INTRODUCTION

Dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), halogenated aromatic hydrocarbons, and polynuclear aromatic hydrocarbons have garnered attention as environmental contaminants because they cause an extensive range of adverse effects in various organisms (Peden-Adams et al., 1998). These pollutants are known to be teratogenic, immunotoxic, and toxic to the reproductive systems of avian species (Larson et al., 1995; Walker et al., 1996; Blankenship et al., 2003). A difference in embryo toxicity, or sensitivity to dioxin-like congeners of ≥1,000 times among avian species, has been reported (Brunström and Reutergårdh, 1986). Chickens have been reported to be the most highly evolved avian species with regard to dioxin sensitivity (Higginbotham et al., 1968). Chickens are affected by dioxin toxicity despite their low position in the food chain, and they are considered to accumulate low amounts of environmental pollutants. In 1976, millions of domestic chickens were killed because they consumed feed that was contaminated with TCDD and halogenated aromatic hydrocarbons (Philp, 2001).

The aryl hydrocarbon receptor (AhR) is a critical factor for dioxin sensitivity. The AhR is a member of the basic-helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of proteins and is a transcription factor activated by ligand binding. The ligand-activated AhR causes transcription of the cytochrome P450 1A5 (CYP1A5) gene in African Green Monkey Cercopithecus aethiops kidney cells (COS-7) treated with Sudan III. In primary cultures of ostrich kidney cells, CYP1A5 expression was induced by Sudan III at a lower (or almost identical) concentration to that observed in the chicken. The present study revealed a new AhR ligand sensitive avian species (i.e., the ostrich).

Key words: ostrich, avian species, aryl hydrocarbon receptor, CYP1A, dioxin

ABSTRACT A 1,000-fold difference has been reported in dioxin sensitivity between avian species. This difference is because the 2 amino acids in the type 1 aryl hydrocarbon receptor (AhR1), at positions 325 and 381, correspond to Leu324 and Ser380 in chickens. The chicken had been reported to be the only avian species to possess a sensitive form of AhR1. This is the first study to reveal that the ostrich (Struthio camelus), a nonchicken species, also has a pair of amino acids (Ile-325 and Ser-381) that show high ligand affinity. However, the alignment of the AhR1 cDNA sequence showed that the AhR sequence in the ostrich was different than that of other avian species even though the critical amino acids were observed at positions 325 and 381. Ostrich AhR1 was also evaluated in a heterologous expression study. Ostrich AhR1 showed very high transcriptional activity of the cytochrome P450 1A5 (CYP1A5) gene in African Green Monkey Cercopithecus aethiops kidney cells (COS-7) treated with Sudan III. In primary cultures of ostrich kidney cells, CYP1A5 expression was induced by Sudan III at a lower (or almost identical) concentration to that observed in the chicken. The present study revealed a new AhR ligand sensitive avian species (i.e., the ostrich).

Key words: ostrich, avian species, aryl hydrocarbon receptor, CYP1A, dioxin

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with an amino acid sequence of 325-Ile and 381-Ser exhibit the highest sensitivity to dioxins (Karcher et al., 2006). Chickens are the only species to possess this type of AhR1, which shows the highest ligand binding affinity to TCDD.

Ostriches are the largest birds with 2 toes (most other birds have 3 toes) and have gained popularity because of their meat. Ostrich meat is considered to be healthy because it is low in fat, calories, and cholesterol. Accordingly, the number of ostrich farms worldwide is increasing. Ostrich products have become so popular that the demand exceeds supply, and ostrich meat has become a major meat product. The main problem with the farming of ostriches is the low rate of egg hatching. The number of pores in the eggshell or shell thickness have been postulated to be the causes of the low rate of egg hatching, but other factors (e.g., chemical contamination) could be the cause. A failure of egg hatching also causes edematous embryos, which are also observed in chicken embryos injected with TCDD (Gonzalez et al., 1999).

In the present study, we found a new highly sensitive AhR from an avian species (i.e., the ostrich). A phylogenomic study revealed that Struthioniformes (ostriches) is the family that branches earliest in the avian stem (Hackett et al., 2008; i.e., the ostrich could have a specific characteristic that all other avian species have lost in the past). Indeed, the ostrich is said to have existed for >2 million years, and is often called a living fossil due to its primitive appearance. We cloned ostrich AhR and compared AhR ligand sensitivity in vitro with that of the chicken AhR. This is the first report to show an avian species other than chickens to possess a highly sensitive AhR with regard to ligand affinity.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

All test agents used were of reagent grade. The TRI reagent was purchased from Sigma-Aldrich (St. Louis, MO). Oligo(dT) primer, reverse-transcription (RT) buffer, and ReverTra Ace were obtained from Toyobo (Osaka, Japan). Primer sets were purchased from Invitrogen (Carlsbad, CA). Ex Taq Polymerase was purchased from TaKaRa (Tokyo, Japan). All other reagents were of analytical grade or the highest quality available and purchased from Wako Pure Chemical Industries (Tokyo, Japan).

**Birds**

All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. Tissues from 4 female ostriches (Struthio camelus; age, 1 to 2 yr) were provided by Hokkaido Ostrich Farm Kuroda (Hokkaido, Japan) in November 2009. Portions of the kidneys were conserved in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) and maintained at 4°C for several hours until use for primary culture. The remaining kidneys and other tissues (brain, heart, lung, liver, intestine, spleen, and ovary) were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

**Primary Culture of the Kidney Cells of Ostriches and Chickens**

The central part of the kidney was extirpated under aseptic manipulation. This part was minced in DMEM and centrifuged at 180 × g for 3 min at room temperature. The pellet was dissolved in PBS containing modified type-I collagenase, agitated for 1 h at 37°C, then centrifuged again at 90 × g for 3 min at room temperature. Precipitated renal epithelial cells were cultured in DMEM containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and a 1% antibiotic mixture (100 U/mL of penicillin and 100 μg/mL of streptomycin) in the presence of 5% CO2 at 37°C.

**CYP1A5 Expression in the Primary Cultures of Kidney Cells of Ostriches and Chickens**

Primary cultures of renal epithelial cells from ostriches and chickens were plated on 24-well plates. Cells were maintained until they reached 80% confluence, then exposed to 0.1% dimethyl sulfoxide (DMSO) or Sudan III (10 nM, 100 nM, 1 μM, and 100 μM) for 12 h. Cell viability was determined using a Cell Counting Kit (Dojindo, Kumamoto, Japan). Total RNA was prepared by the single step method (Chomczynski and Sacchi, 1987), and cDNA was synthesized according to the method of Darwish et al. (2010). Quantitative real-time RT-PCR was undertaken using the Step One Plus Real-Time PCR System (Applied Biosystems, Bedford, MA). The PCR reactions were set in 10-μL volumes. The PCR reaction mixture was prepared with SYBR qPCR Mix (Toyobo), 0.3 μM of each primer, 100 ng of cDNA, 1 × ROX reference dye, and RNase-free water. The mixture was made up to a final volume of 10 μL. The reaction cycle consisted of an initial denaturation at 95°C for 40 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 15 s, and extension at 72°C for 45 s. For CYP1A5, the following primers were set as sense 5′-CAGCAGCTGGGATTTGCGTGTT-3′, antisense 5′-CTGGTAGGATGTCCACATTTGTTGT-3′ (Yasui et al., 2007). For β-actin, the sense primer was 5′-TGGTTATGAGTGACATCGACAC-3′, and the antisense was 5′-CGGATATCCACATCGCATT-3′ (Head and Kennedy, 2007). Quantitative values were calculated from the threshold cycle number of each sample (absolute calibration curve) and normalized to the β-actin content. The mRNA levels of each sample were expressed as a relative expression to that of nontreated cells of each species.
Cloning and Sequencing of AhR

The RNA were isolated from the livers of ostriches and chickens and reverse-transcribed to cDNA primed by Oligo(dT). The full length of AhR1 was cloned by RT-PCR using the primers listed in Table 1. These primers were designed with reference to sequences of other avian AhR, and targeting only AhR1, not AhR2. The PCR conditions for AhR1 started with an initial denaturation at 94°C for 2 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 66°C for 45 s, and extension at 72°C for 3 min, followed by a final extension at 72°C for 5 min. The PCR products were direct-sequenced using avian AhR1–1 to AhR1–4 at an annealing temperature of 50°C. The partial sequence of AhR2 was cloned by AhR-A1

\[ 5' - CGGGATCCARGCICTSAAYGGITT-3' \]

and AhR-B1

\[ 5' - GCTCTAGACATICCRCTYTCICCIGTYTT-3' \]

(Karchner et al., 2000). To obtain information about the DNA sequence of the ligand-binding domain, AhR2-LBD-F

\[ 5' - TCTCCAGACAAAGCACAAGCTGGAC-3' \]

and AhR2-LBD-R

\[ 5' - GTACAGGACTGCTTCCCCGTTG-3' \]

were used. The DNA sequence was confirmed in 4 ostriches.

Phylogenetic Tree

The nucleotide sequences of AhR and AhRR were aligned using ClustalX2 (Larkin et al., 2007). Accession numbers for the sequences used in this phylogeny are listed in Table 2. The spineless isoform of Drosophila melanogaster was added and used as an outgroup. Alignments for only ~300 amino acids (including PAS-A and PAS-B domains) were included; the areas with gaps were excluded. The phylogenetic tree was constructed with a bootstrap neighbor-joining tree and viewed by the neighbor-joining plot (Perrière and Gouy, 1996).

AhR1 and AhR2 Expression Ratio

The PCR products of AhR1 and AhR2 were purified using a QIA Quick PCR Purification Kit (Qiagen, Valencia, CA) and used as standards. Quantitative real-time RT-PCR was estimated as mentioned previously. The sense and antisense sequences of AhR1 primers were 5'-AGAAAGGGAAGGATGGCAG-3' and 5'-CTGGTACCTCCGTTCTCTAC-3', respectively. For AhR2, the sense primer was set as 5'-AACCGCTCCTTTGGGGAGG-3', and the antisense was 5'-TCCCG-

GAGGAGTTATCCAGCAA-3'. The measurement was undertaken in duplicate. Approximately logarithmic curves were made from the threshold cycle of 4 concentrations of the standards. Absolute copy numbers of each AhR were obtained from the standard curve, and an AhR1:AhR2 expression construct was calculated.

Expression Constructs

The PCR products of full-length AhR1 from the ostrich, chicken, and cormorant (Phalacrocorax carbo) were ligated into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). Cormorant Arnt1 (pcDNA-ccARNT1), CYP1A5 promoter containing 6 Xenobiotic Responsive Elements (XRE; pGL4-cCYP1A5–6XRE; Lee et al., 2009), and pRL-SV40 (Promega, Madison, WI) were also used for the reporter assay.

Cell Culture

The African Green Monkey Cercopithecus aethiops kidney cells (COS-7) were purchased from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Cells were maintained in DMEM with 10% FBS in an atmosphere of 5% CO2 at 37°C. Cells were plated on 24-well plates (Yasui et al., 2007). The medium was changed 24 h after plating on to serum-free medium (VP-SFM, Invitrogen).

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common cormorant AhR1</td>
<td>AB109545</td>
</tr>
<tr>
<td>Black-footed albatross AhR1</td>
<td>AB106109</td>
</tr>
<tr>
<td>Common tern AhR1</td>
<td>AF192503</td>
</tr>
<tr>
<td>Chicken AhR</td>
<td>AF192502</td>
</tr>
<tr>
<td>Mouse AhR</td>
<td>M94623</td>
</tr>
<tr>
<td>Human AhR</td>
<td>L19872</td>
</tr>
<tr>
<td>Xenopus laevis AHR1a</td>
<td>AY635782</td>
</tr>
<tr>
<td>Xenopus laevis AHR1b</td>
<td>AY635783</td>
</tr>
<tr>
<td>Killifish AhR1</td>
<td>AF024591</td>
</tr>
<tr>
<td>Killifish AhR2</td>
<td>U29679</td>
</tr>
<tr>
<td>Black-footed albatross AhR2</td>
<td>AB106110</td>
</tr>
<tr>
<td>Common cormorant AhR2</td>
<td>AB287294</td>
</tr>
<tr>
<td>Killifish AhRR</td>
<td>AF443441</td>
</tr>
<tr>
<td>Human AhRR</td>
<td>AB033060</td>
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<tr>
<td>Mouse AhRR</td>
<td>AB015140</td>
</tr>
<tr>
<td>Drosophila melanogaster spineless</td>
<td>AF350630</td>
</tr>
</tbody>
</table>

Table 1. Primers for avian aryl hydrocarbon receptor (AhR)

<table>
<thead>
<tr>
<th>Item</th>
<th>Forward 5’-sequence-3’</th>
<th>Reverse 5’-sequence-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian AhR full</td>
<td>CAGGATGAACCCCAATGTCAC</td>
<td>GTCACATAAATCCACATGAGCTCCA</td>
</tr>
<tr>
<td>Avian AhR1–1</td>
<td>GGATGAACCCCAATGTCACCTA</td>
<td>ATCGTCTGTGAAAATTCATA</td>
</tr>
<tr>
<td>Avian AhR1–2</td>
<td>TCATCTGCAGGTTACGATGCTACACAGACTCATCTTGCCTTA</td>
<td>ACAAGATCTCATCTGCTTTA</td>
</tr>
<tr>
<td>Avian AhR1–3</td>
<td>TGGCCCTTCATGTTGGCCTCGTGTGA</td>
<td>TCCAAATTTGGAACATCCCAT</td>
</tr>
<tr>
<td>Avian AhR1–4</td>
<td>CAGCTCTGTCAAAGAGATGAAA</td>
<td>TTACATAATGCCACTAGA</td>
</tr>
</tbody>
</table>

Table 2. Accession numbers for DNA sequences of aryl hydrocarbon receptor (AhR) and aryl hydrocarbon receptor repressor (AhRR)
Transfection and Luciferase Assay

Transfections were carried out 24 h after changing the medium. Approximately 30 ng of AhR1, 300 ng of pcDNA-ccARNT1, 120 ng of pGL4-ccCYP1A5–6XRE, and 18 ng of pRL-SV40 were transfected per well. The total amount of transfected DNA was kept at 500 ng by the addition of an empty pcDNA vector. The DNA were diluted with OPTI-MEM medium (Invitrogen) and mixed with 1.25 μL per well of FuGENE HD Transfection Reagent (Roche, Basel, Switzerland). Four hours after transfection, cells were exposed to 0.1% DMSO or Sudan III (AhR legend, final concentration, 10 μM; Refat et al., 2008) and diluted with VP-SFM. Cells were lysed after 18 h of exposure, and luminescence was measured using a Dual-Glo Luciferase Assay System (Promega). Final values of luminescence were expressed as a ratio of the luciferase units from the firefly to luciferase units from renilla (Renilla reniformis).

Tissue Distribution of AhR1, AhR2, and CYP1A5 in the Ostrich

The RNA were extracted from the brain, heart, lung, liver, intestine, kidney, spleen, and ovary and reverse-transcribed to cDNA. Quantitative real-time RT-PCR were completed as mentioned previously. The same primers were used for AhR1, AhR2, CYP1A5, and β-actin. The PCR conditions were the same as detailed above. Melting curve analyses and agarose gel electrophoresis confirmed amplification of a single amplicon of the expected size as well as the absence of primer dimers and genomic DNA amplification. Each measurement was performed in duplicate and repeated thrice. The expression of each gene was normalized to the expression of β-actin and calculated relative to the corresponding gene expression in the liver for each bird.

Statistical Analyses

Values are the mean ± SD. Statistical significance was evaluated using the Tukey-Kramer honestly significant difference test (JMP statistical package, SAS Institute Inc., Cary, NC). A P-value <0.05 was considered significant.

RESULTS

Induction of CYP1A5 in the Primary Cultures of the Kidney Cells of Ostriches and Chickens

The CYP1A5 induction was observed in the primary cultures of the kidney cells of ostriches and chickens after treatment with AhR ligand and Sudan III (Figure 1). There were no changes in the viability or morphology of cells between Sudan III-treated and nontreated groups (data not shown). Comparison of the induction of CYP1A5 by Sudan III in the 2 cell cultures revealed that the ostrich cell cultures were more sensitive to AhR ligands than the chicken cell cultures. The concentration of Sudan III required to induce CYP1A5 mRNA was 2-fold higher than the control (100 nM in the ostrich and >1 μM in the chicken, Figure 1). Use of the AhR ligand 3-methylcholanthrene (3-MC) revealed a similar phenomenon (data not shown).

AhR1 Sequence in the Ostrich

The full-length cDNA sequence of ostrich AhR1 encoded 863 amino acid residues. It shared 88% of the cDNA sequence with the chicken AhR and had the greatest alignment with AhR from the common cormorant and black-footed albatross (91%). The chicken and cormorant had a very similar amino acid sequence in the ligand binding domain (except for the 3 amino acids at positions 258, 325, and 381), whereas 7 amino acids were different in this region only in the ostrich (Figure 2).

AhR2 Sequence in the Ostrich

A partial sequence of AhR2 (760 bp) containing the ligand binding domain was confirmed. The AhR2 shared 87% of its nucleic acid sequence with the common cormorant and 80% with the chicken.

Phylogenetic Analyses

To distinguish between AhR isoforms, a phylogenetic tree was constructed. Ostrich AhR1 and AhR2 belonged to the respective AhR1 and AhR2 clades (including those of fishes and mammals; Figure 3).

AhR1 and AhR2 Expression Ratio

Quantitative mRNA expression of AhR1 and AhR2 in the ostrich revealed AhR1 to account for 96.6% of AhR (data not shown).

Luciferase Reporter Assay

The luciferase reporter assay was carried out to compare the transcriptional activity of ostrich AhR1 with that of low/high dioxin-sensitive avian species. Comparison of transfected cells with AhR1 from each species treated with Sudan III revealed that the transcriptional activity of ostrich AhR1-transfected cells (3.4-fold) was significantly higher than that from cormorant (0.9-fold) or mock (1.0-fold)-transfected cells, and was as high as that of chicken (3.5-fold)-transfected cells (Figure 4).

Tissue Distribution of AhR1, AhR2, and CYP1A5 in the Ostrich

The AhR1, AhR2, and CYP1A5 mRNA expressions were observed in all tissues examined. Liver tissue showed the highest expression of AhR1, AhR2, and
CYP1A5. High expression of AhR1 was also observed in the heart (Figure 5a–c).

**DISCUSSION**

The AhR is an intracellular ligand-dependent transcriptional factor. It binds dioxins and dioxin-like compounds, is transactivated, and mediates the toxicity of these compounds by cellular processes (Long and Bonefeld-Jørgensen, 2012).

Ostriches are floor-reared birds, regardless of whether they live on farms or in the wild. Thus, they are at risk of exposure to environmental pollutants via the food they consume. In addition, ostriches have quite long digestive tracts and take a long time to ingest food. This could increase their total intake of environmental pollutants.

In the present study, the sensitivity of ostriches to AhR ligands was tested in comparison with other avian species (Figure 1). In the primary cultures of kidney cells from chickens and ostriches, the ability to induce CYP1A5 expression was greater in ostrich renal cells than in that of equivalent chicken cells, which are known for their considerable ability to induce CYP1A expression. Ostrich renal cells exposed to Sudan III showed high CYP1A5 expression. The concentration of Sudan III required to induce CYP1A5 expression was lower or almost identical in ostrich primary culture cells compared with those of the chicken. Also, CYP1A5 expression at the same concentration of Sudan III was higher in the primary culture of ostrich renal cells (100 nM). Our results correspond with those of Amsallem-Holtzman and Ben-Zvi (1997). Those authors reported that CYP1A-dependent ethoxyresorufin-O-deethyl-ation and methoxyresorufin-O-demethylation in ostrich hepatic microsomes were 10- and 20-fold higher than that reported in the chicken.

Sequence analyses of AhR1 of the ostrich revealed that it was quite different to that of other avian species but similar to that of the chicken (Figure 2). Two critical amino acids affect the ligand affinity of AhR. This pair (Ile at position 325 and Ser at position 381) has been reported to contribute to high ligand affinity (Karchner et al., 2006; Head et al., 2008). Phylogenetic analyses of AhR of the ostrich were in agreement with the sequence analyses; AhR1 of the ostrich was located beside that of the chicken (Figure 3).

To evaluate the function of AhR1, the luciferase assay was carried out (Figure 4). In the present study, chicken AhR1 was the sensitive model, and AhR1 of the common cormorant was the nonsensitive model. Dioxin sensitivities of both species in vivo and in vitro have been reported (Hoffman et al., 1998; Karchner et al., 2006; Yasui et al., 2007). Cells transfected with ostrich AhR1 exhibited much higher luciferase activity in the ligand-treated condition than in the non-ligand-treated condition. The transcriptional ability of ostrich AhR1 was as high as that of chicken AhR1 (which is known for its high transcriptional ability), and significantly higher than that of the common cormorant (which has AhR of low transcriptional ability; Figure 4). In the present study, Sudan III was used as the AhR ligand (Refat et al., 2008, Fujisawa et al., 2012), and similar data were obtained using 3-MC (data not shown).
Figure 2. Alignment of amino-acid sequences of 3 species of avian type 1 aryl hydrocarbon receptor (AhR1). The amino acid sequences of AhR1 were aligned using ClustalX2. The accession numbers are listed in Table 2. The Per-Arnt-Sim (PAS)-A and PAS-B domains are shown by black bars. Boxes indicate the 2 critical amino acids at positions 325 and 381. The ligand-binding domain is in brackets.
This sensitive AhR1 accounts for most of the total AhR expressed in the ostrich. It could be said that dioxin sensitivity in the ostrich is almost entirely because of AhR1. This tendency was found in the liver, brain, heart, lung, intestine, ovary, kidney, and spleen (Figure 5). This result suggests that AhR1 is dominant throughout the body of the ostrich. It has been reported that the dominant AhR (or the expression ratio of AhR) varies among avian species (Kim et al., 2008). Domination of AhR1 has also been reported in the chicken (Kim et al., 2008). With respect to individual AhR, the greatest expression of AhR1 and AhR2 was in the liver, but they were also highly expressed in the heart, intestine, brain, and kidney. In addition, CYP1A5 was expressed in a similar pattern to that of AhR1 and AhR2 (Figure 5).

In conclusion, the present study was the first to reveal that there is an avian species apart from the chicken that has a highly sensitive AhR1. The AhR ligand sensitivity in the ostrich could be as high (or higher) as that observed in the chicken.

ACKNOWLEDGMENTS

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**Figures**

Figure 5. Tissue distribution of aryl hydrocarbon receptor (AhR) and CYP1A5 mRNA expression in ostriches. Tissue distribution of A) AhR1, B) AhR2, and C) CYP1A5 ostrich mRNA in brain, heart, lung, liver, intestine, kidney, spleen, and ovary. Data were normalized to the expression of β-actin in each tissue. Data are means ± SD (n = 4). Values with different letters (a–e) are significantly different (P < 0.05).


