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β-Catenin signaling induces *CYP1A1* expression by disrupting adherens junctions in Caco-2 human colon carcinoma cells

Shuya Kasai [a,b], Takanori Ishigaki [b], Ryo Takumi [b], Toru Kamimura [b], Hideaki Kikuchi [a,b,*]

[a] *Science of Biosources, United Graduate School of Agricultural Science, Iwate University, Morioka 020-8551, Japan*
[b] *Department of Biochemistry and Molecular Biology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan*

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ABSTRACT

Background: The aryl hydrocarbon (Ah) receptor is one of the best known ligand-activated transcription factors. The present study has focused on the wound-healing process on Ah receptor function.
Methods: Depletion of calcium from culture medium of Caco-2 human colon carcinoma cells by transfer to Minimal Essential Medium (Spinner Modification; S-MEM) destroyed adherens junctions and the cells were used as the model of wound-healing process.
Results: Calcium depletion induced both nuclear translocation of the Ah receptor, and increased expression of CYP1A1 and *Slug* mRNAs in Caco-2 cells. However, expression of *Slug* mRNA was not significantly induced by treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Knockdown of the Ah receptor and treatment with Ah receptor antagonists decreased level of CYP1A1 mRNA. The fragment of E-cadherin released by γ-secretase was not involved in induction of CYP1A1 mRNA following S-MEM treatment. Knockdown of β-catenin increased levels of Ah receptor mRNA, which may be attributable to direct or indirect involvement of β-catenin in suppression of the Ah receptor gene.
Conclusions: Our results suggest that mRNA induction of some genes by destruction of adherens junctions depends on the Ah receptor. β-Catenin, one of the components of the adherens junction, was released from the E-cadherin complex, which resulted in its increased interaction with the Ah receptor, and was translocated into the nucleus, and consequently the target genes would be transcribed.
General significance: Our observations suggest that some aspects of the molecular mechanism of wound healing involve the Ah receptor.

1. Introduction

The aryl hydrocarbon (Ah) receptor is an endogenous target molecule for dioxins, and is thought to be a key intermediate in the induction of a variety of types of toxicity [1–3]. The Ah receptor is a ligand-activated transcription factor that belongs to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) protein family. In the absence of ligand, the Ah receptor cytoplasmic complexes with the molecular chaperone heat shock protein 90 (HSP90), the co-chaperone p23, and hepatitis B virus X-associated protein-2 (XAP-2). The primary role of this complex is to contribute to the stability and cytoplasmic retention of the Ah receptor. Following ligand binding and nuclear translocation, the Ah receptor dissociates from the complex and forms heterodimer with another bHLH/PAS transcription factor, Ah receptor nuclear translocator (ARNT), and binds to the xenobiotic-responsive element (XRE) consensus sequence [4,5].

There are several reports that ligand binding is not required for transcriptional activation of the Ah receptor after cells have been subjected to treatments such as the administration of omeprazole [6], destruction of cell–cell adherens junctions [7], and hydrodynamic shearing [8]. Transcriptional activation of Slug, which encodes a zinc finger transcriptional repressor that is crucial for the induction of epithelial–mesenchymal transition (EMT), coincides with nuclear accumulation of the Ah receptor in the HaCaT keratinocyte cell line following incubation in calcium-deficient Minimal Essential Medium, Spinner Modification (S-MEM) [9]. Calcagno et al. used microarray analysis to show that the destruction of adherens junctions by treatment with E-cadherin binding peptide induces expression of the gene that encodes cytochrome P450 1A1 (CYP1A1) in the Caco-2 colon carcinoma cell line [10].

Abbreviations: ARNT, Ah receptor nuclear translocator; bHLH, basic helix-loop-helix; CTF2, E-cadherin carboxy terminal fragment 2; CYP1A1, cytochrome P-450 1A1; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EMT, epithelial–mesenchymal transitions; FoxM1, forkhead box M1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP90, shock protein 90; Lef, lymphoid enhancer factor; MAF, 3-methoxy-4-aminoflavone; PAS, Per-ARNT-Sim; S-MEM, Minimal Essential Medium, Spinner Modification; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XAP-2, hepatitis B virus X-associated protein-2; XRE, xenobiotic-responsive element

* Corresponding author at: Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki 036-8561, Japan. Tel./fax: +81 172 39 3586.
E-mail address: hkikuchi@cc.hirosaki-u.ac.jp (H. Kikuchi).

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Adherens junctions are formed by homophilic binding of the membrane-penetrating E-cadherin protein between neighboring cells in a calcium-dependent manner. The intracellular domain of E-cadherin is anchored by interaction with α-catenin, β-catenin, and p120-catenin [11]. β-Catenin is a mediator of canonical wingless (wg, a fly Wnt gene) signal transduction that is initiated by binding of Wnt to Frizzled. With the discovery that β-catenin binds to the T cell factor/lymphoid enhancer factor class of DNA-binding proteins (TCF/Lef) [12], β-catenin has become known as a transcriptional factor that acts downstream of Wnt signaling and controls the expression of a large number of Wnt target genes. The disruption of the adherens-junction-induced, β-catenin-mediated pathway occurs in processes of wound-healing or epithelial–mesenchymal transition. Furthermore, this pathway plays an important role in processes involved in cell growth and development.

E-cadherin is one of most important molecules involved in tissue morphogenesis, wound healing, and cell–cell contact [13,14]. E-cadherin-mediated cell–cell contact can be disrupted by changing the culture medium from Dulbecco's Modified Eagle's Medium (DMEM) to calcium-depleted S-MEM, which mimics an early stage of wound healing. Such disruption causes nuclear localization of β-catenin and induction of *Slug* expression, followed by inhibition of E-cadherin transcription [15].

In the study reported herein, we focused on the role of the Ah receptor in the transcriptional activation of target genes in conjunction with β-catenin and the mechanism of this activation by examining the effects of disrupting adherens junctions in Caco-2 cells by treatment with S-MEM.

2. Materials and methods

2.1. Cell culture and treatment

The human colon adenocarcinoma cell line, Caco-2, was cultured in DMEM supplemented with 0.1 U/l penicillin, 0.1 g/l streptomycin, and 5% fetal bovine serum. Cells were maintained in a CO_2 incubator at 37 °C in 5% CO_2/95% air at 100% humidity. After cells reached 90% confluence, they were passaged in phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% EDTA.

For all assays, trypsinized cells were inoculated at a density of 10,000 cells/cm^2 and incubated for 1 week, followed by serum deprivation overnight. To disrupt the intercellular junctions of the confluent cell monolayer, cells were washed with PBS and calcium-free S-MEM (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 2 mM L-glutamine was added. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; Cambridge Isotope Laboratories, Andover, MA, USA), PD98059 (Wako Pure Chemical Industries, Osaka, Japan), 3-methoxy-4-aminoflavone (MAF; a gift from Dr. S. Safe, A & T University, Texas, USA), and N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Calbiochem, San Diego, CA, USA), were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and applied to cells after dilution in the growth media.

2.2. Reverse transcriptase and quantitative polymerase chain reaction (PCR)

After treatment, cells were harvested and frozen at −80 °C. Total RNA was extracted in accordance with the acid guanidinium phenol chloroform method [16] and 5 μg of total RNA was reverse-transcribed to cDNA using M-Mul V Reverse Transcriptase (Fermentas, Hanover, MD, USA) in accordance with the manufacturer's instructions. We amplified cDNAs that encoded CYP1A1, Slug, Ah receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using KOD FX Taq polymerase (Toyobo, Osaka, Japan) and the following primer sets: CYP1A1-F, GTATCGGTGAGACCATTGCC and CYP1A1-R, TCTCAAGCAC CTAAGAGCGC; Slug-F, AGACCCTGGTTGCTTCAAGG and Slug-R, TGGAG CAGTTTTTGCACTGG; Ah receptor-F, GGACTTGGGTCCAGTCTAATGCAC and Ah receptor-R, AGCCAGGAGGGAACTAGGATTGAG; GAPDH-F, CATC ACCATCTTCCAGGAGC and GAPDH-R, GGATGATGTTCTGGAGAGCC. Specific amplification of the cDNAs was confirmed by checking the lengths of the PCR products (predicted lengths of 180, 157, 296, and 404 bp, respectively), and by sequencing the PCR products after insertion into the vector pT7Blue (Novagen, Madison, WI, USA). The same plasmid DNA was used as a standard for the quantification of cDNA copy numbers.

2.3. Protein extraction

After treatment, cells were washed and harvested in cold PBS. Total cellular protein was extracted by sonication of the cells in lysis buffer (10 mM Tris, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM dithiothreitol, 10% glycerol, 0.5% Triton X-100, and 1% Nonidet P-40) supplemented with protease inhibitor cocktail (0.2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml antipain, 2 μg/ml elastatin, and 4 μg/ml aprotinin). The insoluble fraction was removed by centrifugation at 20,000 *g* for 10 min at 4 °C.

Subcellular fractionation of the nuclear extract and cytoplasmic fraction was performed using the method of Dignam et al. [17] with minor modifications. Cells were washed and harvested in cold PBS and collected by centrifugation at 1000 *g* for 5 min at 4 °C. Cell pellets were suspended in hypotonic buffer (10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM dithiothreitol, 10% glycerol, and 0.5% Nonidet P-40) supplemented with the protease inhibitor cocktail. The suspension was incubated on ice for 10 min and homogenized by vigorous pipetting. Nuclei were collected by centrifugation at 3300 *g* for 5 min at 4 °C. The supernatant recovered contained the cytoplasmic fraction. The nuclei were washed again with hypotonic buffer without Nonidet P-40, and collected by centrifugation. The nuclear pellet was suspended in high-salt buffer (20 mM Tris, pH 7.5, 0.3 M KCl, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM dithiothreitol, and 10% glycerol) supplemented with a cocktail of protease inhibitors. Nuclear protein was extracted by incubating the suspension on ice for 30 min and vortexing several times. The insoluble fraction was removed by centrifugation at 20,000 *g* for 30 min at 4 °C.

Protein concentrations were determined by the Bradford protein assay using commercial reagents (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Immunoprecipitation

To extract protein under native conditions, cells were homogenized in low-stringency buffer (20 mM Tris, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, 5 mM NaF, 5 mM 2-glycerophosphate, 1 mM Na_3VO_4, 10% glycerol, and 0.1% Nonidet P-40) supplemented with protease inhibitor cocktail. To precipitate the protein complex that contained the Ah receptor, 4 μl of Dynabeads protein G (Invitrogen, Carlsbad, CA, USA) was mixed with 1 μg of either antibody against Ah receptor (Affinity BioReagents, Golden, CO, USA) or normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rotated for 1 h. Beads were washed with PBS, then mixed with 1 mg of extracted protein in 0.5 ml of low-stringency buffer, and rotated overnight at 4 °C. Beads were washed three times with low-stringency buffer and immunopurified protein was eluted by boiling the beads in Laemmli Sample Buffer.

2.5. Immunoblotting and densitometry

Equivalent amounts of extracted proteins were resolved by SDS-PAGE and transferred to a Hybond-P PVDF membrane (GE Healthcare, Buckinghamshire, UK). Ah receptor protein was detected using the protocol of Dr. H. Ashida, Kobe University, Japan [18]. To detect E-cadherin, 3 × FLAG, β-catenin, HSP90, XAP-2, or GAPDH, the membrane was blocked with 5% nonfat dried milk dissolved in PBS containing 0.1% Tween 20. Primary antibodies against E-cadherin (DB Transduction