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Bisphenol A Induces Hepatic Vitellogenin mRNA in Male *Bombina orientalis*

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Chemical contamination continues to be postulated as a contributing factor in the global decline of amphibians (Renner 2002). To date, a number of studies have assessed endocrine disruption by these chemicals in amphibians. Evidence for abnormalities such as feminization of wildlife animals implies contamination by estrogenic compounds at concentrations high enough to be of concern (Purdom et al. 1994; Palmar and Palmar 1995; Hayes and Menendez 1999; Kloas et al. 1999; Mann and Bidwell 2000; Hayes et al. 2002; Hurter et al. 2002; Mosconi et al. 2002; Bevan et al. 2003; Mackenzie et al. 2003; Gye 2004; Kohno et al. 2004). Of these chemicals, bisphenol A (BPA) is among the most widely used placticizers worldwide and has been found in sewage effluents, rivers, estuaries, sediments and animal tissues, including humans and may already be ubiquitous (Khim et al. 2001; Ikezuki et al. 2002). BPA can act at very low detected doses in the environment (Sheehan 2000). Accordingly, BPA in the aquatic environment could induce toxic effects by coming into direct contact with aquatic animals. Moreover, amphibian embryos may be exposed to BPA maternally during oogenesis. Therefore, BPA has the potential to cause direct harm during embryonic as well as larval life in amphibians inhabiting a contaminated aquatic environment. Because of its structural similarity to estrogen, BPA can induce the estrogen-responsive transcription of genes and is associated with adverse effects in amphibian development (Kloas et al. 1999; Lutz and Kloas 1999; Pickford et al. 2003).

In oviparous vertebrate species, vitellogenin (Vg), an egg yolk precursor protein is physiologically synthesized in female liver by estrogen. In the male frog, several xenoestrogens, as well as natural estrogen, stimulate synthesis of estrogen responsive proteins, including liver Vg (Wangh and Knowland 1975; Carnevali and Mosconi 1992; Carnevali et al. 1995; Bogi et al. 2003; van Wyk et al. 2003; Gye 2004). Therefore, an established method to characterize the estrogenic potential of media is the use of induction of the plasma Vg protein or hepatic Vg mRNA as a biomarker in the male (Rotchell and Ostrander 2003). Vg genes occur as a multigene family. In the frog *Xenopus*, four Vg genes are actively expressed and grouped as A and B genes. Of these, genes A1 and A2 have a 95 percent sequence homology in their messenger RNA (Wahli et al. 1981). Recently, in an effort to develop a molecular biomarker for testing estrogen mimicking chemicals, we characterized the Vg A2 cDNA fragment in *Bombina orientalis,* one of the most common amphibians in the world and comprising a large proportion of their total number in Korea (Lee and Gye 2004). This species lives in lakes, ponds,

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swamps, and streams, and spawns in the rice field and ponds from March to April in Korea. Therefore contamination of their aquatic habitats by xenoestrogens including BPA, may threaten this species. To date, however little has been known about the impact of BPA on this species. In the present study we examined the effect of BPA on the expression of hepatic Vg mRNA in adult male *B. orientalis*.

MATERIALS AND METHODS

Frogs (*B. orientalis*) were collected from Hongcheon-Gun, Kangwon-Do in Korea during April 2004. Frogs were put in aquaria maintained at a diurnal 14:10h light: dark cycle at 20-22℃ and fed mealworm three times a week. Adult males with a mean body weight (BW) of 10.0 ± 0.5g were selected and given an intraperitoneal injection of 17ß estradiol (1 mg/kg BW) or BPA (1, 10 and 100 mg/kg BW) dissolved in 100 μL of sesame oil. Vehicle control males were given sesame oil only. Frogs were returned to the aquaria, and sampled at the appropriate time after the injection.

Frogs were anesthetized by inhalation of ether to minimize pain, and livers were dissected. Liver total RNA was isolated using TRI reagent (Moleular Research Center, Inc., Cincinati, Ohio) as directed by the manufacturers. Amounts of RNA were determined and then stored at –85℃ until use. 1 μg of RNA sample was reverse transcribed for 60 min at 42℃ in a 20 μL reaction with 50 units of MuLV reverse transcriptase and 2.5 μM oligo $d(T)_{16}$ primer by the standard protocol from the supplier (Applied Biosystems, Foster City, CA). 25 μL of a PCR mixture containing 0.5 μL of the RT product, 1.25 units of Ex Taq™ polymerase (Takara, Japan), 1X Ex PCR™ Buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP mixture, and 0.4 μM of each primer. Based on the partial cDNA sequence of Vg in *B. orientalis* (Lee and Gye 2004), a pair of specific primers for Vg were designated 5'- TGC TGA TCC ATC TGT CCT GA-3' (forward) and 5'- AAC AGG CTG TGT GAG CTT GA-3' (reverse). For semi-quantitative RT-PCR analysis of Vg mRNA expression, the ß actin mRNA was amplified as an internal control. A set of primers spanning the highly conserved region of the ß actin gene from *Xenopus* were designated 5'-GAG AGG TAT CCT GAC CCT GAA GTA-3' (forward) and 5'-ATA ACC TTC ATA GAT GGG CAC AGT-3' (reverse). These primer sets gave rise to Vg and ß actin diagnostic fragments of 512 and 325 bp, respectively. To determine the optimal annealing temperature for PCR of Vg mRNA and ß actin 30 cycles of PCR were performed with temperature gradient mode using i-Cyler (BioRad, CA). 20 – 40 cycles of PCR amplifications were performed to determine the optimal number of PCR cycles for quantitative analysis. Each cycle consisted of the following: 95℃, 30s; 56℃, 30s; 72℃, 45s. PCR products (20 μL) were run on 2 % agarose gels containing 0.5 μg/μL ethidium bromide and photographed under UV light. The amounts of RT-PCR products were quantified by analyzing the band intensity with Scion Images software (Scion Corporation, Frederick, MD). The Vg mRNA level was expressed as arbitrary units relative to the density of the ß actin mRNA level. Statistical significance was analyzed by the Student's *t*-test and accepted at $p < 0.05$.

RESULTS AND DISCUSSION

PCR annealing temperature was optimized at 56℃ for both Vg and ß actin primers sets (Fig. 1A). At this condition, PCR product for Vg was first detected at 32 cycles of amplification and linearly increased up to 40 cycles in control male liver, suggesting scarcity of Vg mRNA in male liver. On the contrary, ß actin was linearly amplified between 24 and 34 cycles of amplification (Fig. 1B). Thereafter RT-PCR was conducted at 32 and 26 cycle for Vg and ß actin, respectively.

The BPA dosing at 100 mg/kg evoked neither death nor narcosis at 5 days after injection, suggesting that the dose of BPA examined in this study did not result in a systemic acute toxic effect on *B. orientalis*. Five days after drug treatment, BPA (100 mg/kg) as well as 17ß estradiol (1 mg/kg) apparently induced hepatic Vg mRNA in male frogs, and which was not largely different from the expression level in female liver (Fig. 2). This suggests that BPA has an estrogen mimicking effect on adult male *B. orientalis*. Five days after drug treatment, BPA induced hepatic Vg mRNA in a dose-dependent manner. At 1 mg/kg the Vg mRNA level slightly increased but was not statistically significant. At 10 mg/kg, there was a significant increase in Vg mRNA level compared to the vehicle control. In 100 mg/kg dosing group, there was a large induction of Vg mRNA (Fig. 3A). Therefore it is suggested that the single lowest effective dose level for BPA to induce Vg mRNA is between 1-10 mg/kg in male *B. orientalis*. According to the guideline from the National Toxicology Program (NTP), it can be calculated from our result that an acceptable exposure value for BPA lacking overt toxic effects on the frogs is 1-10 μg/kg/day which is much higher than the environmentally relevant concentration of BPA (< 2 μg/kg). However, a second injection of BPA at 1 mg/kg BW with a 5 day interval resulted in a marked increase in Vg mRNA (Fig. 3B). This suggests that the primary injection of BPA did not evoke observable Vg expression but it may sensitize the transcriptional machinery required for hepatic Vg expression, allowing for a response to second injection of BPA in male frogs. Repeated exposure of frogs to BPA is most likely unavoidable to frogs in the contaminated habitat. BPA is frequently found in environmental media together with other xenoestrogens, such as alkylphenols. Therefore it should be emphasized that BPA even at a no observable effect concentration (NOEC) may elicit endocrine disruption in this frog species when other xenoestrogens are also present in environment.

Previously it was reported that Vg synthesis in male hepatocytes is less sensitive to estrogen during the refractory period in *Rana esculenta* (Carnevali and Mosconi 1992). Because our experiment was carried out using frogs adapted in animal husbandry for several months, the sensitivity of Vg mRNA induction to BPA might be altered compared to that of a natural population. Therefore, for the analysis of field samples using this method, seasonal differences in the sensitivity of Vg mRNA induction by an estrogenic compound should be considered. The present study may provide additional clues on factors responsible for the decline in the amphibian populations and may explain the increased incidence of abnormalities in situ for some frog species. Furthermore, an optimized protocol for semiquantitative RT-PCR analysis of hepatic Vg mRNA levels in *B. orientalis* males will be useful for the monitoring xenoestrogen contamination in the Korean freshwater environment.

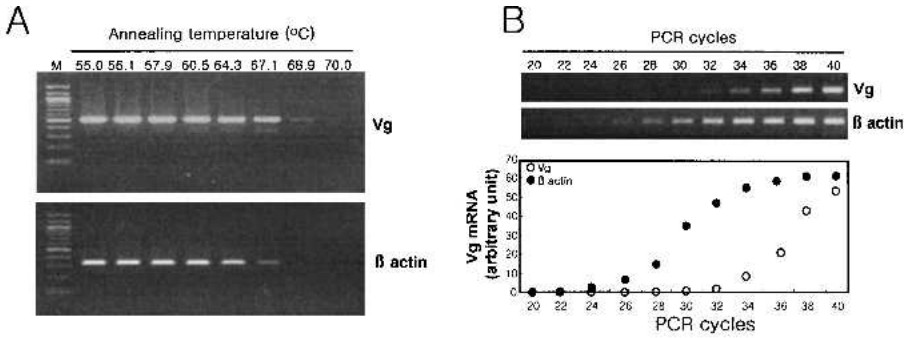

Figure 1. Optimization of semiquantitative RT-PCR of Vg mRNA in male liver from *B. orientalis*. Optimization of annealing temperature (A) and PCR cycle (B).

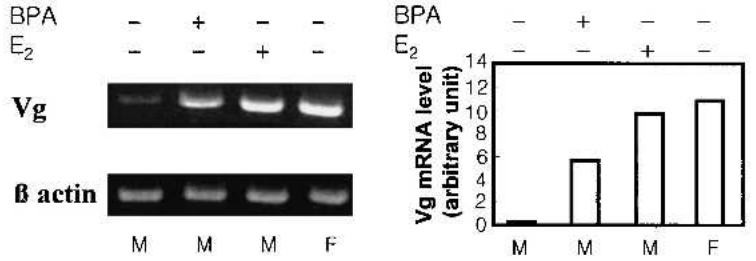

Figure 2. Effects of bisphenol A and estrogen on Vg mRNA level in liver from *B. orientalis*. RT-PCR of Vg and ß actin mRNA following a single injection of BPA (100 mg/kg) or 17ß estradiol (E_2, 1 mg/kg). M, male; F, female.

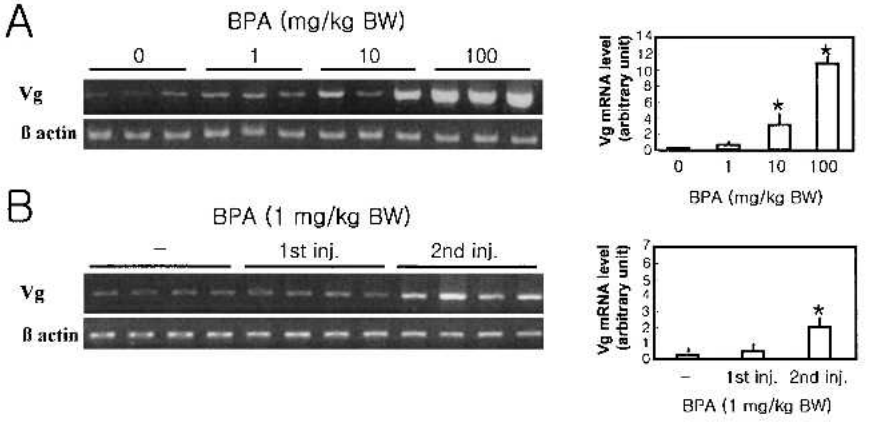

Figure 3. Effects of bisphenol A on the expression of Vg mRNA in male liver from *B. orientalis*. (A) RT-PCR of Vg and ß actin mRNA following a single injection of BPA (1, 10, 100 mg/kg). (B) RT-PCR of Vg and ß actin mRNA following two injections of BPA (1 mg/kg) with a 5 day interval. Error bar = SD (n > 3). *, significantly different from vehicle control by Student's *t* test.

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REFERENCES

Bevan CL, Porter DM, Prasad A, Howard MJ, Henderson LP (2003) Environmental estrogens alter early development in *Xenopus laevis*. Environ Health Perspect 111:88-96

Bogi C, Schwaiger J, Ferling H, Mallow U, Steineck C, Sinowatz F, Kalbfus W, Negele RD, Lutz I, Kloas W (2003) Endocrine effects of environmental pollution on *Xenopus laevis* and *Rana temporaria*. Environ Res 93:195-201

Carnevali O, Mosconi G (1992) In vitro induction of vitellogenin synthesis in *Rana esculenta*: role of the pituitary. Gen Comp Endocrinol 86:352-358

Carnevali O, Sabbieti MG, Mosconi G, Polzonetti-Magni AM (1995) Multihormonal control of vitellogenin mRNA expression in the liver of frog, *Rana esculenta*. Mol Cell Endocrinol 114:19-25

Gye M (2004) Two-dimensional gel electrophoresis analysis of liver proteins following nonylphenol treatment of *Bombina orientalis* Boulenger. Bull Environ Contam Toxicol 73:411-415

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc Natl Acad Sci USA 99:5476-5480

Hayes TB, Menendez KP (1999) The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic reedfrog (*Hyperolius argus*: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. Gen Comp Endocrinol 115:188-199

Hurter E, Pool EJ, Van Wyk JH (2002) Validation of an ex vivo *Xenopus* liver slice bioassay for environmental estrogens and estrogen mimics. Ecotoxicol Environ Saf 53:178-187

Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y (2002) Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum Reprod 17:2839-2841

Khim JS, Lee KT, Kannan K, Villeneuve DL, Giesy JP, Koh CH (2001) Trace organic contaminants in sediment and water from Ulsan Bay and its vicinity, Korea. Arch Environ Contam Toxicol 40:141-150

Kloas W, Lutz I, Einspanier R (1999) Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. Sci Total Environ 225:59-68

Kohno S, Fujime M, Kamishima Y, Iguchi T (2004) Sexually dimorphic basal water absorption at the isolated pelvic patch of Japanese tree frog, *Hyla japonica*. J Exp Zool A Comp Exp Biol 301:428-438

Lee MS, Gye MC (2004) Partial characterization of vitellogenin cDNA in *Bombina orientalis*. Ann Conf Kor Soc Environ Biol, Pusan, p 19

Lutz I, Kloas W (1999) Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. Sci Total Environ 225:49-57

Mackenzie CA, Berrill M, Metcalfe C, Pauli BD (2003) Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. Environ Toxicol Chem 22:2466-2475

Mann RM, Bidwell JR (2000) Application of the FETAX protocol to assess the

developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 251:19-29

Mosconi G, Carnevali O, Franzoni MF, Cottone E, Lutz I, Kloas W, Yamamoto K, Kikuyama S, Polzonetti-Magni AM (2002) Environmental estrogens and reproductive biology in amphibians. Gen Comp Endocrinol 126:125-129

Palmer BD, Palmer SK (1995) Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environ Health Perspect 103 Suppl 4:19-25

Pickford DB, Hetheridge MJ, Caunter JE, Hall AT, Hutchinson TH (2003) Assessing chronic toxicity of bisphenol A to larvae of the African clawed frog (*Xenopus laevis*) in a flow-through exposure system. Chemosphere 53:223-235

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP (1994) Estrogenic effects of effluents from sewage treatment works. Chem Ecol 8:275-285

Renner R (2002) Amphibian declines. Conflict brewing over herbicide's link to frog deformities. Science 298:938-939

Rotchell JM, Ostrander GK (2003) Molecular markers of endocrine disruption in aquatic organisms. Toxicol Environ Health B Crit Rev 6:453-496

Sheehan DM (2000) Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: initial results confirmed. Proc Soc Exp Biol Med 224:57–60

van Wyk JH, Pool EJ, Leslie AJ (2003) The effects of anti-androgenic and estrogenic disrupting contaminants on breeding gland (nuptial pad) morphology, plasma testosterone levels, and plasma vitellogenin levels in male *Xenopus laevis* (African clawed frog). Arch Environ Contam Toxicol 44:247-256

Wahli W, Dawid IB, Ryffel GU, Weber R (1981) Vitellogenesis and the vitellogenin gene family. Science 212:298-304

Wangh LJ, Knowland J (1975) Synthesis of vitellogenin in cultures of male and female frog liver regulated by estradiol treatment in vitro. Proc Natl Acad Sci USA 72:3172-3175