Cadmium Disrupts the Diethylstilbestrol Effect on Very-Low-Density Apolipoprotein II Gene Transcription in the Liver of Japanese Quail (*Coturnix japonica*)

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To explore the possible endocrine-disrupting effect of cadmium (Cd), we have tested its role on very-low-density apolipoprotein II mRNA (apoVLDL II) expression by diethylstilbestrol (DES) in Japanese quail. Three-week-old immature male quail were intraperitoneally injected with DES (10 mg/kg body wt and 24 hrs before sacrifice) to induce apoVLDL II mRNA in the liver. The same birds were intraperitoneally injected with 0.001, 0.01, 0.1, 1 or 3 mg/kg body wt of CdCl₂ at 48 hrs before DES injection. A reverse-transcription polymerase chain reaction (RT-PCR) was applied to quantify the mRNA level. We found that 0.1 mg/kg body wt of Cd injection contributed an additive effect in DES-stimulated apoVLDL II mRNA expression, whereas Cd injection above this level suppressed the effect. The present results suggest that Cd has biphasic effects on estrogen-dependent gene transcription, and 0.1 mg/kg body wt of Cd administration can mimic the estrogen functions in Japanese quail.

Key words: cadmium, diethylstilbestrol, Japanese quail, liver, very-low-density apolipoprotein II

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Introduction

Cadmium (Cd) is a toxic heavy metal and ubiquitous environmental pollutant. Recently, it has received the increased attention of researchers as a possible endocrine disrupter. It has been reported that steroidogenesis and steroid hormone actions can be altered variably by Cd exposure (Paksy *et al.*, 1992; Olsson *et al.*, 1995), depending upon the doses of Cd used or other factors (Henson and Chedrese, 2004). In contrast, Shimada *et al.* (1998) reviewed that steroid compounds or some steroid antagonists can modify the adverse effects of Cd exposure. However, it is still controversial what type of effect is exerted by Cd on endocrine functions at different exposure levels and in different species (Silva *et al.*, 2006).

Tagiguchi and Yoshihara (2006) discussed the estrogen- and androgen-like activities of Cd *in vivo* and *in vitro* by directly binding to estrogen and androgen receptors. One major avian yolk protein gene, encoding very-low-density apolipoprotein II (apoVLDL II) remains in-

active prior to stimulation with estrogen (Cochrane and Deeley, 1988). The transcription of this gene is specific to laying females and can be induced by estrogens in immature males (Hanafy *et al.*, 2006). There are reports that Cd interferes with the estrogen action on some yolk protein gene expression in fish (Olsson *et al.*, 1995) and insects (Hahn *et al.*, 2002), but such information on an avian model is not available.

In this study, we used a synthetic estrogenic compound, diethylstilbestrol (DES), to induce the apoVLDL II gene in immature male Japanese quail (*Coturnix japonica*) and investigated the possible role of Cd in the transcriptional effect of DES to express this gene. We have evaluated the Cd effects by measuring the relative expression of apoVLDL II mRNA in liver tissues using a semiquantitative RT-PCR technique.

Materials and Methods

Animal management and chemicals

Three-week-old immature male quail were used in this experiment. They were maintained under a 24-hr continuous lighting program with free access to commercial quail diet (Tokai-Kogyo, Toyohashi, Japan) and drinking water. DES (4,4'-(1,2-diethyl-1,2-ethene-diyl)-bisphenol; Sigma, St. Louis, MO, USA) and CdCl₂·2.5H₂O

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(Wako Pure Chemical Industries Ltd., Japan) were used as the test chemicals. Care and sacrificing of animals were done under the approved rules of the Animal Care and Use Committee of Shizuoka University, Japan.

Experimental plan

The whole experiment was done in two separate steps. In step one; we established a semi-quantitative RT-PCR condition for the study of DES-induced apoVLDL II mRNA expression in the liver of immature male quail. To do this, we first administered DES (10 mg/kg body wt) by intraperitoneal injection with sesame oil (0.1 ml/100 g body wt) and the birds were sacrificed 6, 12, 24, 48 or 72 hrs after the injection. To determine the time course of the effect of DES on apoVLDL II mRNA expression, we monitored the dose-response trail of DES using doses ranging from 0 to 10 mg/kg body wt. Control birds received only oil in their injections.

In step two, we investigated the role of Cd in the induction of apoVLDL II mRNA by DES. First, we observed the time effects of Cd administration on DES-induced apoVLDL II mRNA expression in birds that were intraperitoneally injected with 3 mg/kg body wt Cd (in saline solution and 0.2 ml/100 g body wt) from 0 to 72 hrs before DES injection. We then tested the effects of 0 to 3 mg/kg body wt Cd at the most effective time point of Cd injection on DES-induced apoVLDL II mRNA expression. The control birds received both the saline and oil in the same amounts as the Cd-injected birds.

Tissue preparation and RNA extraction

Birds were decapitated, and the livers were extracted, flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted by RNeasy (Takara, Japan) according to manufacturer's protocol. The isolated RNA pellet was dissolved in diethyl pyrocarbonate-treated water, and the RNA concentration and purity were determined by measuring the light absorption of RNA at 260 and 280 nm using a spectrophotometer (Gene Quant; Pharmacia Biotech, Uppsala, Sweden). RNA samples exhibiting an A_{260} to A_{280} ratio of 1.8 or more were used in the next portion of the experiment.

Semiquantitative RT-PCR

We quantified the level of apoVLDL II mRNA expression in different samples using a semiquantitative RT-PCR protocol (Uzbekova *et al.*, 2000) with necessary modifications to satisfy our experimental conditions in birds. Briefly, 2 μg of total RNA was reverse-transcribed according to the protocol of the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using oligo(dT) primer. The genomic DNA-contaminated RNA samples were avoided by checking them in RT-negative (no reverse transcriptase) reactions. The cDNA was amplified by PCR reaction with primer sets of 5'-GGTACAATACAGGGAATTGGTGATA-3' (forward) and 5'-AACTAGTAGCCTAGTTTCCGTTCC-3' (reverse) for apoVLDL II (product size, 318 base pairs; GenBank accession no. S82591) and 5'-CCAGGGCTGCTGTCCTTTCT-3' (forward) and 5'-CAGAGACAT

TGGGGGTTGGC-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size, 669 base pairs; GenBank accession no. Z19086).

The total reaction volume was 50 μl containing 1 μl of cDNA, 10 pmol of each forward and reverse primer, 4 μl of dNTPs (2.5 mM each), 5 μl of 10X PCR buffer and 1.25 U of Taq DNA-polymerase (Takara Bio Inc, Japan). The PCR profile consisted of an initial denaturation at 94°C for 1 min, followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 45 sec, extension at 72°C for 1 min for apoVLDL II and 20 cycles of denaturation at 94°C for 1 min, annealing at 59.5°C for 1 min and extension at 72°C for 1 min for GAPDH. The PCR products were then subjected to electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide and photographed under ultraviolet illumination. Intensity of the fluorescence, which is related to the concentration of DNA in the gel, was determined using image analysis software (ImageJ, NIH, USA).

The result was expressed as the ratio of apoVLDL II mRNA signal intensity and that of GAPDH as the standard. To obtain the actual difference in mRNA expression, we chose the PCR cycle number at a linear phase of amplification for both genes.

Statistical analyses

Data obtained from replicated experiments were analyzed using analysis of variance to find out significant difference and the Tukey's multiple-range test (Snedecor, 1956) to identify the difference between groups.

Results

We found that 3-week-old male quail maintain a very low level of apoVLDL II mRNA in their liver. To avoid the influence of the basic mRNA on the interpretation of the exact level of apoVLDL II expression by DES stimulation only, we fixed the suitable amplification cycle number in the PCR reaction so that expression was near the threshold.

Results obtained from the time course experiment of DES effect (Fig. 1) showed that a single injection of 10 mg/kg body wt of intraperitoneal DES stimulated the liver of immature male quail to induce a significant level of apoVLDL II mRNA within 6 to 24 hrs after injection, which reached a negligible level at 48 hrs after injection. Data presented in Fig. 2 demonstrated that DES injection enhanced the apoVLDL II mRNA expression dose-dependently, whereas 1 mg/kg body wt and higher doses of DES induced apoVLDL II mRNA significantly at 24 hrs after administration.

We selected the 10 mg/kg body wt dose of DES for subsequent experiments, as it produced a strongly significant difference ($p < 0.01$) in the level of apoVLDL II mRNA among the doses used as compared to the control (Fig. 2). In spite of the significant difference observed in apoVLDL II mRNA expression as early as at 6 hrs after DES injection (Fig. 1), we selected the 24-hr time period of DES administration because it facilitated the longer

Fig. 1. Time course of diethylstilbestrol (DES) effect on the expression of very-low-density apolipoprotein II (apoVLDLII) mRNA in the liver of immature male Japanese quail. Three-week-old quail were intraperitoneally injected with 10 mg/kg body wt DES, and the mRNA expressions relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by RT-PCR at the indicated time period after DES injection. Values are the mean $\pm$ SE of at least 3 replications. ** $p < 0.01$, when compared to the control. The scanned autoradiograph shown in the upper row is a representative of the replicated results.

Fig. 2. Dose-response of very-low-density apolipoprotein II (apoVLDLII) mRNA expression in the liver of immature male Japanese quail. Three-week-old quail were intraperitoneally injected with indicated doses of diethylstilbestrol (DES), and the mRNA expressions relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by RT-PCR at 24 hrs after injection. Values are the mean $\pm$ SE of 3 replications. ** $p < 0.01$ and * $p < 0.05$, when compared to the control. The scanned autoradiograph shown in the upper row is a representative of the replicated results.

co-exposure of DES and Cd in the next experiments.

We found that a 3 mg/kg body wt injection of Cd at 48 hrs before DES administration suppressed the apoVLDL II mRNA expression significantly compared to that found in birds that received only DES at 24 hrs after DES injection (Fig. 3). The results also showed an insignificant positive influence of Cd on the effect of DES to induce this gene when the birds received Cd at 0 hr before DES administration (co-injection). The data shown in Fig. 4 indicated that different doses of Cd at 48 hrs before DES administration induced apoVLDL II mRNA differentially. A pretreatment of birds with lower doses of Cd increased the DES action for apoVLDL II mRNA induction, and the 0.1 mg/kg body wt of Cd injection significantly increased the apoVLDL II mRNA expression compared to that found in birds injected with only DES. On the other hand, Cd administration above 0.1 mg/kg body wt suppressed the expression with higher magnitudes as the doses of Cd increased. Different doses of Cd administered simultaneously with DES injection did not significantly influence the DES effect to induce apoVLDL II mRNA (Fig. 5). We also investigated the only Cd-injection effect on apoVLDL II mRNA expression in liver tissues and found no changes in the basal level of this mRNA until 5 days after injection (data not shown).

Discussion

The exogenous estrogen-stimulated expression of apoVLDL II mRNA in the male bird liver has been confirmed by some researchers. The dose-response of DES (Fig. 2) to induce a significant level of apoVLDL II mRNA was similar to that found by Hanafy *et al.* (2006) in immature male quail, but the exposure time, 6 hrs after DES injection, was earlier to induce a significant level of apoVLDL II mRNA compared to the 24 hrs found by them. The maximum synthesis of hepatic apolipoprotein B at 12–16 hrs after estrogen treatment in cockerel (Capony and Williams, 1980) strongly supported our results. However, differences in the method of mRNA detection or doses of DES used might be the causes of this slight variable expression pattern.

In the present study, we found an additive effect of Cd on DES action in apoVLDL II mRNA expression at lower doses. We hypothesize that Cd shows this effect either by representing itself as an estrogenic compound or by enhancing the activity of transcription factors involved in this transcription channel. The estrogenic activity of Cd reported by Martin *et al.* (2002) and Safe (2003) in the rat supported the first part of this assumption, but the lack of changes in apoVLDL II mRNA expression found in our only Cd injection experiment denied the potentiality of Cd

Fig. 3. Effects of cadmium administration on diethylstilbestrol (DES)-induced very-low-density apolipoprotein II (apoVLDLII) mRNA expression in the liver of immature male Japanese quail. Three-week-old quail were intraperitoneally injected with 3 mg/kg body wt cadmium before the indicated time of 10 mg/kg body wt DES injection. The mRNA expressions relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by RT-PCR 24 hrs after DES injection. Values are the mean $\pm$ SE of at least 3 replications. * p < 0.05, when compared to DES only. The scanned autoradiograph shown in the upper row is a representative of the replicated results.

as an estrogen. Further, the lack of estrogenic activity of Cd found by Silva *et al.* (2006) in their yeast estrogen screen assay strongly supported the nonestrogenic action of Cd alone found in the present study.

The latter part of the hypothesis, that is, that Cd exerts its effect via a transcription factor, might be strengthened by some recent in-depth studies on Cd action using cell line cultures. Estrogen receptors are the transcriptional regulator that become activated upon response to estrogen and bind to it to form a hormone-receptor complex and regulate the transcription of estrogen-responsive genes. To support this idea, Stoica *et al.* (2000) have reported that lower doses of Cd mimic estradiol effects in the COS-1 cell line by binding with the hormone-binding domain of estrogen receptors. In addition, Henson and Chedrese (2004) discussed the possible mechanism of Cd action that is substitutive for the Zn role in the Zn-finger motif structure in the hormone-binding domain of estrogen receptors.

The suppression of apoVLDL II mRNA in DES-injected birds by the administration of more than 0.1 mg/kg body wt of Cd might be due to the blocking of DES action

Fig. 4. Effects of cadmium administration on diethylstilbestrol (DES)-induced very-low-density apolipoprotein II (apoVLDLII) mRNA expression in immature male quail. Three-week-old quail were intraperitoneally injected with the indicated doses of cadmium at 48 hrs before 10 mg/kg body wt of DES injection. The mRNA expressions relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by RT-PCR 24 hrs after DES injection. Values are the mean $\pm$ SE of at least 3 replications. ** p < 0.01 and * p < 0.05, when compared to DES. The scanned autoradiograph shown in the upper row is a representative of the replicated results.

by Cd or due to alterations in the DNA domain encoded for apoVLDL II mRNA replication. In support of the former idea, Shimada *et al.* (1999) reported that steroid function can be modulated by Cd. In addition, a reduction in the estradiol-stimulated vitellogenin mRNA level by Cd has been reported in the rainbow trout liver (Hwang *et al.*, 2000; Vetillard and Bailhache, 2005). And, the latter assumption agrees with the evidence of Cd-induced DNA damage reported by Bagchi *et al.* (1996). They noted that the reactive oxygen species produced by Cd-mediated cellular toxicity causes this damage. Furthermore, the inhibition of DNA repairs by Cd, through unbalancing of necessary enzymes, is even clearer evidence of DNA damage by Cd exposure (Giaginis *et al.*, 2006).

The total Cd exposure period of 72 hrs, the sum of 48 hrs pre-exposure and 24 hrs post-exposure time of the DES injection (Fig. 3), needed to show a significant effect was within the normal range of time courses required for mineral metabolism in birds. The time required to show Cd effects on estrogen-responsive gene expression in fish was similar (Vetillard and Bailhache, 2005) to this study result. Pretreatment of Cd at longer than 48 hrs before DES administration did not show any effect, which might

Fig. 5. Co-injection effect of cadmium administration along with 10 mg/kg body wt diethylstilbestrol (DES) to induce very-low-density apolipoprotein II (apo-VLDLII) in the liver of three-week-old immature male Japanese quail. The mRNA expressions relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by RT-PCR 24 hrs after injection. Values are the mean $\pm$ SE of 3 replications. The scanned autoradiograph shown in the upper row is a representative of the replicated results.

be due to the complete excretion of the one-time injection of a smaller amount of Cd or might be due to the binding of Cd with metallothionein induced by Cd stimulation (Klaassen *et al.*, 1999).

Finally, the results found in this study together with the details described above suggest that Cd administration in Japanese quail disrupts estrogen effects biphasically to express the apoVLDL II gene in liver tissues. And, the lower doses of Cd administration modulated the estrogen-induced gene expression not by acting as an estrogenic compound, but rather by disrupting the gene transcription via transcription factors.

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