TISSUE DISPOSITION AND CYP 1A GENE EXPRESSION IN FISH EXPOSED TO INDIGO VIA AMBIENT WATER

Kai Gao*, Maria E. Jönsson, Ingvar Brandt

Department of Environmental Toxicology, Uppsala University, Norbyvägen 18A, SE-752 36 Uppsala, Sweden
Kai.gao@ebc.uu.se

Introduction

Indigo is a classic dye used for fabrics. Based on an annual production of about 5 billion pairs of blue jeans, about 50 000 tonnes of indigo will be required. Large amounts of indigo may be released with the wastewater from the dyeing process and from the washing of fabrics. Due to the low water solubility, indigo can reside in the effluent of secondary sludge, which makes it resistant to sewage treatment degradation. Indigo is also utilized as an ingredient in Chinese traditional medicine (Danggui Longhui Wan) intended for treatment of chronic myelocytic leukemia (Hoessel et al., 1999, Xiao et al., 2002). Along with the isomer indigorubin, indigo has been isolated from human urine following hydrolysis, and both compounds are potent activators of the aryl hydrocarbon receptor (AhR) in a yeast-based system (Adachi et al., 2001, Guengerich et al., 2004). The information on the tissue distribution, metabolic degradation, and excretion of indigo from the body is, however, very limited.

In a previous study we observed that indigo is an efficient inducer of gill EROD activity in rainbow trout gill filaments following water-borne exposure in vivo (Jönsson et al., 2006). In the present study we aimed to determine the pattern of induction of various CYP1A and EROD activity in gills and liver in three-spined stickleback. In addition, we used autoradiography to clarify the absorption, tissue distribution, and excretion paths for 14C-indigo following water-borne exposure in rainbow trout.

Material and Methods

Three-spined stickleback were caught along the coast near Skanör in south-western Sweden. Rainbow trout were purchased from Näs fiskodling AB, By Kyrkby, Sweden. Both species were held in tanks at 11° C.
3,3',4,4',5-Pentachlorobiphenyl (PCB 126; Cas number: 57465-28-8) and indigo (Cas number: 482-89-3) were obtained from Larodan (Malmö, Sweden) and Sigma-Aldrich (St Louis, MO, USA), respectively . ^{14}C-indigo (specific activity: 55 mCi/mmol; radiochemical purity: 98 %) was prepared by OncoTargeting AB, Uppsala, Sweden.

Gill EROD induction; concentration-response

Stock-solutions were prepared using DMSO as solvent for indigo and acetone as a solvent for PCB 126. Dilutions of the stock solutions were added to the water of beakers yielding nominal concentrations ranging from 1 pM to 10 µM for indigo and from 1 to 100 nM for PCB 126. The concentrations of the carriers were 50 ppm DMSO and 100 ppm acetone. Stickleback were exposed to indigo or DMSO for 6 hours. Exposure to PCB 126 and acetone lasted for 24 hours after which the fish were transferred to clean water and kept for another 24 hours. The fish were killed by decapitation. One gill arch was dissected and put into ice-cold HEPES-Cortland (HC) buffer (Jönsson et al., 2002) for analysis of EROD activity.

Ten-day time-course induction of EROD activity and CYP1A gene expression

Groups of stickleback were exposed to indigo (1 nM) or PCB 126 (10 nM) for 3, 6, 9, or 24 hours, while another two groups were exposed for 24 hours and subsequently held in clean water for 2 or 9 days. After each time point six fish were randomly sampled from each exposure group. EROD activity and CYP1A gene expression were determined.

Autoradiography

Exposure regimen			
Exposure group	Day 1	Day 2[a]	Day3[b]
^{14}C-indigo	Vehicle	^{14}C-indigo	Depuration; fresh water
^{14}C-indigo + PCB 126	PCB 126	^{14}C-indigo	Depuration; fresh water

a After adding indigo, one fish was randomly sampled from each group at 20 min, 2 hours, 6 hours, 12 hours, 24 hours.

b One per group was sampled after depuration in clean water for 24 hours .

Rainbow trout were exposed to ^{14}C-indigo according to the above protocol. To determine whether the disposition of ^{14}C-indigo was influenced by CYP1 induction, both non-induced and PCB 126-induced fish were examined. Following exposure either to PCB 126 or to the vehicle for 24 hours, the fish were transferred to water containing ^{14}C-indigo (18 nM) end exposed for 20 minutes, or 6, 12 or 24 hours before being killed and frozen for autoradiography. Fish exposed for 24 hours to ^{14}C-indigo, were transferred to clean water for depuration. These fished were killed following 24 hours or five days in the clean water. One fish from each group was sampled at the respective exposure time and killed by benzocaine in water. The fish then were embedded in an aqueous gel of carboxymethyl cellulose (1%) and frozen in hexane cooled with solid carbon dioxide (-75° C). Whole-body sections (20 µm) from different levels were collected onto tape, using a PMV cryostate microtom according to Ullberg (1977). Freeze-dried sections were exposed to X-ray film for autoradiographic exposure at -20 °C. Autoradiograms were developed at various intervals ranging from 3-6 weeks to illustrate tissues of interest.

Quantitative PCR

Gill and liver RNA were prepared using Aurum™ total RNA fatty and fibrous Tissue kit (Bio-Rad Laboratories Inc., Hercules CA, USA), and cDNA was synthesized by the iScript cDNA Synthesis kit (Bio-Rad Laboratories inc.). Gene-specific quantitative primers for *CYP1A* and reference genes (Gao et al., 2011) were synthesized by Eurofins MWG Operon (Huntsville, AL, USA) and Sigma-Aldrich Company Ltd (St. Louis, MO, USA). Quantitative PCR was conducted by using the iQ SYBR Green Supermix and a Rotor Gene 6000 Real-Time PCR Machine (Corbett Life Science, Concorde, NSW, Australia). Samples were analyzed in duplicate with the following the protocol: 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 62°C for 45 sec. At the end of each PCR run a melt curve analysis was performed in the range from 55°C to 95°C.

Calculations and Statistics

Relative mRNA expression of target genes was calculated by $E^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Statistical analysis was performed using Prism 4 by GraphPad Software Inc. (San Diego, CA, USA) with log-transformed data.

Results and Discussion

Concentraion-response

As illustrated in Figure 1, indigo was a highly potent inducer of gill EROD activity, giving rise to a significantly increased enzyme activity in the 1-10 pM concentration range and a maximal induction in the 0.01-1 nM range. For a comparison, maximal PCB 126-induced EROD activity was observed at 10-100 nM and a lower but significant induction at 1-3 nM concentration. The calculated EC_{50} values for indigo and PCB 126 were 0.09 and 6 nM, respectively. However, since the measurements were performed following exposure via water, the results do not necessarily reflect the true intrinsic induction potencies of the compounds because ofdifferent hydrophobicities of indigo and PCB 126 (Hou et al., 1991, Kong et al., 2007).

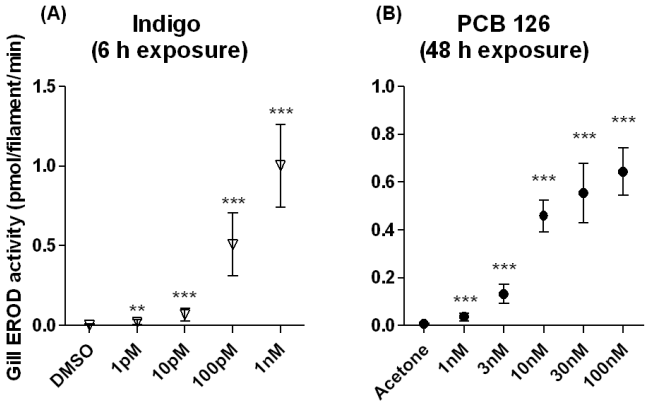

Fig 1. Concentration–response relationships of EROD activity in gill filaments of three-spined stickleback exposed to indigo (A) or PCB 126 (B). Fish were exposed to indigo (1 pM–1 nM) plus 50 ppm DMSO (carrier) or 50 ppm DMSO only for 6 hours. Other fish were exposed to PCB 126 (1–100 nM) plus 50 ppm acetone (carrier) or 50 ppm acetone only for 24 hours and subsequently held in clean water for 24 hours. Statistical differences were determined by one-way ANOVA followed by Dunnett's test (Mean±SD, ***=p<0.001 and **=p<0.01). Bars for 10 and 30 nM PCB 126 represent data from five replicates. All other bars represent six replicates.

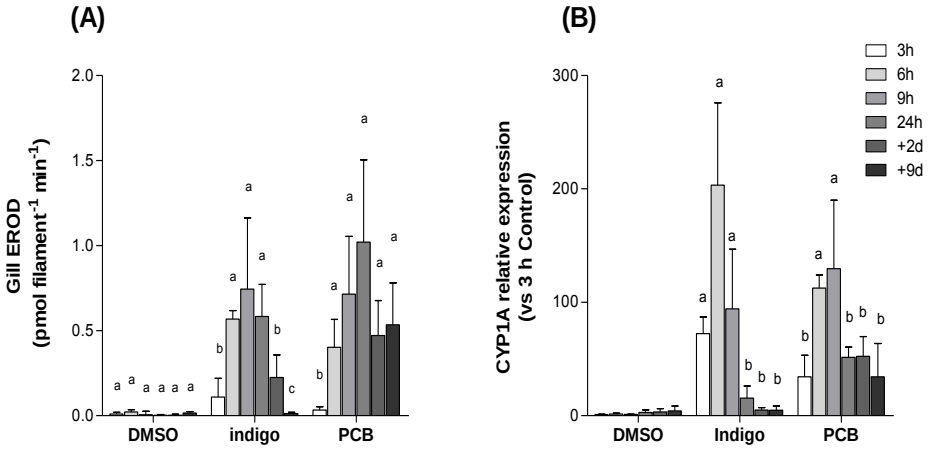

Figure 2. Ten-day time course for EROD activity (A) and *CYP1A* relative mRNA expression (B) in gill filaments of stickleback exposed to the carrier (50 ppm of DMSO), indigo (10^{-9} M), or PCB 126 (10^{-8} M). Statistically significant differences were determined by one-way ANOVA followed by Tukey's post hoc test after log transformation (Mean ± SD, $p<0.05$; n = 5-6 (A) and 3-4 (B)) and are indicated by different letters.

Ten-day time course study

Given that indigo (6 hours of exposure) was a more potent inducer than PCB 126 (48 hours exposure), we determined the persistency of induction over time. Three-spined sticklebacks were exposed to indigo (1 nM) or PCB 126 (10 nM) for 3, 6, 9, or 24 hours, while another two groups were exposed for 24 hours and subsequently held in clean water for 2 or 9 days. Gill EROD activity and CYP1A mRNA expression patterns in gills and liver were determined. In fish exposed to indigo and subsequently transferred to clean water, gill EROD activity was markedly reduced after 2 days, and had returned to the control level after 9 days. The level of EROD induction in PCB 126-exposed fish tended to decrease after transfer to clean water, but between day 2 and day 9 in clean water there was no further change in the EROD level.

Indigo induced maximal gill CYP1A mRNA induction after 6 hours of exposure; and expression of *CYP1A* started to decrease and was back to normal level in the end, while PCB 126 induced maximal expression after 6-

9 hours. After 24 hours, *CYP1A* expression was back to the control level in indigo-exposed fish while it had declined to about 50% of maximum in PCB 126-exposed fish. Compared to PCB 126, indigo had a rapid clearance from the body, resulting in a transient effect. The reduced EROD activity following transfer of the fish to clean water probably reflects both metabolic clearance and redistribution of the PCB 126 compounds from the gills to internal organs, or back to the water.

Autoradiography

There was a rapid uptake of indigo in the fish, with a pronounced uptake radioactivity in the gill filaments already after 20 minutes. During the whole investigative period, the autoradiograms were characterized by a strong uptake in gills and liver and a very high enrichment of radioactive substance into the bile ducts of the liver, the gall bladder and the intestinal contents. In addition, a strong labeling of distinct spots in the kidney was observed, presumably in the kidney tubules. Also notable was the strong uptake and retention of bound radioactivity in the gill filaments. The cause of this observation warrants further studies to define whether indigo is biotransformed into tissue-bound metabolites. Taken together with the induction data, these kinetic data are compatible with the conclusion that indigo is a transient CYP1 inducer due to a rapid degradation and elimination from the tissues in fish exposed via ambient water (Gao et al., 2011, Jönsson et al., 2006).

References

Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., Miller Iii, C., Kato, T., Saeki, K., Matsuda, T., 2001. Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. Journal of Biological Chemistry 276, 31475-31478.

Gao, K., Brandt, I., Goldstone, J.V., Jönsson, M.E., 2011. Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 154, 42-55.

Guengerich, F., P, Martin, M., McCormick, W., Nguyen, L., Glover, E., Bradfield, C., 2004. Aryl hydrocarbon receptor response to indigoids in vitro and in vivo. Archives of Biochemistry and Biophysics 423, 309-316.

Hoessel, R., Leclerc, S., Endicott, J., Nobel, M., Lawrie, A., Tunnah, P., Leost, M., Damiens, E., Marie, D., Marko, D., 1999. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat. Cell Biol 1, 60-67.

Hou, M., Baughman, G.L., Perenich, T.A., 1991. Estimation of water solubility and octanol/water partition coefficient of hydrophobic dyes : Part II: Reverse-phase high performance liquid chromatography. Dyes and Pigments 16, 291-297.

Jönsson, E., Abrahamson, A., Brunström, B., Brandt, I., 2006. Cytochrome P4501A induction in rainbow trout gills and liver following exposure to waterborne indigo, benzo [a] pyrene and 3, 3 , 4, 4 , 5-pentachlorobiphenyl. Aquatic Toxicology 79, 226-232.

Jönsson, E., Brandt, I., Brunström, B., 2002. Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. Environmental Science and Technology 36, 3340-3344.

Kong, X.Q., Shea, D., Baynes, R.E., Riviere, J.E., Xia, X.-R., 2007. Regression method of the hydrophobicity ruler approach for determining octanol/water partition coefficients of very hydrophobic compounds. Chemosphere 66, 1086-1093.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Ullberg, S., 1977. The technique of whole body autoradiography. Cryosectioning of large specimens. Special Issue on Whole-Body Autoradiography, Science Tools, LKB Instrument Journal, 2-29.

Xiao, Z., Hao, Y., Liu, B., Qian, L., 2002. Indirubin and meisoindigo in the treatment of chronic myelogenous leukemia in China. Leukemia and Lymphoma 43, 1763-1768.