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Proteasome affects the expression of aryl hydrocarbon receptor-regulated proteins

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ABSTRACT

The effect of proteasome inhibition with *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) on the protein expression regulated by aryl hydrocarbon receptor (AhR) was studied in T47D breast tumor cells. The luciferase reporter gene assay using a construct which has the xenobiotic responsive element showed that the inducible expression of the reporter with AhR ligands was significantly reduced by co-treatment with ALLN. The same suppressive effect by ALLN was observed for ethoxyresorufin *O*-deethylase (EROD) activity induced by an AhR ligand, 3-methylcholanthrene (3MC). Despite the above effects, the induced expression of CYP1A1 and CYP1B1 mRNAs was unaffected by ALLN. While lactacystin, another proteasome inhibitor, exhibited the same effect as ALLN on EROD activity induced by 3MC, leupeptin, which is one of the cysteine protease inhibitors, had no such effect. Based on the evidence obtained, it appears that proteasome inhibition results in a reduction in the expression of AhR-regulated proteins.

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1. Introduction

The aryl hydrocarbon receptor (AhR) belongs to a family of ligand-dependent basic helix–loop–helix transcription factors, and it is well known as the receptor for halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). A number of studies on the physiological function of AhR have suggested that this receptor plays a role in the development of embryo, liver and immune functions, as well as cell growth and differentiation (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Kolluri et al., 1999). On the other hand, the adverse effects exerted by AhR signaling are also well established: for example, the adverse effects of TCDD and related compounds are believed to require AhR activation followed by changes in the expressions of some functional proteins (see review of Poland and Knutson, 1982). The observation that AhR-null mice are resistant to the toxic effects of TCDD agrees with the above concept (Fernandez-Salguero et al., 1996; Mimura et al., 1997).

Although the key role of AhR in dioxin toxicity is well established, the regulation of AhR-signaling has not been fully elucidated. Following ligand binding, the receptor translocates to the nucleus and heterodimerizes with the AhR nuclear translocator (Arnt). This complex subsequently binds to a cognate regulatory element, named the xenobiotic responsive element (XRE), on the promoter region of target genes, followed by induction of some functional proteins (Bacsi et al., 1995). Accumulating evidence suggests that AhR signaling is governed by a number of regulatory proteins, including chaperones, partner receptors, kinases, phosphatases and co-activators (see review of Carlson and Perdew, 2002). Among them, heat-shock protein (HSP) 90 is thought to be one of the most important factors for the stabilization of AhR and for the retention of AhR in the cytoplasm (Pongratz et al., 1992). In addition, one of the mechanisms regulating ligand-activated transcriptional factor is the control by degradation. For example, inhibition of the degradation of peroxisome proliferator-activated receptor α by a proteasome inhibitor, MG132, increases its transcriptional activation and the expression of its target genes (Blanquart et al., 2002). In the case of AhR, the consensus obtained is that its degradation by 26S proteasome affects its own stability and subcellular distribution (Pollenz, 2002). Some studies have shown that the inhibition of AhR degradation by proteasome inhibitors increases the ligand-dependent or -independent nuclear translocation of AhR (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Santiago-Josefat and Fernandez-Salguero, 2003; Ohtake et al., 2007). However, little is known whether proteasome affects AhR signaling, especially the expression of AhR-regulated protein, after nuclear accumulation of this protein. To address this issue, we examined the effect of proteasome inhibitors, including *N*-

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acetyl-leucyl-leucyl-norleucinal (ALLN) (Zhou et al., 1996), on the expression in AhR-regulated protein. The results obtained suggest that the proteasome regulates the expression of AhR-governed proteins without affecting their mRNA levels. Although we failed to clarify its mechanism, this study provided new insight into the regulation of AhR-regulated proteins.

2. Materials and methods

2.1. Materials

TCDD (AccuStandard Inc., New Haven, CT, U.S.A.), 3-methylcholanthrene (3MC, Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) and ALLN (Roche Diagnostic, F. Hoffman-La Roche, Ltd., Basel, Switzerland) were purchased from the sources indicated. 2,2′,5,5′-Tetrachlorobiphenyl (PCB52) was synthesized in this laboratory. Human breast tumor-derived T47D cells were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Trans-IT LT-1, a cationic liposome for the transfection of plasmid, and Ex Taq™ DNA polymerase were obtained from TaKaRa Bio Inc. (Ohtsu, Japan). Mouse monoclonal antibodies against human AhR (Affinity BioReagents, Inc., Gordon, CO, U.S.A.) and *Achlya ambisexualis* HSP90 (Stressgen Biotechnologies Corp., Victoria, BC, Canada) were supplied by the sources indicated. Rabbit polyclonal antibody against rat cytochrome P450 (CYP) 1As was prepared in this laboratory (Nagata et al., 1985). NADPH, 7-ethoxyresorufin (Sigma, St. Louis, MO, U.S.A.) and resorufin sodium salt (Aldrich Chemical Co., Inc. Milwaukee, WI, U.S.A.) were purchased from the sources indicated. Deoxyribonuclease I (Amplification grade) was purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.). QuantiLum® recombinant luciferase was purchased from Promega Corp. (Madison, WI, U.S.A.). All other chemicals were of reagent grade and commercially available.

2.2. Construction of AhR-driven reporter gene

Plasmid carrying firefly luciferase reporter gene ligated with mouse XRE was constructed using pGL3-promoter vector (Promega Corp.). Of the XREs present in the dioxin-inducible *Cyp1a1* gene, one (−994 to −974 bp, which contains one XRE sequence) of them was selected as the active XRE (Lusska et al., 1993). The forward and reverse oligonucleotides of this region were synthesized by attaching restriction tags for cloning at both ends (Fig. 1, wild-type XRE). Another pair of nucleotides was also prepared to obtain mutated XRE in which two bases were changed from the wild-type XRE sequence (Fig. 1, mutant-type XRE). The pGL3-promoter vector originally contains an XRE-like sequence at its cloning site (Mlu 1 site) as shown by the broken underline in Fig. 1. Therefore, we also disrupted this site in the mutant-type XRE. Synthetic oligonucleotides were annealed, and inserted into the pGL3-promotor vector using the restriction tags attached. The constructed vector was transfected to competent *Escherichia coli* JM109 which was prepared by treatment with $CaCl_2$, and the transformed cells were selected by ampicillin resistance. Presence of the desired plasmid in the positive clone was further confirmed by PCR, although the details are not given here. Recombinant cells were purified once by culturing on ampicillin-containing Luria–Bertani (LB) plates, and stored at −80 °C until use in the presence of 23% glycerol. Following large scale culturing in ampicillin-containing LB medium (200 mL), the amplified plasmid in positive cells was purified using a Wizard Plus Maxipreps DNA purification kit (Promega Corp.).

Wild-type XRE

Mlu I

Forward: 5′-CGCGTGGCTCTTCTCACGCAACTCCGA -3′

Reverse: 3′- ACCGAGAAGAGTGCGTTGAGGCTCTAG-5′

Bgl II

Mutant-type XRE

Forward: 5′- CTTAC**TATTT**GCTCTTCTC**A**A**T**CAACTCCGA -3′

Reverse: 3′-TCGAGAATG**ATAAA**CGAGAAGAG**T**T**A**GTTGAGGCTCTAG-5′

Sac I Bgl II

Fig. 1. Sequences of an XRE-containing region of mouse *Cyp1a1* gene which was ligated upstream of luciferase reporter plasmid (pGL3-promoter). The XRE sequence of the *Cyp1a1* gene is underlined. In the mutant-type XRE, two changes of base indicated in bold were introduced. The XRE-like sequence present in the cloning site of the pGL3-promoter vector is shown by the broken underline. Brackets show the restriction tags attached to the ends of the XRE-containing oligonucleotide. Both wild-type and mutant-type XREs were inserted in the frame to the pGL3-promoter vector at these restriction sites.

2.3. Reporter gene assay

The T47D cells were grown on a 6-well plate at a density of 1.5×10^6 cells/well in RPMI 1640 medium (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) containing 10% (v/v) fetal bovine serum (FBS). Throughout this study, culture of T47D cells was performed at 37 °C in an atmosphere of 5% CO_2 in air. Following culturing for 72 h

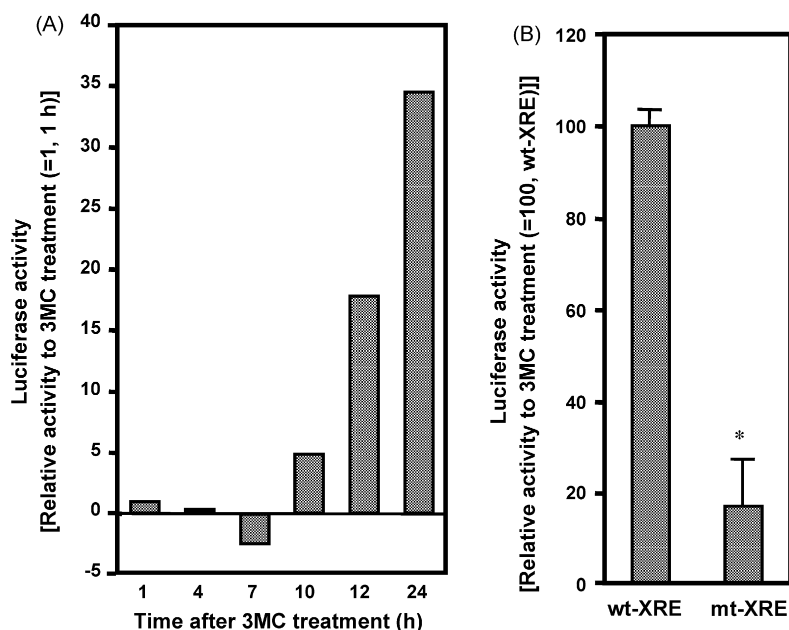

Fig. 2. Expression of luciferase gene following treatment with 3MC (A) and its dependence on XRE (B). In the experiment of panel A, T47D cells were pre-cultured for 24 h after co-transfection with pGL3-promoter vector carrying XRE and pRL-TK vector. The cells were further cultured in medium containing 1 μM 3MC for the periods indicated, and the luciferase activity was determined. The activity of firefly luciferase coded in XRE-carrying gene (pGL3-promoter vector) was divided by the activity of *Renilla* luciferase (pRL-TK vector), an internal standard for the correction of transfection efficiency. The data are expressed as values relative to the activity (=1) after 1 h culture. Each bar represents the mean of two dishes. In panel B, T47D cells were transfected with pRL-TK vector and pGL3-promoter vector following insertion of either wild-type or mutant-type XRE (abbreviated as wt-XRE and mt-XRE, respectively, in the figure). After pre-culture for 24 h, the cells were further cultured for 24 h in medium containing 1 μM 3MC. The data are shown as a percentage of the activity of wild-type XRE treated with 3MC. Each bar represents the mean ± S.E. of three dishes. (*) Significant difference from wild-type XRE ($p < 0.001$).

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