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Estrogenic Response in Male Bullfrog (*Rana catesbeiana*) Hepatocytes After Single or Combined Exposure to Cadmium (Cd) and 17beta-Estradiol (E2)

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Abstract Contamination by heavy metals and sex hormones in a water environment is an important health issue. In this study, we investigated the estrogenic effects of cadmium (Cd) administration alone and in combination with 17beta-estradiol (E2) on the hepatocytes of male Bullfrog (*Rana catesbeiana*). Their vitellogenin (VTG) expression and reactive oxygen species (ROS) were analyzed upon exposure to Cd alone or to both Cd and E2. Our results suggest that the VTG levels induced by the co-treatment of 100 nM E2 and 100 nM $CdCl_2$ were significantly higher than those induced by 100 nM E2 alone ($p < 0.05$), and were comparable to vitellogenin induction observed with 1 μM E2. A similar result was observed by western blot analysis in the culture medium of hepatocytes. Meanwhile, Cd (but not E2) increased the ROS levels. These results suggest that Cd has a cooperative effect with E2 in the induction of VTG, thus acting as an estrogenic disruptor. Cd also causes oxidative stress that occurs with the enhanced vitellogenesis.

Keywords Bullfrog · Hapatocytes · Cadmium · VTG · 17beta-estradiol (E2)

The contamination of fresh water ecosystems by heavy metals is one of the main environmental issues today (Iavicoli et al. 2009). Fish and amphibians present in such a contaminated water environment can accumulate heavy metals by a process known as bioaccumulation. These heavy metals are present throughout the food web, potentially endangering human health. Heavy metals present in a water environment are either naturally occurring or originate from the exhaust emissions of industrial effluents, agricultural chemicals or urban refuse. Recent studies have concluded that heavy metals, such as hydrargyrum (Hg), plumbum (Pb), cadmium (Cd), and copper (Cu), can cause male infertility, since gonadal dysfunction and congenital malformation are the main alterations caused by these substances (reviewed in Queiroz and Waissmann 2006). Thus, heavy metal elements were classified as a type of endocrine disruptor (ED) which can bind to the relevant receptors and, thus, further disrupt the physiologic functions of hormones and interfere with the action of endogenous steroid hormones. The element cadmium (Cd) is of special importance, since it is widely used in pesticides, batteries, rubber processing, the production of pigments, and galvanizing (Järup and Åkesson 2009). It is bioaccumulative and persistent in the environment (with a half-life of 10–30 years). Some studies have also found that Cd has worrisome estrogenic effects in cell culture (McLachlan 2001).

Vitellogenin (VTG), an egg yolk precursor that is normally produced in oviparous females as a response to estrogens circulating in blood plasma, is secreted in the liver of female organisms in the reproductive stage (Sole et al. 2001). VTG proteins are usually not synthesized in male animals, although they also contain the VTG gene. Exposure to either natural or synthetic estrogens [such as 17beta-estradiol (E2)] can trigger the over-expression of VTG proteins (Sumpter and Jobling 1995). The most potent estrogen, 17beta-estradiol (E2), can enter cells freely and bind to estrogen receptor (ER). The ER-E2 complex then activates the transcription of the VTG genes by binding to estrogen response element (ERE). In a previous study, the synthesis of VTG was induced by Cd in

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males of Antarctic fish *Trematomus bernacchii* (Canapa et al. 2007).

Frog vitellogenin (VTG) has been used as a sensitive biomarker for monitoring environmental estrogenic activity (Mitsui et al. 2003; Li et al. 2006). A bullfrog VTG quantification system was established in our laboratory (Li et al. 2006). The bullfrog is situated at a high level in the food web and bullfrog-VTG was selected as a biomarker for estrogenic activity in this study. The aim of this study was to investigate the influence of Cd on the aquatic environment by verifying whether it is capable of inducing estrogenic activity in male bullfrog hepatocytes and oxidative stress in hepatocytes. Moreover, we want to know if there are relationships between ROS and VTG expression.

Materials and Methods

Bullfrog hepatocytes were isolated according to the procedure of Kim et al. (2003). Briefly, mature male bullfrogs with average weights of 150–200 g were bought from a local market. After anesthetizing the bullfrog with 0.2 g/l of MS222 (Sigma), the liver was carefully excised from the abdominal cavity. The liver was cleared of blood by means of pre-perfusion with a sterile Ca^{2+}-free hepatocyte buffer (HB, 136.9 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5.0 mM $NaHCO_3$, pH 7.6) for 10 min at room temperature. The pre-perfusion buffer was replaced with HB containing collagenase (Wako Pure Chemicals, Osaka, Japan) at a concentration of 0.3 mg/ml. The softened liver was mechanically dissociated and the cells were first filtered through a 200 μm nylon mesh and then through a 50 μm nylon mesh. The cells were centrifuged 4 times at 50× *g* for 90 s at 8°C with HB containing 1.5 mM $CaCl_2$. After the last wash, the cell pellet was resuspended in Leibovitz-15.

After their isolation, the cells were seeded in a Microtest^{TM}tissue culture plate at a density of 1×10^6/ml, at 150 μl/well, and cultured in Leibovitz-15 medium (L-15, Sigma, St. Louis, MO) supplemented with 5 mM $NaHCO_3$, 1 μl/ml penicillin–streptomycin, 10 μg/ml polymixin B, 0.25 μg/ml fungizone and hormone-free fetal bovine serum (10%). The cells were incubated at 25°C, 5% CO_2 and saturated humidity. After 24 h of cell-culture, the bullfrog hepatocytes were exposed to the ethanol control and predetermined amounts of 17beta-estradiol (E2) and/or $CdCl_2$ for 48 h. Cell viability of the primary bullfrog hepatocytes was determined by the MTT assay (Carmichael et al. 1987). This assay is based on the ability of viable cells to convert MTT, a soluble tetrazolium salt, into an insoluble formazan precipitate, which is quantitated by spectrophotometry after solubilization in DMSO.

After 48 h of exposure, the media of cultured hepatocytes were electrophoresed by 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The electroblotted SDS–PAGE samples included the medium containing the 100 nM E2 treated hepatocytes, the medium containing the untreated medium, the 100 nM $CdCl_2$ treated hepatocytes and the medium used for the co-treatment of 100 nM $CdCl_2$ and 100 nM E2. After being blocked with 3% of bovine serum albumin (BSA) in tris-buffered saline (TBS), the membrane was incubated with primary antibodies against VTG produced by our laboratory (Li et al. 2006) at approximately 1 mg/ml in TBS, and then with secondary antibodies (goat anti-mouse IgG antibody conjugated with alkaline phosphatase) at a dilution of 1:5,000. The bands were developed using phosphatase substrate solutions.

A sandwich enzyme-linked immunosorbent assay system (ELISA) was developed for the quantitative detection of bullfrog VTG, and the VTG levels of the culture media were measured by ELISA (Li et al. 2006). The production of ROS was assessed in the 96-well microplates, as described by the method of Wang and Joseph (1999), using 100 μM of 2,7-dichlorofluorescein diacetate. The measurements were performed on a Molecular Devices SpectraMax microtiter plate reader with $\lambda_{ex} = 495$ nm and $\lambda_{ex} = 530$ nm.

Finally, a statistical analysis was performed using the SPSS (version 11.0) program. Significant differences between the means were studied using a one-way analysis of variance (ANOVA). Their statistical significance ($p < 0.05$) was determined by Tukey's multiple-comparison post hoc test.

Results and Discussion

Chemicals present in the environment have the capacity to interfere in the functioning of the endocrine system (Colborn 2002; Kloas 2002). Among the endocrine active compounds, the heavy metal, Cd, is of particular current concern, because it can be found in the air, in the ground and in water in proximity to industrial areas (Queiroz and Waissmann 2006). Cd has also been proved to be a potent carcinogen, as well as a potential cause of reproductive damage in humans and animals (Hsiao and Stapleton 2004). There are several pathways in which chemicals can affect the endocrine system: for example, estrogenic compounds bind to estrogen receptors (Kloas et al. 1999). Recently, it has also been shown that Cd has potent estrogen- and androgen-like activities in vivo and in vitro, by directly binding to estrogen and androgen receptors (Takiguchi and Yoshihara 2006).

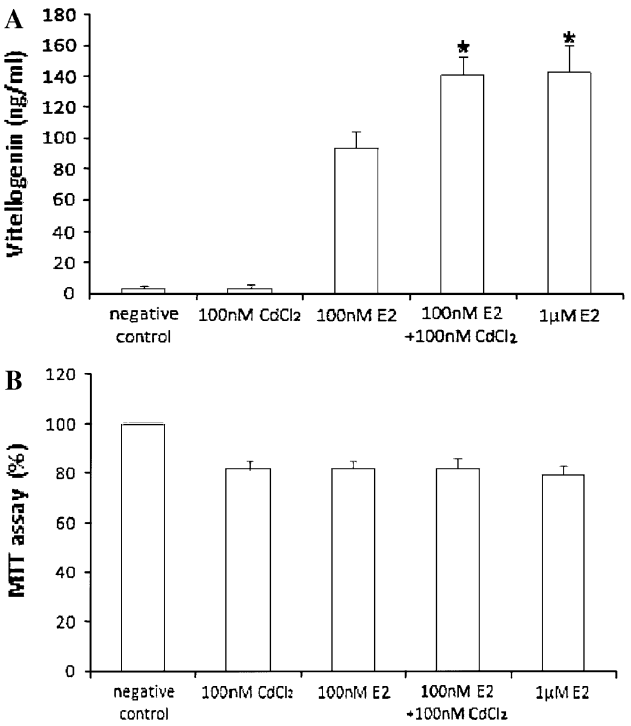

Fig. 1 **a** VTG levels of bullfrog hepatocytes treated with E2 or $CdCl_2$ after 48 h using a sandwich ELISA Kit. The results are expressed in ng/ml and means ± SD * $p < 0.05$ versus 100 nM E2. *White bars* show VTG production. **b** MTT assay results for cell viability of bullfrog hepatocytes treated with 100 nM E2, 100 nM $CdCl_2$, 1 μM E2, or co-treated with 100 nM $CdCl_2$ and 100 nM E2 for 48 h. The results are means ± SD and expressed in % versus negative control. *Error bars* represent ± SD

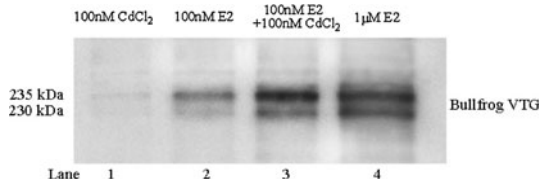

Fig. 2 Western blot analysis of hepatocytes culture medium. The molecular weights (230 and 235 kDa) of the immunoreactive bands are indicated on the *left*

In this study, we examined whether Cd metal could disrupt the endocrine system of male hepatocytes from the Bullfrog (*Rana catesbeiana*). E2 (17beta-estradiol) was chosen as a positive control and the solvent ethanol as the negative control. The results in Fig. 1a show that VTG was not induced by 100 nM $CdCl_2$ after 48 h exposure, but was induced by E2. Interestingly, the male hepatocytes co-exposed to a mixture of 100 nM $CdCl_2$ and 100 nM E2 for 48 h showed a significantly higher VTG level than those exposed to 100 nM E2 alone ($p < 0.05$) (similar to those exposed to 1 μM E2) (Fig. 1a). Moreover, we examined their cell viabilities using the MTT assay and obtained similar results (Fig. 1b), suggesting that the effect of these chemicals on the cell viabilities of male hepatocytes is independent of the dose. E2 (but not Cd) significantly increased the VTG levels of the male hepatocytes. However, the co-treatment with 100 nM $CdCl_2$ and 100 nM E2 resulted in a higher VTG level in the male hepatocytes than that with 100 nM E2 alone, suggesting that there is a synergic effect between E2 and Cd.

Furthermore, bullfrog VTG proteins were examined by western blots in the culture mediums under the same conditions, and similar results were obtained with the sandwich Elisa assay (Fig. 2).

Heavy metals were found to induce oxidative stress in hepatocytes in *rainbow trout* fish (Risso-de Faverney et al. 2004), since estrogenic compounds are capable of producing free radicals (Wyllie and Liehr 1997). Ait-Aissa et al. (2003) reported that E2-induced VTG was not altered by heavy metals (such as Hg, Pb, and Cu) (Ait-Aissa et al. 2003), but Canapa et al. (2007) observed that VTG mRNA was induced by Cd. In order to identify whether exposure to Cd influences the ROS level of hepatocytes, the occurrence of oxidative stress was examined in the present study, and was confirmed after 48 h exposure (Fig. 3). Co-treatment of $CdCl_2$ and E2 was found to induce ROS. A previous study found that the production of free oxygen radicals appears to be related to the induction of VTG (Radice et al. 2002; Radice et al. 2004). In male bullfrog hepatocytes, VTG and ROS levels, which were induced by 100 nM $CdCl_2$ and 100 nM E2, did not show any inhibition of their estrogenic activity. Co-treatment with 100 nM $CdCl_2$ and 100 nM E2 induced the same level of VTG as that with 1 μM E2 alone. It is important to note that E2 and Cd can both induce ROS in male bullfrog hepatocytes.

It is known that in cells ligand-free estrogen receptors (ER) interact directly with established chaperone function proteins (Hsp90, Hsp70), stabilizing the receptor in an inactive state. In the existence of endocrine disrupting chemicals, these heterocomplexes are dissociated to allow the ligands to bind the receptor followed by the gene expression of vitellogenin (Guevel et al. 2000). In other studies, it was also reported that the production of reactive oxygen species (ROS) generates misfolded proteins (Kim et al. 2007). These misfolded proteins then can combine with the molecular chaperone such as Hsp70 and Hsp90 forming protein complexes. This may lead to a competition between ER heterocomplexes and misfolded proteins resulting in the dissociation of heterocomplexes. This may explain the results in our study. First, E2 has the ability to bind ER but Cd does not. So VTG was not induced by $CdCl_2$ but was induced by E2 although both of Cd and E2 can induce ROS. In the co-treatment of E2 and $CdCl_2$, the formation of ROS did increase slightly compared to the treatments alone but not significantly. It is thought that this

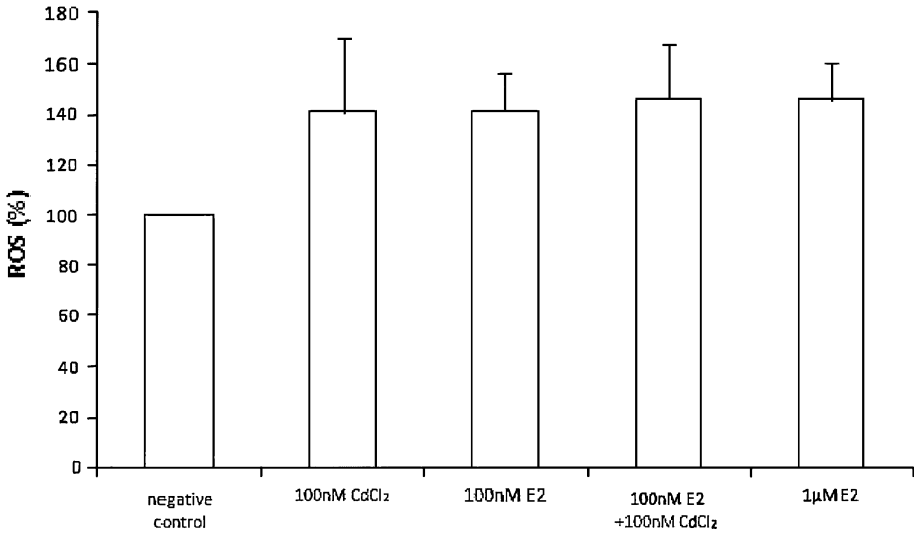

Fig. 3 ROS in bullfrog hepatocytes treated after 48 h. The results are means ± SD and expressed in % versus negative control

slight increase in free oxygen radicals in the co-treatment released more ER to bind to E2, suggesting that ROS is an important mediator in vitellogenesis.

In conclusion, we demonstrated that $CdCl_2$ did not suppress vitellogenesis in male bullfrog hepatocytes, but in combination with E2 did increase ROS production slightly above levels for either $CdCl_2$ or E2 alone. It is thought that this slight increase in ROS production may have been the mechanism by which Cd metal appeared to have acted cooperatively with E2 to induce VTG production in male bullfrog hepatocytes.

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