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The tertiary structures of porcine AhR and ARNT proteins and molecular interactions within the TCDD/AhR/ARNT complex

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that can be activated by structurally diverse synthetic and natural chemicals, including toxic environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In the present study, homology models of the porcine AhR-ligand binding domain (LBD) and the porcine aryl hydrocarbon receptor nuclear translocator-ligand binding domain (ARNT-LBD) were created on the basis of structures of closely related respective proteins *i.e.*, human Hif-2α and ARNT. Molecular docking of TCDD to the porcine AhR-LBD model revealed high binding affinity (−8.8 kcal/mol) between TCDD and the receptor. Moreover, formation of the TCDD/AhR-LBD complex was confirmed experimentally with the use of electrophoretic mobility shift assay (EMSA). It was found that TCDD (10 nM, 2 h of incubation) not only bound to the AhR in the porcine granulosa cells but also activated the receptor. The current study provides a framework for examining the key events involved in the ligand-dependent activation of the AhR.

1. Introduction

Aryl hydrocarbon receptor (AhR) was initially recognized as a mediator of toxicological actions of halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAHs) and PAH-like chemicals (for review see Refs. [1,2]). AhR is a ligand-activated transcription factor belonging, together with AhR nuclear translocator (ARNT) and hypoxia-inducible factor 2α (Hif-2α), to the big basic-helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family [2,21]. Among many exogenous AhR agonists, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent. TCDD and other dioxins are present in air, soil, fresh and salt water sediments as well as in plants and animals. Because of their high lipid solubility and chemical stability, dioxins show an extremely high potential for retention in human and animal tissues. The half-life of TCDD in humans was estimated to be 7–10 years [23,26].

The unliganded AhR is located in cytosol and bound to chaperone proteins (*e.g.*, hsp90, p23, and AhR-interacting protein 1) [6,19,32]. Upon ligand binding, the ligand/AhR complex is translocated to the nucleus, where it combines with ARNT [7,35]. The ligand-AhR-ARNT complex binds to a cognate dioxin-responsive element (DRE) present in regulatory regions of target genes, inducing their transcription [17].

The AhR molecule is composed of multiple functional domains [38]. In the N-terminal end, the AhR contains bHLH domain which is involved in DNA binding, dimerization with ARNT and association with chaperone proteins. The PAS domain, which is situated next to the bHLH domain consists of two structural repeats: PAS-A and PAS-B [32]. PAS-A is involved in the receptor dimerization with ARNT, PAS-B, in turn, is responsible for ligand and chaperone binding [38]. Similarly to AhR, ARNT is composed of bHLH, PAS-A and PAS-B domains, and all domains are required for optimal dimerization with AhR [2].

The understanding of molecular events that underlay the ligand-AhR binding requires a detailed knowledge concerning three dimensional (3D) structures of AhR and ARNT, especially of their ligand binding domains (LBDs). In recent years, molecular modeling was successfully used to create AhR-LBD models for humans [3], mice [3,15,29], rats [3,30], rabbits [3,30], hamsters [3,30], guinea pigs [3,30], zebrafish [3], beluga whales and seals [30]. Despite the fairly broad representation of AhR-LBD models in humans or laboratory and aquatic animals, the lack of knowledge regarding farm

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animals is apparent. This is particularly important in view of the fact that TCDD toxicity varies among animal species. The guinea pig, for example, is much more sensitive to TCDD (LD_{50} = 0.6 μg/kg of body weight),[1] than the hamster (LD_{50} = 5000 μg/kg of body weight) [36]. Such diversity in the animal sensitivity to TCDD may result either from differences in TCDD affinity to AhR or TCDD metabolism. The TCDD-AhR affinity, in turn, is closely associated with a structure of LBD. Therefore, the lack of information concerning TCDD-AhR-ARNT interactions in the pig justifies our interest in this species in the current study. This interest is strengthened by the significance of pigs manifested by using the species as a model animal for the study of many physiological phenomena as well as in human transplantation and nutrition.

The complete mRNA sequences of the porcine *AhR* and *ARNT* which were obtained in our previous experiments ([20]; Sadowska et al., unpublished) were used to construct reliable spatial models of AhR-LBD and ARNT-LBD in the pig by means of homology modeling. To perform this analysis, the crystalline protein structures of the PAS domains of human Hif-2α [10] and ARNT [5] were used in the present study as modeling templates. The aim of the current study was to predict the structure of the porcine AhR- and ARNT-LBD by means of homology modeling. Moreover, the molecular docking was used to examine molecular interactions within the TCDD/AhR/ARNT complex. Finally, the ability of the TCDD/AhR/ARNT complex to bind to the DRE sequence was examined in porcine granulosa cells.

2. Materials and methods

In silico analysis (homology modeling and molecular docking) was performed on the basis of porcine AhR and ARNT cDNA sequences. The sequences were established experimentally by next generation sequencing (NGS) and were submitted to GenBank under the following accession numbers: KM817031.1. (*AhR*) and KP735786.1 (*ARNT*). In addition, transcriptional activity of the TCDD/AhR/ARNT complex in porcine granulosa cells was examined *via* electrophoretic mobility shift assay (EMSA).

2.1. Homology modeling of the AhR-LBD

The predicted amino acid sequence of porcine AhR protein (AIY35111.1) was created on the basis of the cDNA nucleotide sequence of the porcine *AhR*. The AhR-LBD was established within the protein sequence using Pfam tool in the EMBL-EBI database [34]. Three-dimensional structure of porcine AhR-LBD was created by homology modeling. Two crystalline structures of the human Hif-2α protein: apo (Protein Data Bank – PDB ID: 3F1P) and holo (PDB ID: 3H82) were chosen as templates. Both of the X-ray structures are complete and characterized with very low resolution [4]. The current model was created with the presence of THS020, an AhR ligand used previously for Hif-2α modeling. To assure the stability of the modeled structure, the ligand was placed within a binding pocket. Alignment of the amino acid sequence of porcine AhR and the Hif-2α templates was performed by MUSCLE software [11]. The constructed alignment was exported to MODELLER 9v12 program [8], and models with the lowest DOPE (Discrete Optimized Protein Energy) values, generated by MODELLER, were selected for further analysis. The accuracy of each model was verified with ProSAII [37,41], Verify3D [9] and PROCHECK [24] software. The created models were positioned in the 'favored' or 'unfavored' region of Ramachandran plot, and the best model was chosen for further analysis. The structural accuracy of the generated model of porcine AhR-LBD was assessed by its pairwise superpositioning with each of the two human Hif-2α models (UCFF Chimera program) followed by calculating the RMSD (Root Mean Square Deviation) value [31].

2.2. Homology modeling of the ARNT-LBD

The predicted amino acid sequence of porcine ARNT (AKV89672.1) protein was created on the basis of the cDNA nucleotide sequence of porcine *ARNT*. The domain responsible for the AhR binding (ARNT-LBD) was established within the protein sequence using Pfam tool in the EMBL-EBI database. Three-dimensional structure of porcine ARNT-LBD was created by homology modeling, and the crystalline structure of the human ARNT protein (PDB ID: 2HV1) was chosen as a template. Alignment of the amino acid sequence of porcine ARNT and the template was performed by MUSCLE software, and the constructed alignment was exported to MODELLER 9v12 program. Models with the lowest DOPE values, generated by MODELLER, were selected for further analysis. The accuracy of each model was verified with ProSAII and PROCHECK software. The created models were positioned in the 'favored' or 'unfavored' region of Ramachandran plot, and the best model was chosen for further analysis.

2.3. TCDD/AhR-LBD docking study

TCDD was docked *in silico* into the binding site of AhR-LBD with the use of AutoDock Vina 1.1.2 program [40]. Ligand was manually placed inside the receptor's binding site. Next, the hydrogen atoms and Gasteiger charges were added using the MGL tool. The grid-box was constructed around the TCDD/AhR-LBD complex with parameters (X,Y,Z) set to 15 Å, 15 Å, 15 Å. The docking analysis was performed with exhaustiveness of 32. The AutoDock Vina 1.1.2 program generated 20 results presenting the best positions of the TCDD within the receptor binding site. A model, with the lowest binding energy, was selected to represent the best spatial orientation of TCDD and the AhR-LBD, ensuring their proper molecular interactions.

2.4. AhR/ARNT-LBD docking study

The ability of ARNT and AhR proteins to interact with each other was examined by means of sequence interaction analysis performed with STRING 9.1 program [13]. Next, a protein-protein docking was carried out in ClusPro tool [22] in order to obtain a spatial arrangement of the porcine AhR/ARNT-LBD complex. To estimate specific interactions between the amino acid residues, the docked complex was analyzed with Protein Interaction Server (PIC) [39].

2.5. Transcriptional activity of the TCDD/AhR/ARNT complex

2.5.1. Isolation of cells

The study was carried out in accordance with principles and procedures of the Local Ethical Commission for Animal Experiments of the University of Warmia and Mazury in Olsztyn. Porcine ovaries with medium (5–7 mm) follicles were collected in a local slaughterhouse (Biskupiec, Poland). The ovaries were transported to the laboratory in cold buffered physiological saline (PBS, 4 °C) supplemented with gentamycin and nystatin. Granulosa cells were isolated from the follicles as previously described [28]. Cell viability was determined by trypan blue dye exclusion, and it was always greater than 90%.

[1] LD_{50} (lethal dose, 50%) – dose of a toxin, radiation or pathogen that is required to kill half of the members of a tested population after a specified duration of an exposure.