biol Lett 2:87-105
Livingstone DR (1990) Cytochrome P-450 and oxidative metabolism in invertebrates. Biochem Soc Trans 18: 15-19
Nebert DW, Jones JE (1989) Regulation of mammalian cytochrome p450 (CYPIA1) gene. Int J Biochem 21:243-252
Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez F, Guengerich FJ, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, Waxman DJ (1991) The P-450 superfamily: update on new sequence, gene mapping and recommended nomenclature. DNA Cell Biol 10: 1-14
Payne JF (1977) Mixed function oxydases in marine organisms in relation to petroleum hydrocarbon metabolism and detection. Mar Pollut Bull 8: 112-116
Stegeman JJ (1989) Cytochrome P450 forms in fish: catalytic, immunological and sequence similarities. Xenobiotica 19: 1093-1110
Stegeman JJ, Kloepper-Sams PJ (1987) Cytochrome P-450 isoenzymes and monooxygenase activity in aquatic animals. Environ Health Perspect 71: 87-95
Stegeman JJ, Hahn ME (1994): Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cyto-

chrome P450 in aquatic species. In: Malins DC, Ostrander GK (eds) Aquatic toxicology, biochemical and cellular perspectives. Lewis Publ., Boca Raton, Ann Arbor, London, Tokyo, 87-206

Triebskorn R (1991) The impact of molluscicides on enzyme activities in the hepatopancreas of *Deroceras reticulatum* (Müller). Malacologia 33:255-272

Waters LC, Zelhof AC, Shaw BJ, Chang LY (1992) Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila.* Proc Natl Acad Sci USA 89: 4855