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Aromatase mRNA expression in the brain of adult *Xenopus laevis* exposed to Lambro river water and endocrine disrupting compounds

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ABSTRACT

Aromatase P450 (P450 arom; Cyp19) is a key enzyme for vertebrate reproduction and brain development that catalyzes the conversion of androgens to estrogens. The aim of this study was to improve the knowledge on EDC effects by analysing their potential impact on brain P450 arom in adult *Xenopus laevis* exposed for 4 weeks to an environmental sample, the water of the river Lambro (LAM), the most polluted tributary of the Po river in North Italy. Other groups were exposed to individual compounds 10^{-8} M tamoxifen (TAM), ethinylestradiol (EE2), flutamide (FLU) and methyldihydrotestosterone (MDHT) known for their (anti)estrogenic and (anti)androgenic modes of action. Expression of CYP19 was evaluated in brain extracts by quantitative RT-PCR, using a pair of primers located in the open reading frame (ORF) that allowed the simultaneous amplification of all transcripts (Aro-ORF) and a pair of primers specific for brain aromatase (Aro-B). Significant increase in Aro-ORF and Aro-B mRNA levels were observed in both females and males exposed to LAM. Different changes were observed for the model compounds using two pairs of primers. Aro-ORF mRNA expression was significantly increased in EE2 and MDHT exposed males and in FLU-exposed females, while it was significantly decreased in TAM exposed females. Aro-B mRNA was significantly increased in both sexes exposed to FLU and decreased in TAM exposed females. In conclusion, aromatase mRNA in the brain of *X. laevis* was regulated differentially in a gender specific manner by certain (anti)estrogenic and (anti)androgenic EDCs, supporting previous hypotheses that diverse compounds present in the river Lambro may induce feminization and demasculinization effects.

1. Introduction

Aromatase cytochrome P450 (P450 arom), the product of the aromatase gene *Cyp19*, is the terminal enzyme in the estrogen biosynthetic pathway that catalyzes the transformation of testosterone to estradiol-17β (E2). The gonads are considered to be the major source of estrogens in the body, however, local estrogen production in other sites, especially in the brain, is also very important, since it is essential for gonad development as well as for other diverse physiological processes. In the brain, E2 regulates multiple functions like control of neuroendocrine system, sex differentiation, activation of sexual behavior and stimulation of gonadal activity by increasing gonadotropin secretion in the pituitary (Balthazart and Ball, 1998; Forlano et al., 2006; Guerriero et al., 2000; Hutchinson, 1993; Lephart, 1996, 1997; McEwen, 1997). In amphibians, P450 aromatase is encoded by one CYP19 gene leading to two gonad and one brain P450 aromatase transcripts which differ in their 5-untranslated region (UTR) but contain an identical open reading frame (Iwabuchi et al., 2007). These transcripts have been detected at different expression levels in brains and gonads. Brain P450 arom is mainly expressed in the embryonic and adult brain, but also in undifferentiated gonads and in adult ovaries and testis. Gonad P450 aroms 1 and 2 are expressed in undifferentiated gonads and in adult ovaries and testis, but also weakly in the brain (Iwabuchi et al., 2007).

Aromatase is considered as one of the potential targets of the many environmental chemicals, called Endocrine Disrupting Compounds (EDC) that can interfere with the endocrine system affecting the reproductive biology of aquatic vertebrates (Hogan et al., 2008; Kloas, 2002; Kloas et al., 2009; MacKenzie et al., 2003; Thibaut and Porte, 2004). Changes in aromatase expression may alter the rate of estrogen and androgen production and disturb the local and systemic levels of these steroids (Cheshenko et al., 2008; Kazeto et al., 2004; Kishida and Callard, 2001; Mann et al., 2009; Murphy et al., 2006; Sanderson et al., 2002).

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Xenopus laevis has been widely used as a sensitive model for the screening of EDCs *in vitro* (Kloas et al., 1999; Lutz et al., 2005) and *in vivo* (Bögi et al., 2002; Kloas, 2002; Levy et al., 2004; Urbatzka et al., 2006). In this study, adult *X. laevis* were exposed to 17α-ethinylestradiol (EE2) as estrogenic compound, tamoxifen (TAM) as anti-estrogenic compound, 17α-methyldihydrotestosterone (MDHT) as androgenic compound, and flutamide (FLU) as anti-androgenic compound, in a 4 week exposure at the concentration of 10^{-8} M. The condition of exposure where chosen in order to mimic environmental exposure conditions to both EDC mixtures and individual EDCs as previously described (Cevasco et al., 2008; Urbatzka et al., 2007a). Furthermore, adult *X. laevis* were exposed to Lambro river water to assess the interference of an environmental mixture. The river Lambro is a heavily polluted tributary of the river Po that drains a sub-basin densely inhabited, characterized by many industrial activities and extensive plain agriculture (Camusso et al., 1995). Recent studies have demonstrated that water and sediment of the Lambro river are mainly polluted by substances exhibiting estrogenic (Viganò et al., 2008) and anti-androgenic mode of actions (MOAs) (Urbatzka et al., 2007b). The aim of the study was to compare the possible effects of (anti)estrogenic and (anti)androgenic EDC model compounds and of Lambro river water samples on the level of aromatase mRNA in the brain of adult *X. laevis*. Both total (Aro-ORF) and brain (Aro-B) transcripts were evaluated by quantitative real-time PCR assays.

2. Materials and methods

2.1. In vivo exposure

All exposure protocols were performed as previously described (Urbatzka et al., 2007a; Cevasco et al., 2008) within the activities of the EU-project EASYRING (Contract No. QLK4-CT-2002-02286). Adult *X. laevis* (3–4 years old) were taken from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin. The frogs were fed twice a week and kept under a 12 h light:12 h dark cycle.

For the exposure, adult male and female *X. laevis* were transferred to aerated 10 L tanks containing reconstituted tap water using distilled water supplemented with 2.5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany). *X. laevis* were exposed to tamoxifen (TAM), ethinylestradiol (EE2), flutamide (FLU) and methyldihydrotestosterone (MDHT), chosen as (anti)estrogenic and (anti)androgenic compounds, respectively, and to Lambro river water. Chemicals were dissolved in ethanol (Roth, Karlsruhe, Germany) and a solvent control (0.001% ethanol) was included in the experimental design of the study. The treatment lasted 4 weeks and consisted of eight females and eight males per compound equally distributed into two 10 L tanks containing four females and four males, respectively. The test concentration of the chemicals was 10^{-8} M. Rearing water and chemicals were renewed completely every Monday, Wednesday and Friday. During exposure, *X. laevis* were fed once a week with commercial fish diet (Fisch-Fit, Interquell, Wehringen, Germany) and water temperature was adjusted to 22 ± 1 °C.

2.2. Chemicals and Lambro sampling

All test chemicals (EE2, TAM, MDHT, FLU) were purchased from Sigma (Taufkirchen, Germany). A composite sample (total volume 280 L) of Lambro river water was sampled using 2 L glass vessels on March 18th 2004 during the whole day, about 60 km south of Milano (Lat: 45:09:57N, Long: 9:31:55E) and transported to Germany. The Lambro water sample was kept at 4 °C and exposure started immediately after arrival at the IGB in Berlin.

2.3. Sampling and RNA-isolation

At the end of exposure, all *X. laevis* frogs were sacrificed. Whole brain ($n = 6$ for treatment) including the pituitary was dissected, transferred to an Eppendorf tube and snap-frozen in liquid nitrogen. Isolation of total RNA was carried out using the phenolic reagent TRIZOL (Invitrogen, Karlsruhe, Germany) according to the manufacturers instructions as described in Bögi et al. (2002). The amount of isolated total RNA ranged between 10 and 32 μg per tissue sample and was adjusted to 1 μg total RNA per 8 μl RNAse free water (diethyl pyrocarbonate-treated water). First-strand cDNA was synthesized with 0.5 μg RNA using the SuperScript first-strand synthesis system (Invitrogen, San Diego, CA) and oligo(dT) primers. The resulting cDNA was used in PCR experiments.

2.4. RT-Q-PCR

The expression levels of Cyp19 gene was quantified by using a Chromo 4TM System real-time PCR apparatus (Biorad, Milan, Italy). Real-time quantitative PCR (q-PCR) reactions were performed in triplicate in a final volume of 20 μl containing 10 ng cDNA, 10 μl of iTaq SYBR Green Supermix with ROX (Biorad), and 0.25 μM of each primer pairs. The GAPDH mRNA was used as housekeeping gene to normalize the expression data. For normalization, expression of GAPDH mRNA was used since no differences were found between all treatment groups. Similar results were obtained using the EF-1α, elongation factor-1 alpha-chain (not shown). Expression of CYP19 was evaluated in brain extracts using a pair of primers located in the open reading frame (ORF) that allowed the simultaneous amplification of all transcripts (Aro-ORF) and a pair of primers specific for brain aromatase (Aro-B). Aro-ORF primers were designed in our laboratory on the basis of the sequence reported in GenBank and location is indicated in Table 1. Aro-B primers were used as designed by Iwabuchi et al. (2007). The accession numbers of the genes used in the study, the primer sequences and the product sizes are given in Table 1. The thermal protocol included an enzymatic activation step at 95 °C (3 min) and 40 cycles of 95 °C (15 s), 60 °C (30 s) and 72 °C (20 s). The melting curve of the PCR products (55–94 °C) was also recorded to check the reaction specificity. Relative expression of target genes in comparison with that of the GAPDH mRNA reference gene was conducted following the comparative C_t threshold method (Pfaffl et al., 2002) using the Biorad software tool Genex-Gene Expression Macro™ (Vandesompele et al., 2002). The normalized expression was then expressed as relative quantity of mRNA (fold induction) with respect to the control sample.

2.5. Statistical analyses

Data ($n = 6$/treatment) are presented as the mean ± SD of analyses in triplicate. Statistical analysis was performed by using ANOVA followed by Tukey ad post hoc test (INSTAT software, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Males

The effects of different conditions of exposure on the transcription of Aro-ORF and Aro-B in male brain are reported in Fig. 1(A and B). Exposure to Lambro river water (LAM) induced a significant increase in the level of mRNA of both Aro-ORF (Fig. 1A) and Aro-B (Fig. 1B), that was apparently higher for Aro-B than for Aro-ORF (with relative expressions of 2.42 ± 0.51 and 1.67 ± 0.15, respectively). Exposure to the estro-