Study on the novel effects of indole-3-acetic acid on colorectal cancer cells

（大腸癌細胞に対するインドール-3-酢酸の新規作用に関する研究）

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Chapter-1

General Introduction

1. Colorectal cancer

1.1 Epidemiology

Colorectal cancer (CRC) has gained attention recently because it is one of the most commonly diagnosed cancers in the world, and it is ranked third in the growing incidence of 1.9 million new CRC cases per year and rated as second in the increased mortality rate (Sung et al., 2021). CRC usually occurs in the large intestine, and it is considered a life-threatening disease due to its high metastasis and recurrence rates. The adenoma-carcinoma sequence is commonly acknowledged as describing the sequential evolution from normal epithelia to a malignant tumor linked to the accumulation of various genetic changes (Medema and Vermeulen, 2011). CRC incidences vary geographically, and socioeconomic status is one of the critical factors associated with CRC (Thanikachalam and Khan, 2019). Age-specific higher CRC incidence and mortality rates are found in men in comparison to women. The most increased occurrence is seen in the developed countries and is predicted to increase 2.2 million new cases yearly by 2030 in developing countries (Arnold et al., 2017; Brenner et al., 2007). CRC occurs in different portions of the colon, such as the proximal colon (around 41%), distal colon (about 22%), and rectum (approximately 28%) (Cheng et al., 2011).

1.2 Risk factors associated with CRC

Studies revealed that both lifestyle and genetic factors play an essential role in the initiation and progression of CRC (Ahmed, 2006; Khan et al., 2010). Crucial risk factors involving colon cancer syndrome are dietary factors (Flood et al., 2008), personal (Imperiale et al., 2000) and familial history of CRC (Johns and Houlston, 2001), aging (Imperiale et al., 2000), infectious diseases (Burnett-Hartman et al., 2008), and inherited CRC syndromes (Al-Sukhni et al., 2008; Garber and Offit, 2005; Rustgi, 2007). Lifestyle factors such as obesity, particularly abdominal obesity (Thygesen et al., 2008), a diet rich in red and processed meat (Oba et al., 2006), consumption of alcohol (Mizoue et al., 2008), and cigarette smoking (Botteri et al., 2008; Buc et al., 2006; Paskett et al., 2007) can increase the risk of CRC. Moreover, disease states such as inflammatory bowel disease, diabetes mellitus, insulin resistance, and coronary artery disease are also associated with CRC (Ferlay et al., 2010).

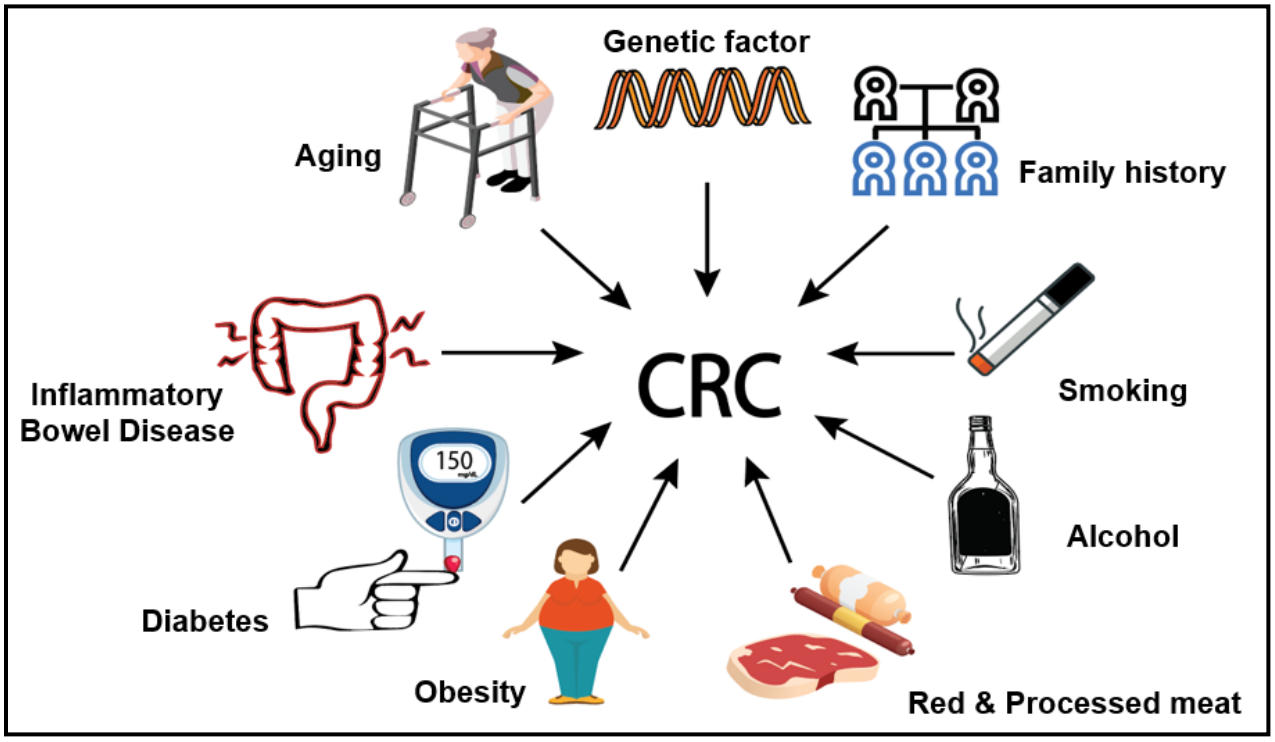


Figure 1. Risk factors associated with colorectal cancer.

2. Tumor necrosis factor α as an inflammatory cytokine in CRC

Researchers have indicated that the risk of CRC development is directly linked with the cytokine-mediated inflammatory process (Nieminen et al., 2014; Yehuda-Shnaidman and Schwartz, 2012). Tumor necrosis factor α (TNFα), a pro-inflammatory cytokine mainly produced and activated by infiltrating cells, is considered the pivotal mediator for inflammatory processes and incitement of oncogene and is regarded as a risk for cancer progression (Balkwill, 2009, 2006; Szlosarek et al., 2006). TNFα, which is a ligand that belongs to a large superfamily of type II transmembrane proteins and a representative inflammatory cytokine, located on the human chromosome 6, and the soluble TNFα cleaved as a 17-KDa protein at the cell surface from a transmembrane precursor (pro-TNFα, 26 KDa) by the specific protease known as TNF-converting enzyme (TACE) (Black et al., 1997; Zidi et al., 2010). TNFα binds as a homotrimer to two distinct homotrimeric cell surface receptors, namely tumor necrosis factor receptor 1(TNFR1) (also called p55 or CD120a) and tumor necrosis factor receptor 2 (TNFR2) (also called p75 or CD120b) (Bazzoni and Beutler, 1996; MacEwan, 2002; Vandenabeele et al., 1995). Downstream of TNFα signaling involving the binding of the TNFα ligand to its receptors, which requires multiple

adapter proteins such as RIP1 (Receptor interacting protein), TRAF-2 (TNFR-associated factor 2), and FADD (Fas-associated DD) (Chen, 2002; Szlosarek et al., 2006; Zidi et al., 2010).

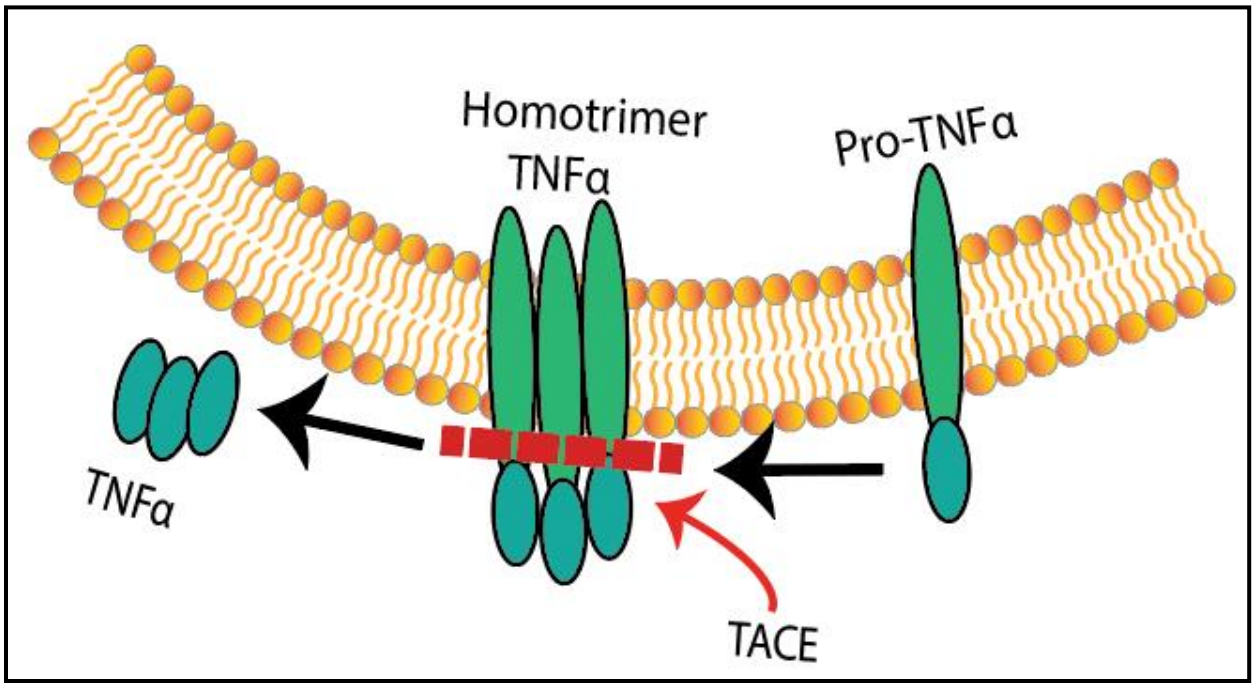


Figure 2. Release of TNFα from the cell membrane.

Ample documentation showed that TNFα is expressed by CRC cells (Al Obeed et al., 2014; Zins et al., 2007) and is involved in the growth of CRC (Zidi et al., 2010) as well as the induction of the epithelial-mesenchymal transition, which plays an essential role in promoting CRC invasion and metastasis (Wang et al., 2013). In addition, increased TNFα expression is associated with advanced CRC progression (Al Obeed et al., 2014) and tumor recurrence in patients with metastases of CRC (Grimm et al., 2011). Furthermore, TNFα expression is increased in serum from patients with CRC (Csiszár et al., 2004). Moreover, researchers also demonstrated that the TNFα mediated c-myc oncogene upregulation occurs through β-catenin and mitogen-activated protein kinases (MAPKs) (ERK and p38) dependent pathway in Caco-2 cells (Tselepis et al., 2002).

3. Aryl hydrocarbon receptor

3.1 AhR signaling and sensitivity in human and mice

Aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor of the basic region helix-loop-helix-Per/Arnt/Sim (bHLH/PAS) homology family (Bersten et al., 2013), presents in the cytoplasm as a protein complex with a dimer of 90-kDa heat shock protein (hsp90) (Denis et al., 1988; Perdew, 1988). The hepatitis virus X-associated protein -2 (XPA2) (Meyer et al., 1998) and one molecule of 23-kDa co-chaperone protein (p23) (Johnson et al., 1994) attached with the N-terminal ATP binding domain of hsp90 (Grenert et al., 1997), having a total mass of about 280-kDa for AhR (Denison et al., 1986). After binding with the ligand, conformational changes occur in the AhR receptor and thus opens the nuclear localization sequence (NLS) by dissociating the dimer of hsp90 along with p23 (Ikuta et al., 1998). For the nuclear localization, the minimum length of AhR NLS is 27 amino acid residues, from Arg^{13} to His^{39} of the NH_2 terminal, and the three essential amino acids required for the translocation of AhR to the nucleus are Arg^{13}, Lys^{14}, and Arg^{15} has been described previously (Ikuta et al., 1998). This consequence exposing the NLS region shifts AhR into the nucleus leaving the cytoplasmic complex behind (Beischlag et al., 2008). After that, the aryl hydrocarbon translocator (Arnt) protein inside the nucleus binds with the AhR to form a transcriptionally active heterodimer (Puga et al., 2009), directly or indirectly interacting with DNA by binding to recognition sequences located in the 5′- regulatory region of AhR-responsive genes. Some researchers described the sequence (5′-TNGCGTG-3′) as the core sequence necessary for AhR/Arnt protein DNA interaction (Denison et al., 1989; Fujisawa-Sehara et al., 1988). However, Whitlock et al. mentioned the part of the core sequence that is 5′- CGTG-3′ as an absolute requirement for the inducible interaction of AhR/Arnt protein with DNA (Whitlock et al., 1996). Amino-terminal that contains the ligand-binding domain of the AhR found 85% of the sequence homology in the amino acid sequence when comparing between human and murine receptors (Ah^{b-1} allele), which leads to significant differences in the selectivity and ligand binding affinity between the species (Hubbard et al., 2015). Researchers observed a 10-fold increase in the relative ligand affinity in mAhR $^{b-1}$ than human AhR. Moreover, they revealed that a single amino acid mutation, valine at 381 to alanine, will increase the ligand-binding affinity in human AhR (Ramadoss and Perdew, 2004).

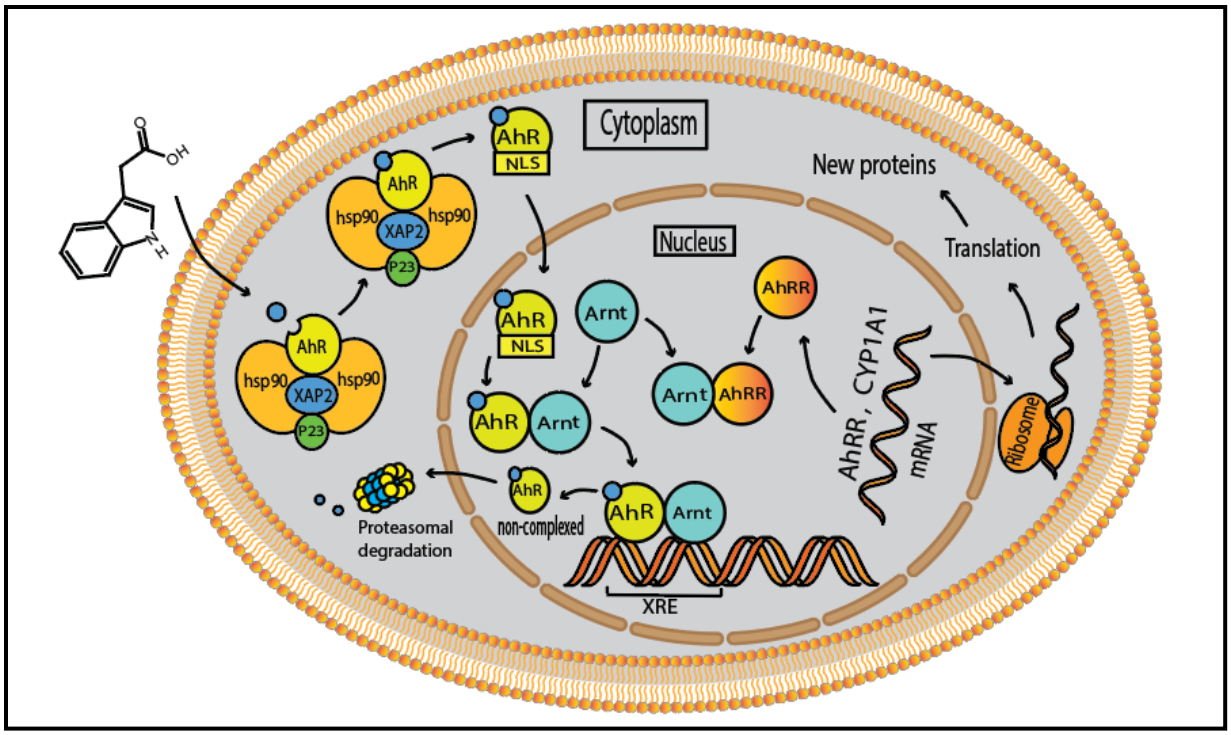


Figure 3. Canonical pathway of AhR-mediated transcription after binding with IAA.

3.2 Pathophysiological significances of AhR

The various physiological significance of AhR activation includes intestinal homeostasis by developing and regulating intraepithelial lymphocytes, and innate lymphoid cells (Kiss et al., 2011; Li et al., 2011; Qiu et al., 2012), innate and adaptive immunity by differentiating the regulatory T-cells lymphocytes, and B-cell (Bhattacharya et al., 2010; Funatake et al., 2008, 2005; Mohinta et al., 2015), as well as the progression of carcinogenesis by increasing AhR activity with non-small cell lung cancer have been described (Su et al., 2013). The upregulation of AhR expression has also been reported in lung carcinomas (Lin et al., 2003), pancreatic cancer (Koliopanos et al., 2002), and prostate cancer (Richmond et al., 2014) by numerous studies. Furthermore, AhR global knockout mice have observed some developmental anomalies like the problem with the closure of the portal ductus venous in neonatal mice (Lahvis et al., 2005). Additionally, in aged AhR global knockout mice, flawed organ development was also reported and suggested a comprehensive physiological role of AhR in mammalian development (Hubbard et al., 2015). However, researchers have reported the development of cecal cancer in AhR global knockout mice, implying that AhR has a tumor inhibitory effect (Kawajiri et al., 2009). Moreover, AhR has been linked to the prevention of tumor growth (Ikuta et al., 2016). The ligands responsible

for this AhR-mediated action may occur in different ways, such as dietary consumption, synthesis of various metabolites from dietary compounds by gut microbes, free radical production as well as endogenous enzyme activity (Hubbard et al., 2015).

3.3 AhR-mediated biological responses

Most of the endogenous AhR ligands come from the metabolism of dietary tryptophan and indole derivatives (Bittinger et al., 2003; Chung and Gadupudi, 2011). Indole-3-carbinol (I3C) and indole-3-acetonitrile (I3ACN) from cruciferous vegetables can act as AhR ligands (Bjeldanes et al., 1991; Ito et al., 2007; Loub et al., 1975; Shapiro et al., 2001), and I3C has been shown to inhibit carcinogenesis in a variety of target tissues, including the liver, thyroid, skin, lung, and colon (Fares, 2014). Moreover, I3C or its metabolites from dietary sources have been shown to exhibit anti-inflammatory (Safa et al., 2015) and antimicrobial activities (Monte et al., 2014). Microbiota-derived AhR ligands such as indole-3-acetic acid (IAA) are produced through the indole-3-acetamide pathway by the tryptophan monooxygenase and indole-3-acetamide hydrolase enzymes (Tsavkelova et al., 2012).

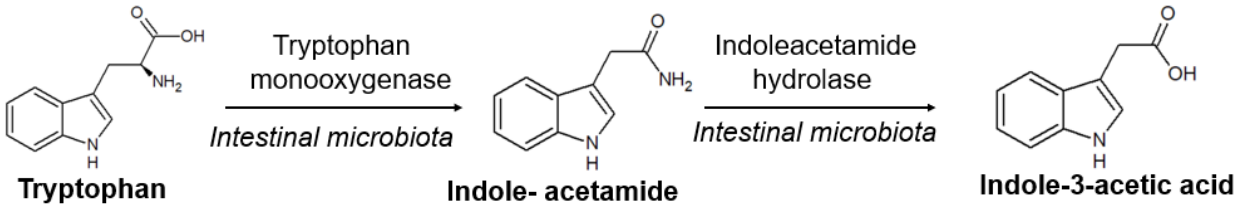


Figure 4. Conversion of indole-3-acetic acid from dietary tryptophan by intestinal microbiota.

The IAA and tryptamine produced by the commensal microorganism have been demonstrated to modulate the AhR-mediated induction of different genes in CaCo-2 cells (Jin et al., 2014a). The typical AhR-mediated target genes are a group of drug-metabolizing enzymes, including cytochrome P450 1A1 (CYP1A1), 1A2 (CYP1A2), and 1B1 (CYP1B1) (Nukaya et al., 2009; Rowlands and Gustafsson, 1997; Strom, 1992; Zhang et al., 1998). However, inflammatory genes such as interleukin-6 (IL-6), interleukin-22 (IL-22), and prostaglandin-endoperoxide Synthase 2 (PTGS2) are also reported to be regulated by AhR (DiNatale et al., 2010; Lahoti et al., 2013; Monteleone et al., 2011). Apart from the endogenous compounds, xenobiotic compounds such as 2,3,7,8-tetrachloride dibenzo-*p*-dioxin (TCDD), biphenyls, polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HAs) (Denison and Heath-Pagliuso, 1998; Kafafi et al., 1993; Mimura and Fujii-Kuriyama, 2003; Safe, 1994) have shown several biological responses,

including suppression of immune response, induction of different genes as well as promotion of tumorigenesis (Chung and Gadupudi, 2011; Pollenz and Barbour, 2000).

3.4 Aryl hydrocarbon receptor repressor

The AhR repressor (AhRR) is a major target gene activated in the AhR genomic pathway (Baba et al., 2001; Korkalainen et al., 2005). The AhRR protein is identical to AhR. However, at the N-terminal of AhRR, the PAS B domain is missing, which prevents it from binding with the ligands (Korkalainen et al., 2004; Mimura et al., 1999a). In addition, the C-terminal domain of AhRR is a transrepression domain rather than a transactivation domain in AhR and Arnt (Hahn et al., 2009). Thus, it allows corepressors to bind with Arnt, acting as a negative regulatory loop for AhR. AhRR decreases the AhR activity by binding to Arnt (AhRR-Arnt complex) and xenobiotic responsive element (XRE) (Hahn, 2002; Hahn et al., 2009). As a result, AhRR suppresses the transcription of AhR-dependent genes. In such a way, this negative regulatory loop, as well as the unbounded receptor's proteasomal degradation, refrains AhR from overexpressing the targeted gene expression and hence brings control of the AhR signaling pathway.

4. Xenobiotic metabolizing enzymes

4.1 Phase I and phase II enzymes

Metabolism of xenobiotics such as drugs and environmental pollutants by xenobiotic-metabolizing enzymes (XMEs) can be classified into two groups, namely phase I and phase II reactions. The phase I or functionalization reactions are involved in the metabolic activations of xenobiotics by adding functional groups to the compounds. Sometimes this process generates electrophilic intermediates such as reactive oxygenated species, which can be mutagenic through binding with DNA and protein. On the other hand, phase II or conjugation reactions occur by the coupling of drugs or their metabolites with molecules like acetate, methyl, sulfate, glucuronide, glutamine, glycine, thiocyanate, and so on. This conjugation process makes the moieties more hydrophilic that can easily be excreted from the body (Nebert and Dalton, 2006; Williams, 1971).

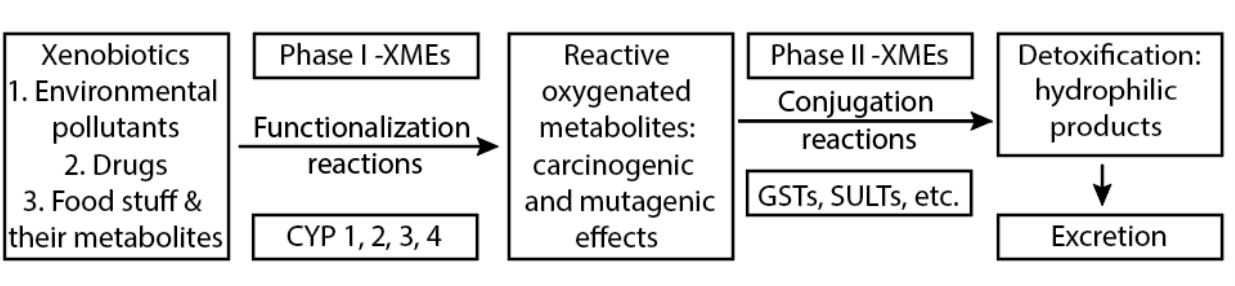


Figure 5. Conversion of xenobiotics by phase I and phase II xenobiotic-metabolizing enzymes.

4.2 Cytochrome P450 1A1 and CRC

The cytochrome P450s, a multigene superfamily (Nebert and Gonzalez, 1987; Nelson et al., 1996) of constitutive and inducible metabolic enzymes, attract considerable interest due to their various cellular functions, such as oxidative activation or deactivation of endogenous substrates and xenobiotics, including many drugs and carcinogens (Gonzalez and Gelboin, 1994; Guengerich and Shimada, 1991). Xenobiotic procarcinogens like PAHs are primarily hydrophobic, and CYP1A1 is one of the significant P450 inducible enzymes involving the process of metabolic activation of these xenobiotics (Bartsch et al., 2000; Nebert et al., 2004). Usually, CYP1A1 is expressed by the intestinal cells (McKay et al., 1993; Mercurio et al., 1995). Epidemiological studies described the risk of colorectal cancer (CRC) due to genetic polymorphism with the CYP1A1 gene. The most commonly studied single nucleotide polymorphisms (SNPs) in the CYP1A1 concerning CRC is $Ile^{462}Val$ polymorphism, which results from an amino acid substitution isoleucine to valine at codon 462 of the CYP1A1 gene with increased CYP1A1 enzymatic activity (Hayashi et al., 1991; Kiss et al., 2000; Sivaraman et al., 1994). Meta-analysis data relating to CYP1A1 $Ile^{462}Val$ polymorphism showed that this SNP may increase the risk of CRC (Gil et al., 2014; Jin et al., 2011; Xu and Wei, 2015; Zheng et al., 2012).

Previously, an association between red meat consumption, particularly broiled or grilled meat, and CRC was shown (de Verdier et al., 1991; Sugimura, 1985; Zhang et al., 1992). More notably, high-temperature exposure to this dietary element can produce PAHs such as Benzo[a]pyrene (BaP) and other HAs (Conney, 1982). Researchers demonstrated that PAHs metabolized by CYP1A1 could either activate proto-oncogene or deactivate tumor suppressor gene, and the expression level of the CYP1A1 enzymes can also induce by PAHs through AhR (Hankinson, 1995). Since PAHs are the substrates for the CYP1A1 enzymes (Thakker et al., 1981), and metabolism of PAHs can generate mutagenic diol epoxides in the colon (Alexandrov et al.,

1996) therefore, it is conceivable that the increased expression of the CYP1A1 gene is directly proportional to the development and progression of CRC. Furthermore, recently it has been shown that BaP induces CYP1A1 in the mouse colon (Uno et al., 2008), and CYP1A1 is involved in the generation of genotoxic BaP metabolites in HCT116 cells (Kabátková et al., 2015). However, Uno et al. have shown that in CYP1A1 global knockout mice, metabolic clearance of BaP is considerably slower, resulting in more BaP-DNA adduct formation (Uno et al., 2001). Thus, the importance of studying the CYP1A1 expression in the initiation and progression of CRC is a must.

5. Toll-like receptor 4 signaling pathway and its expression in the regulation of inflammatory cytokines

Toll-like receptors (TLRs) play central roles in the innate immune responses of vertebrates (Fitzgerald and Kagan, 2020). Each TLR recognizes a specific microbial product and activates each signaling pathway. For instance, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from gram-negative bacteria can activate toll-like receptor 4 (TLR4) (Fitzgerald and Kagan, 2020). However, TLR4 alone cannot recognize LPS. Thus, other proteins are required to recognize the ligand. Series of events occur during activation of TLR4 by LPS that involves several associated proteins (Gioannini and Weiss, 2007; Miyake, 2007). LPS firstly interacts with the soluble shuttle LPS-binding protein (LBP) (Tobias et al., 1986; Wright et al., 1989), which helps LPS binding to the soluble anchored cluster of differentiation 14 (CD14), another glycosylphosphatidylinositol protein (Wright et al., 1990). After that, CD14 transfers LPS to a large hydrophobic pocket in a soluble myeloid differentiation 2 (MD-2) (Park et al., 2009). When LPS is non-covalently bound to MD-2, it interacts with the extracellular domain of TLR4 to form a heterodimer (LPS-MD-2-TLR4) to initiate intracellular signaling (Shimazu et al., 1999).

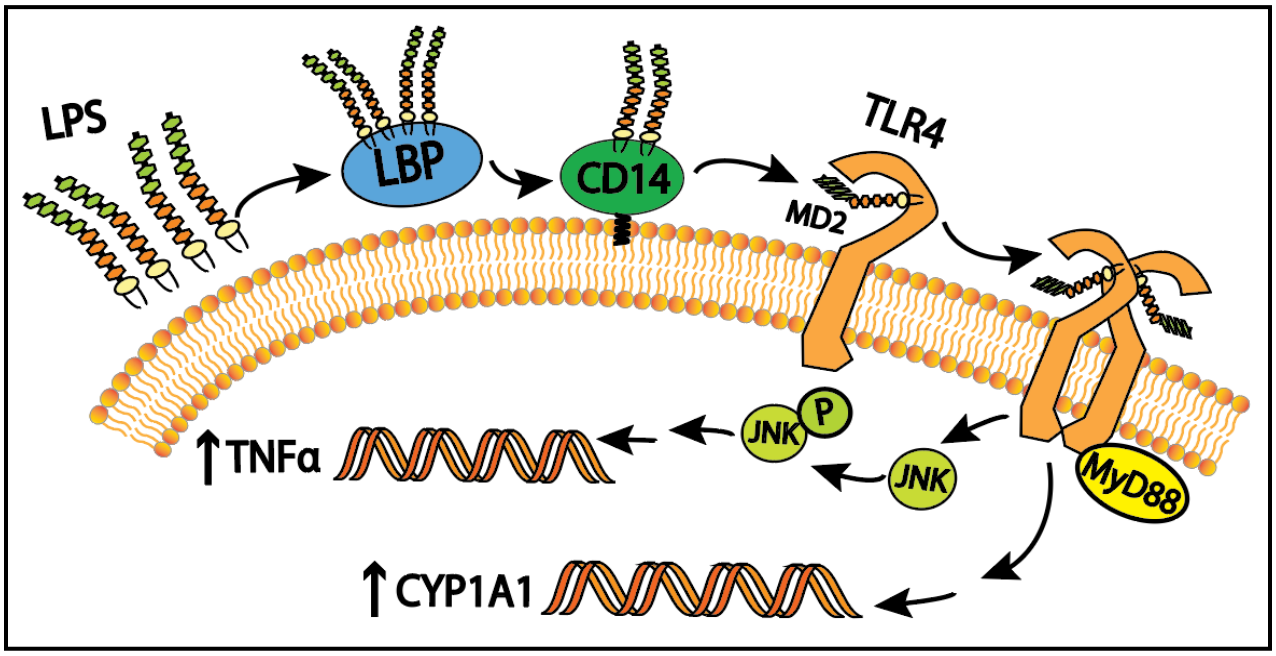


Figure 6. TLR4 signaling through lipopolysaccharide released by Gram-negative bacteria.

The presence of microorganisms has a significant impact on TLRs expression. For example, the highest expression of TLR4 occurs in the colon due to the maximum bacterial load in this part (Ortega-Cava et al., 2003; Price et al., 2018; Wang et al., 2010). Usually, a low level of TLR4 is expressed by the various intestinal epithelial cells in human colon (Abreu, 2010; Abreu et al., 2001; Cario and Podolsky, 2000; Otte et al., 2004). The expression of TLR4 in Caco-2 cells has also been described (Ruemmele et al., 2002). In addition, TLR4 is a receptor for pyrimido[5,4-*b*]indole containing an indole ring (Chan et al., 2013). Furthermore, Tian et al showed the AhR-independent induction of CYP1A1 expression in lungs by LPS (L. Tian et al., 2020), suggesting the involvement of TLR4 signaling cascade in the expression of CYP1A1. Previously, intestine-specific constitutively active TLR4 were shown to be more susceptible to chemically induced colitis (Fukata et al., 2011) and expressed proinflammatory cytokines and chemokines several folds at the baseline in the intestinal epithelial cells (Shang et al., 2008). However, others described the protective functions of TLR4 signaling against bacterial invasion, epithelial injury, and inflammation (Fukata et al., 2005; Rakoff-Nahoum et al., 2004). Since these contradictory findings, where both low and high-level of TLR4 signaling advocate intestinal inflammation, therefore, it is vital to know whether the tryptophan metabolite IAA has any effects through TLR4 signaling or not on Caco-2 cells.

6. c-Jun-N-terminal kinase involvement in AhR and TLR4 regulation

MAPKs are key intracellular signaling mediators that govern gene expression by phosphorylating and modulating the actions of transcription factors (Davis, 1995). c-Jun-N-terminal kinase (JNK) is one of the MAPKs for cell signaling, apoptosis, carcinogenesis (Weston and Davis, 2007). Recently, Moreover, researchers have shown that in monocytic cell lines, binding LPS-LBP to CD14 causes rapid phosphorylation of p38 and JNK (Dziarski et al., 1996; Hambleton et al., 1996).

Chapter-2

Suppression of TNFα expression induced by indole-3-acetic acid is not mediated by AhR activation in Caco-2 cells

1. Introduction

Colorectal cancer (CRC) has recently attracted considerable attention because it is one of the most prevalent malignancies, the second leading cause of cancer-related deaths globally, and 1.5 million patients per year are diagnosed with CRC worldwide (Bray et al., 2018). In CRC tissues, elevated expression of tumor necrosis factor α (TNFα), which is a ligand that belongs to a large superfamily of type II transmembrane proteins and a representative inflammatory cytokine, is involved in the growth of CRC (Zidi et al., 2010) and the induction of the epithelial-mesenchymal transition, which plays an essential role in promoting CRC invasion and metastasis (Wang et al., 2013). In fact, increased TNFα expression is associated with not only advanced CRC progression (Al Obeed et al., 2014), but also tumor recurrence in patients with metastases of CRC (Grimm et al., 2011). Furthermore, TNFα expression is increased in serum from patients with CRC (Csiszár et al., 2004).

The laboratory I belong to has focused on the control of organ functions by indole compounds synthesized by intestinal flora from tryptophan in dietary proteins. For instance, indoxyl sulfate produced in the liver from indole promotes the aggravation of chronic kidney disease (CKD) (Saito et al., 2014; Shimizu et al., 2013a, 2013b, 2013c, 2012a, 2012c, 2011b, 2011a, 2010a) and it correlates with the pathogenesis and progression of CKD complications such as atherosclerosis (Shimizu et al., 2012b, 2009). In addition, skatole synthesized from indole-3-acetic acid (IAA) derived from tryptophan in dietary proteins by intestinal flora causes the dysfunction of intestinal epithelial cells (Kurata et al., 2019). On the other hand, all that is known about the effects of IAA on CRC cells is only than that it suppresses colonic tumor formation in $APC^{min/+}$ mice by causing β-catenin degradation through activating the aryl hydrocarbon receptor (AhR), which is a representative receptor IAA (Kawajiri et al., 2009). Therefore, the present study aimed to determine whether IAA is involved in the regulation of TNFα expression by activating AhR in CRC cells.

2. Materials and methods

2.1 Materials and reagents

Antibodies and reagents were obtained from various suppliers as follows: anti-β-actin, Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); anti-TNFα, and anti-rabbit and anti-mouse IgG-HRP-linked antibodies, Cell Signaling Technology Inc. (Danvers, MA, USA); penicillin and streptomycin, and Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, Wako Pure Chemical Industries Ltd. (Osaka, Japan); fetal bovine serum (FBS), Biowest Inc. (Nuaillé, France); Trypsin-EDTA, GIBCO (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), Sigma-Aldrich Co. (St. Louis, MO, USA): CH223191 (AhR antagonist), Cayman Chemical (Ann Harbor, MI, USA); Indole-3-acetic acid, Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan),

2.2 Cell culture

The human colon cancer cell line Caco-2 cells purchased from the RIKEN Cell Bank (Tsukuba, Japan) was maintained in DMEM supplemented with high glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under a 7% CO_2 atmosphere. Before starting experiments, the cells were seeded and incubated in serum-free DMEM for 24 h. Dimethyl sulfoxide (DMSO; final concentration, 0.1% v/v) was the control as well as the solvent for IAA and CH223191.

2.3 Quantitative real-time PCR

For quantitative real-time PCR, serum-starved Caco-2 cells (3×10^5/3.5-cm dish) were incubated without or with various concentrations of IAA for various periods. Total RNA was isolated from Caco-2 cells using Trizol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized from template RNA (1 μg) derived from Caco-2 cells using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio Co. Inc., Shiga, Japan). Quantitative real-time PCR was proceeded using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) and the Thermal Cycler Dice Real-Time System III (all from Takara Bio Co. Inc.) as described by the manufacturer, and oligonucleotide primers (Table 1). Amplicons were quantified using a calibration curve, and mRNA expression was measured as the ratio of each mRNA to RPLP0 mRNA.

Table 1. Forward and reverse (5′→3′) primer sequences of target human genes.

Gene	Forward	Reverse
CYP1A1	ACATGCTGACCCTGGGAAAG	GGTGTGGAGCCAATTCGGAT
TNFα	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
RPLP0	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG

2.4 Immunoblotting

Serum-starved Caco-2 cells (3×10^5/dish) in 3.5-cm dishes were incubated with or without antagonists and inhibitors before stimulation with or without IAA. Proteins fractionated from cell lysates by SDS-PAGE were transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore Sigma Co., Ltd., Burlington, MA, USA) for immunoblotting. Levels of TNFα were normalized to that of β-actin. Protein bands were visualized using enhanced Chemi-Lumi One L (Nacalai Tesque Inc., Kyoto, Japan). The resulting bands were analyzed using an ImageQuant LAS 4010 densitometer (GE Healthcare Life Sciences, Uppsala, Sweden).

2.5 Statistical analysis

The results are expressed as means ± SE. Data were statistically analyzed by Student *t*-tests (Fig. 7b and c) and Tukey-Kramer tests (Fig. 8b-e) using Excel 2011 (Microsoft Corp., Redmond, WA, USA) and Statcel 4 (OMS Publishing Co., Saitama, Japan). Results were considered statistically significant at $P < 0.05$.

3. Results

3.1 Indole-3-acetic acid increased CYP1A1 expression but suppressed TNFα expression in Caco-2 cells

Since human feces contain 100 μg/g of skatole, a metabolite of IAA synthesized by intestinal bacteria (Gao et al., 2018), and assuming that the water content in feces is between 60% and 80%, the maximum fecal concentration of skatole is considered to be 1000-1200 μM. Therefore, the maximum fecal concentrations for IAA are likely to be in a similar range. In addition, AhR agonistic activity of IAA in Caco-2 cells is reportedly in the range of 500-1000 μM (Shiizaki et al., 2019). Therefore, the present study was conducted mainly using 1000 μM IAA. Figure 7a shows the experimental procedure. Since cytochrome P450 1A1 (CYP1A1) is well known as a representative target gene of AhR, I similarly checked whether AhR is activated by IAA using increased CYP1A1 mRNA expression as an indicator. Figure 7b shows that the stimulation of Caco-2 cells with 1000 μM IAA resulted in a significant increase in the mRNA levels of CYP1A1 expression for 1-8 days, suggesting that AhR is activated by IAA. On the other hand, incubating Caco-2 cells with 1000 μM IAA for 2-8 days resulted in significantly reduced TNFα mRNA expression (Figure 7c). Furthermore, TNFα protein levels decreased after incubation for 8 days with 1000 μM IAA (Figure 7d). These results indicated a correlation between mRNA and levels of TNFα protein induced by IAA.

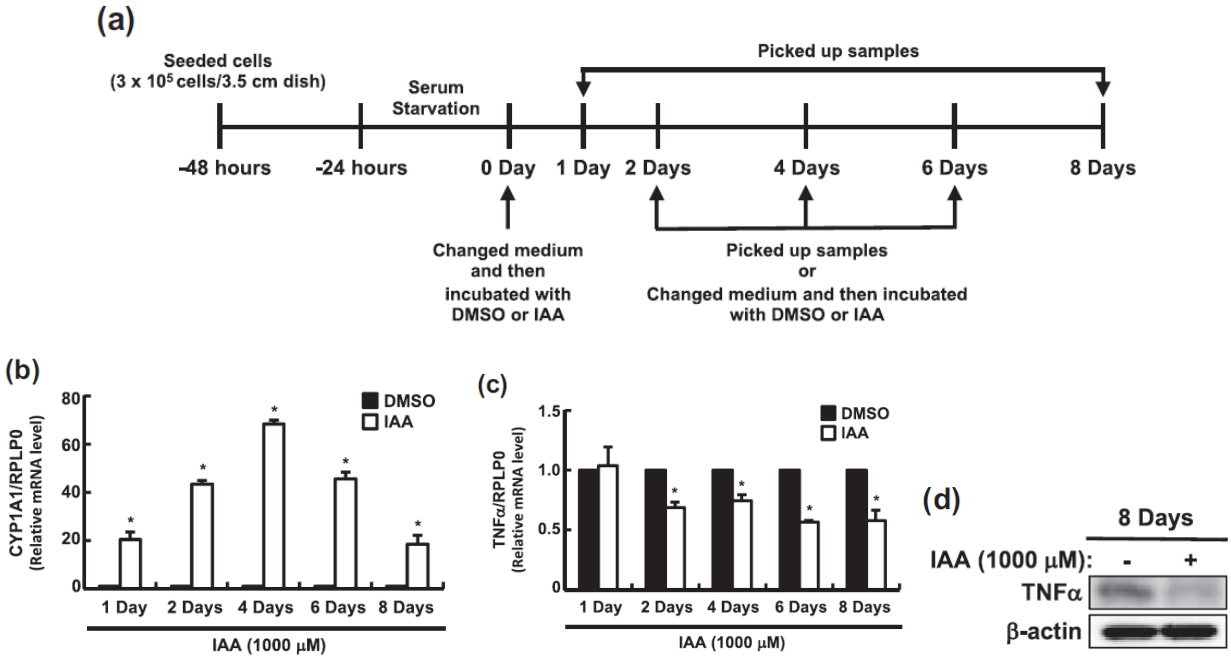
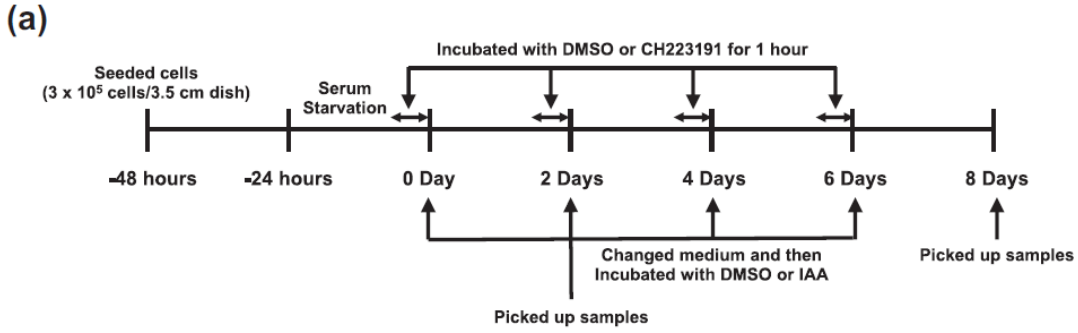


Figure 7. IAA induces CYP1A1 but suppresses TNFα expression in Caco-2 cells. (a) Experimental procedure. Samples were collected on 1, 2, 4, 6, and 8 days after stimulation with DMSO or IAA. (b) Serum-starved Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 1000 μM IAA for 1 and 2 days. Subsequently, the medium was replaced with fresh medium every 2 days for 8 days. After collecting samples on indicated days, levels of CYP1A1 mRNA expression were determined using real-time PCR. (c) Experimental conditions were as described in (b). Levels of TNFα mRNA expression were measured in samples using real-time PCR. (d) Experimental conditions were as described in (b). Cell lysates were immunoblotted using anti-TNFα and anti-β-actin antibodies. Data are expressed as means ± SE of three independent experiments each for (b) and (c). Representative data are shown from several independent experiments that yielded similar results for (d). **P* <0.05 vs. vehicle only-treated cells.

3.2 Activation of AhR is not involved in suppression of TNFα expression induced by IAA in Caco-2 cells

Based on the above findings, I assessed the effects of AhR antagonists on the suppression of TNFα expression induced by IAA using TNFα mRNA levels as an indicator. Figure 8a shows the experimental procedure. Before examining the effects of the AhR antagonist, CH223191, on the relationship between TNFα expression and IAA, I confirmed whether CH223191 could inhibit the induction of CYP1A1. CH223191 significantly repressed the IAA-induced expression of CYP1A1 mRNA at 2 and 8 days after stimulation (Figure 8b and d). However, CH223191 had no antagonistic action on the IAA-induced suppression of TNFα expression in Caco-2 cells on any day (Figure 8c and e). Taken together, these results suggested that AhR is not involved in suppressing TNFα expression induced by IAA in Caco-2 cells.



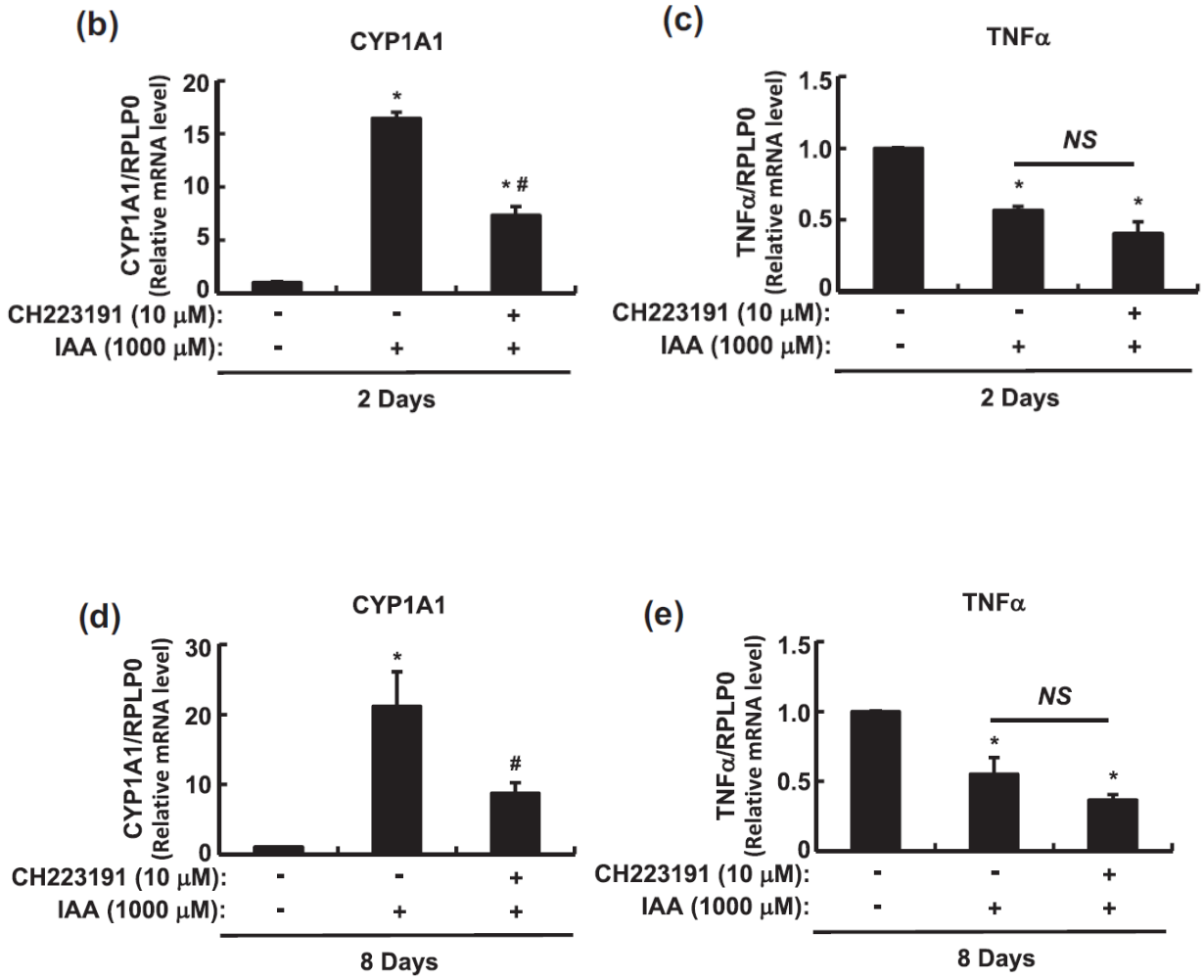


Figure 8. Activation of AhR is not involved in the suppression of TNFα expression induced by IAA in Caco-2 cells. (a) Experimental procedure. Samples were collected at 2 and 8 days after stimulation with DMSO or IAA. (b) Serum-starved Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 10 μM CH223191 (AhR antagonist) for 1 h, followed by vehicle or 1000 μM IAA for 2 days. After collecting samples, CYP1A1 mRNA expression was measured in samples using real-time PCR. (c) Experimental conditions were as described in (b). Levels of TNFα mRNA expression were measured using real-time PCR. (d) Serum-starved Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 10 μM CH223191 (AhR antagonist), followed by vehicle or 1000 μM IAA for 2, 4, and 6 days. CYP1A1 mRNA expression was measured on 8 days using real-time PCR. (e) Experimental conditions were as described in (d). Levels of TNFα mRNA expression were measured using real-time PCR. Data are expressed as means ± SE of three independent experiments each for (b) and (c) and of four independent experiments each for (d) and (e). $^{*}P$ <0.05 vs. vehicle only-treated cells; $^{\#}P$ <0.05 vs. IAA-treated cells.

4. Discussion

I assessed relationships between known factors involved in the initiation and progression of CRC, such as inflammatory bowel disease (IBD), the consumption of high-fat and high-meat diets, and the present findings. First, the amount of IAA is lower in the feces of patients with IBD and in the intestinal contents of model mice with IBD induced by dextran sodium sulfate (DSS) than in healthy humans and mice, respectively (Lamas et al., 2016; Shiomi et al., 2011). Because a reduction in IAA negatively correlates with increased inflammation of the intestinal tract, levels of IAA production in the intestinal tract might be involved in the initiation and progression of inflammation. Furthermore, although others have proposed that IAA produced by transplanted *Lactobacillus* strains in DSS-induced IBD model mice attenuates the progression of colitis via AhR activation (Lamas et al., 2016), low levels of AhR expression in tissues with trinitrobenzene sulfonic acid (TNBS)- and DSS-induced colitis have been reported (Monteleone et al., 2011). In addition, low levels of AhR are also expressed in the intestinal tract of patients with IBD (Monteleone et al., 2011). The present results together with these findings support the notion that IAA inhibits the progression of the inflammatory response via receptors other than AhR through a specific pathway in the gut. I then considered the relationship between the initiation and progression of CRC induced by a high fat diet and IAA. The consumption of a high-fat diet results in increased bile acids, including deoxycholic acid, which promotes CRC progression (Reddy et al., 1976; Yoshitsugu et al., 2019), in the gut and a decreased ratio of *Bacteroidetes*, which produce IAA in the gut (Lee et al., 2020). In fact, the intestines of mice fed with a high-fat diet have lower levels of IAA (Krishnan et al., 2018). Furthermore, the consumption of high-fat diets leads to increased TNFα expression in intestinal tissues, which in turn inhibits β-catenin degradation and subsequently induces an increase in the expression of c-myc, a proto-oncogene that leads to colonic cell proliferation (Liu et al., 2012). On the other hand, IAA suppresses CRC carcinogenesis through AhR-induced β-catenin degradation (Kawajiri et al., 2009), and the present findings suggested that IAA leads to the suppression of TNFα expression via AhR-independent pathways. Taken together, these results suggested that the initiation and progression of CRC associated with consumption of a high-fat diet might involve a decrease in intestinal IAA production. This would be due to decreased *Bacteroidetes* population caused by increased intestinal bile acids, accompanied by reduced β-catenin degradation due to both upregulated TNFα expression and attenuated AhR activity. I finally predicted the relationship between the initiation and progression

of CRC induced by the consumption of red and processed meat, and IAA. Since IAA is a precursor of skatole, it should also be protein concentration-dependently produced in the gut (Gao et al., 2018). However, increased intake of red and processed meat as a source of protein is associated with the initiation and progression of CRC (Godfray et al., 2018). The cause of this discrepancy might be the accelerated metabolism of IAA to skatole, resulting in a decrease in the amount of IAA in the gut. In fact, the amount of skatole in the large intestines of healthy individuals is about 35 μg/g, whereas some patients CRC have an average 75-100 μg/g skatole in their large intestines (Karlin et al., 1985). Indoleacetate decarboxylase is an enzyme involved in the metabolism of IAA to skatole (Liu et al., 2018). Therefore, an increased ratio of intestinal bacteria to indoleacetate decarboxylase might accelerate the development and progression of CRC because the amount of IAA in the gut is reduced. That is, inhibiting indoleacetate decarboxylase activity might confer protective and delayed effects on the development and progression of CRC induced by excessive consumption of red and processed meat.

5. Conclusion

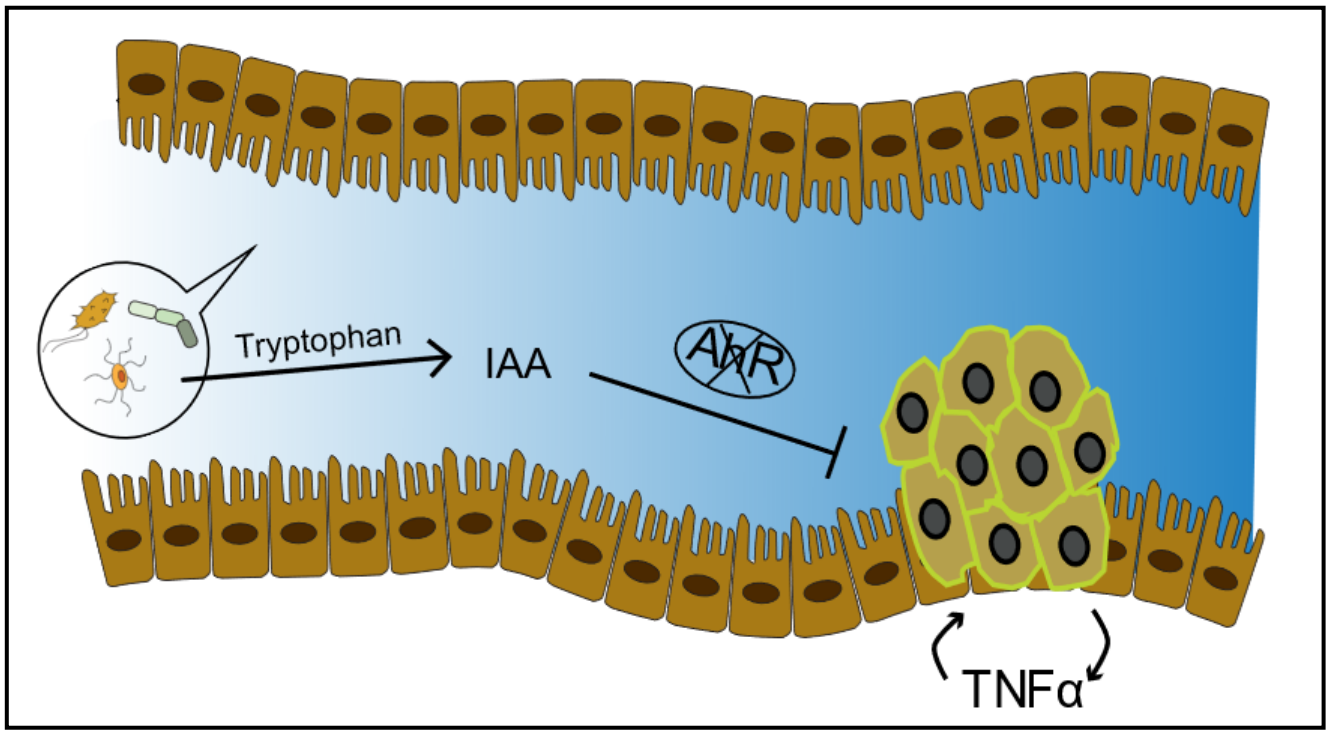


Figure 9. Indole-3-acetic acid suppresses TNFα expression in Caco-2 cells via AhR-independent pathway.

In addition to the identification of AhR-independent suppression mechanisms of TNFα expression induced by IAA, the finding of intestinal bacteria with indoleacetate decarboxylase and a mechanism for reducing IAA might be very important to understand the relationship between the gut environment and the initiation and progression of CRC.

Chapter-3

TLR4 may be a novel indole-3-acetic acid receptor that is implicated in the regulation of CYP1A1 and TNFα expression depending on the culture stage of Caco-2 cells

1. Introduction

Metabolites produced by intestinal microbiota influence host physiological functions. The laboratory I belong to has long focused on indole and indole derivatives that are synthesized by intestinal microbiota from tryptophan in dietary proteins and play an important signaling role between the microbiota and the host. The mechanism through which indoxyl sulfate, which is produced in the liver from indole, accelerates the exacerbation of chronic kidney disease (CKD) (Saito et al., 2014; Shimizu et al., 2013a, 2013b, 2013c, 2012a, 2012c, 2011b, 2011a, 2010a) and contributes to the initiation and progression of CKD complications such as atherosclerosis has been clarified (Shimizu et al., 2012b, 2009). The laboratory I belong to previously reported that skatole metabolized by intestinal microbiota from indole-3-acetic acid (IAA) aryl hydrocarbon receptor (AhR)-dependently and independently causes functional impairment of intestinal epithelial cells (Kurata et al., 2019), and IAA AhR-independently induces the suppression of tumor necrosis factor α (TNFα) expression in Caco-2 cells (Chapter 2).

Indole and indole derivatives are ligands for AhR, which belongs to a family of basic helix-loop-helix transcription factors (Stockinger et al., 2021). Inactive AhR is usually localized in the cytoplasm as a stable protein complex of a dimer comprising 90-kDa heat shock protein (hsp90) (Denis et al., 1988; Perdew, 1988) and hepatitis B virus X-associated protein-2 (XPA2) (Meyer et al., 1998), with co-chaperone p23 (Nair et al., 1996). The structure of the AhR complex changes upon ligand binding, then dissociates from the complex, exposing the amino-terminal nuclear localization sequence and an adjacent nuclear export sequence. The ligand-AhR complex is then translocated into the nucleus (McGuire et al., 1994), where it binds to the aryl hydrocarbon translocator (Arnt) to form a transcriptionally active heterodimer that binds to xenobiotic responsive elements (XREs) in the regulatory region of AhR target genes such as cytochrome P450 1A1 (CYP1A1) and AhR repressor (AhRR), and controls their expression (Matsushita et al., 1993; Mimura et al., 1999b; Reyes et al., 1992). The suppression of AhR transcriptional activity regulated by AhRR and proteolytic degradation of AhR are involved in the negative feedback regulation mechanism of AhR activity. The activation of AhR by its ligands induces the expression of AhRR, which dimerizes with Arnt. This inhibits the formation of AhR-Arnt dimers, thus preventing binding of the AhR-Arnt complex to XREs (Evans et al., 2008; Mimura et al., 1999b). The proteolytic degradation of AhR (Davarinos and Pollenz, 1999), involves 2,3,7,8-

Tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible poly (ADP-ribose) polymerase (TIPARP/ARTD14/PARP7)-mediated ribosylation (MacPherson et al., 2013).

Toll-like receptors (TLRs) play central roles in the innate immune responses of vertebrates (Fitzgerald and Kagan, 2020). Each TLR recognizes a specific microbial product and activates each signaling pathway. For instance, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from gram-negative bacteria can activate TLR4 (Fitzgerald and Kagan, 2020). However, TLR4 alone cannot recognize LPS; thus, other proteins are required to recognize the ligand. LPS firstly interacts with the soluble shuttle LPS-binding protein (LBP) (Tobias et al., 1986; Wright et al., 1989), then helps LPS binding to soluble anchored cluster of differentiation 14 (CD14), another glycosylphosphatidylinositol protein (Wright et al., 1990). Thereafter, CD14 transfer LPS to a large hydrophobic pocket in a soluble myeloid differentiation 2 (MD-2) (Park et al., 2009). When LPS is non-covalently bound to MD-2, it interacts with the extracellular domain of TLR4 to form a heterodimer (LPS-MD-2-TLR4), to initiate intracellular signaling (Shimazu et al., 1999).

Phase I and II metabolic enzymes are involved in the activation and detoxification of xenobiotics. Cytochrome P450s (CYPs) are phase I enzymes that are involved in the oxidative activation and inactivation of endogenous substrates, including drugs and carcinogens (Pelkonen et al., 2008). As a representative gene of CYPs that is normally expressed in intestinal cells (Ding and Kaminsky, 2003), CYP1A1 is involved in the metabolic activation of xenobiotic carcinogens such as polycyclic aromatic hydrocarbons (PAHs) (Mescher and Haarmann-Stemmann, 2018). The most representative transcriptional activation of CYP1A1 is caused by the binding of the AhR/Arnt heterodimer to the XRE in the 5′ flanking region of CYP1A1 (Matsushita et al., 1993; Reyes et al., 1992). In fact, Chapter 2 and Jin et al have shown that IAA leads to increased CYP1A1 expression by activating AhR in Caco-2 cells (Jin et al., 2014b). On the other hand, CYP1A1 expression is elevated in the lungs of AhR global knockout mice induced by LPS (Tian et al., 2020). In addition, TLR4 is a receptor for pyrimido[5,4-*b*]indole containing an indole ring (Chan et al., 2013). Because I previously found that IAA also binds to receptors other than AhR (Chapter 2), the present study aimed to clarify whether TLR4 could be an IAA receptor using increased CYP1A1 expression induced by IAA as an indicator. I also investigated the relationship between suppressed TNFα expression and TLR4 activation induced by IAA.

2. Materials and methods

2.1 Materials and reagents

Antibodies and other reagents were obtained from the following suppliers: anti-aryl hydrocarbon receptor and anti-β-actin (C4) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-rabbit and anti-mouse IgG-HRP-linked antibody (Cell Signaling Technology, Inc., Danvers, MA, USA); Protease Inhibitor Cocktail (EDTA free) (100×), phosphatase inhibitor cocktail, and indole-3-acetic acid (Nacalai Tesque Inc., Kyoto, Japan); penicillin-streptomycin solution (100×), Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, and SP600125 (Wako Pure Chemical Industries Ltd., Osaka, Japan); TAK-242 (Chemscene, Monmouth Junction, NJ, USA), and fetal bovine serum (FBS) (Biowest S.A.S., Nuaillé, France).

2.2 Cell culture

The human colon cancer cell line Caco-2 (RIKEN Cell Bank, Tsukuba, Japan) was maintained in DMEM supplemented with high glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C under a 7% CO_2 atmosphere. Caco-2 cells (3×10^5/3.5-cm dish) were starved in serum-free DMEM for 24 hours before starting all experiments, then incubated them with or without TAK-242 or SP600125, and then stimulated them with or without IAA for various periods. Dimethyl sulfoxide (DMSO; final concentration, 0.1% v/v) served as the control and vehicle for IAA, CH223191, TAK-242, and SP600125.

2.3 Quantitative real-time PCR

Total RNA was isolated from Caco-2 cells using Trizol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized from template RNA (1 μg) derived from Caco-2 cells using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio Co. Inc., Shiga, Japan). Quantitative real-time PCR proceeded using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) and a Thermal Cycler Dice Real Time System III (all from Takara Bio Co. Inc.) as described by the manufacturer, and oligonucleotide primers (Table 2). Amplicons were quantified using a calibration curve, and mRNA expression was measured as the ratio of each mRNA to RPLP0 mRNA.

Table 2. Forward and reverse (5′→3′) primer sequences of target human genes.

Gene	Forward	Reverse
CYP1A1	ACATGCTGACCCTGGGAAAG	GGTGTGGAGCCAATTCGGAT
AhR	ACATCACCTACGCCAGTCG	CGCTTGGAAGGATTTGACTTGA
AhRR	GGGGCAGAGAAGTCCAACC	CCCGGAGGTAACTGACACTG
TNFα	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
RPLP0	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG

2.4 Immunoblotting

Caco-2 cells were lysed in 1% NP-40 buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10% glycerol, Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail), then resolved by SDS-PAGE. The resolved proteins were transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore Inc., Bedford, MA, USA) and immunoblotted. The AhR and phospho-JNK values were normalized to those of β-actin. Protein bands were visualized using the enhanced Chemi-Lumi One Super (Nakalai Tesque Inc.) and analyzed using an ImageQuant LAS 4000 densitometer (GE Healthcare Life Sciences Corp., Uppsala, Sweden).

2.5 Statistical analysis

Results are expressed as means ± SE. Data were statistically analyzed by Student *t*-tests (Fig. 10b and c), Dunnett (Fig. 11b and c), and Tukey-Kramer tests (Fig. 12b and c, 13, 14a, 15c, 16b and c, and 17) using Excel 2011 (Microsoft Corp., Redmond, WA, USA) and Statcel 4 (OMS Publishing Co., Saitama, Japan). Values were considered statistically significant at $P< 0.05$.

3. Results

3.1 Indole-3-acetic acid increased CYP1A1 expression in Caco-2 cells

Since the moisture content of normal feces is 60%-80%, and the maximum fecal concentration of skatole, an IAA metabolite synthesized by intestinal microbiota, is 1000-1200 μM (Yokoyama and Carlson, 1979; Gao et al., 2018), the maximum fecal IAA concentration may be similar. Therefore, the present study mainly applied 1000 μM IAA as I described in Chapter 2. I initially assessed CYP1A1 expression to investigate IAA responses in Caco-2 cells. Figure 10a shows the experimental procedure. Figure 10b shows that CYP1A1 mRNA expression started to significantly increase from 3 hours after stimulation with 1000 μM IAA, and gradually decreased from 4 days, but continued for 2-8 days (Fig. 10c), which agrees with my previous findings (Chapter 2). Figure 11d shows that the abundance of CYP1A1 expression was similar at 3 hours after stimulation with 50 to 1000 μM IAA. On the other hand, CYP1A1 expression at 8 days after stimulation with IAA dose-dependently increased (Fig. 11c). Having reconfirmed that IAA increased CYP1A1 expression, I aimed to identify a new receptor for IAA using this as an indicator.

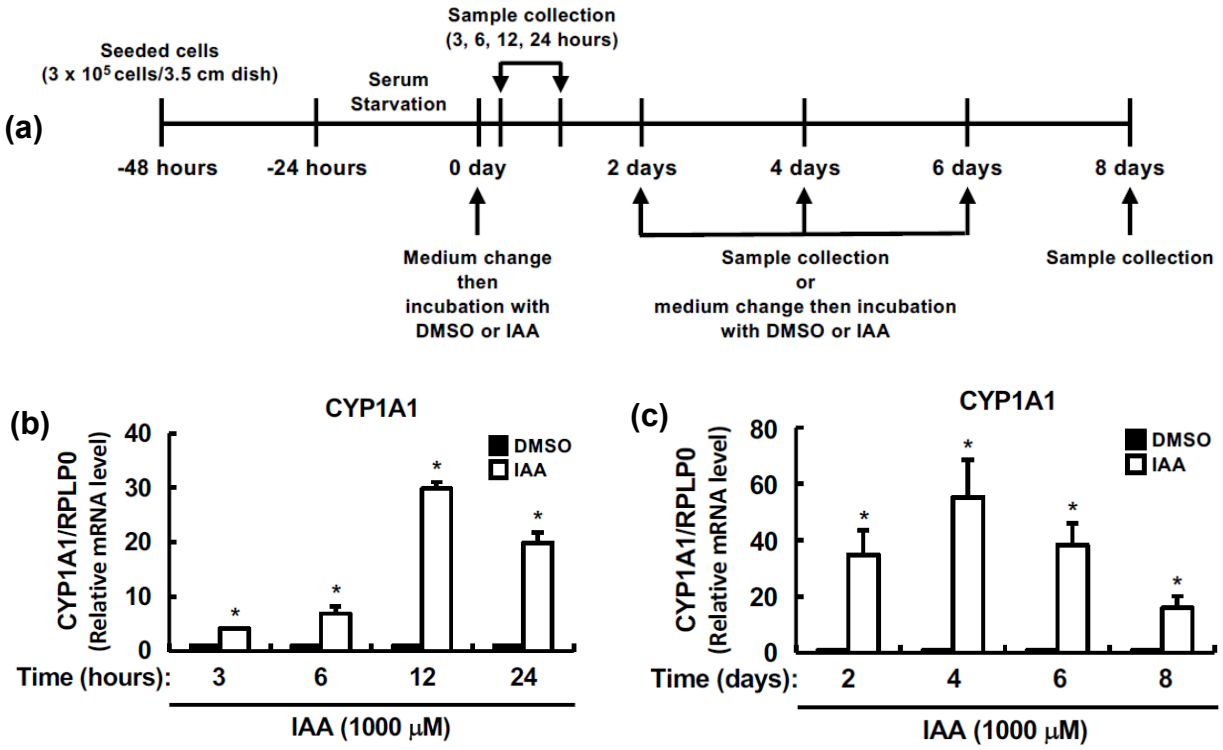


Figure 10. Time-dependent changes in CYP1A1 mRNA expression induced by IAA in Caco-2 cells. (a) Experimental procedure. Samples were collected at various times. Expression of CYP1A1 mRNA

determined by real-time PCR in samples collected at indicated times (b) and after 2 days (c) of incubation with 0.1% DMSO (vehicle) or IAA (1000 μM). Medium was replenished every 2 days for 8 days. Data are expressed as means ± SE of three for (b) and four (c) independent experiments *P < 0.05 vs. vehicle only-treated cells.

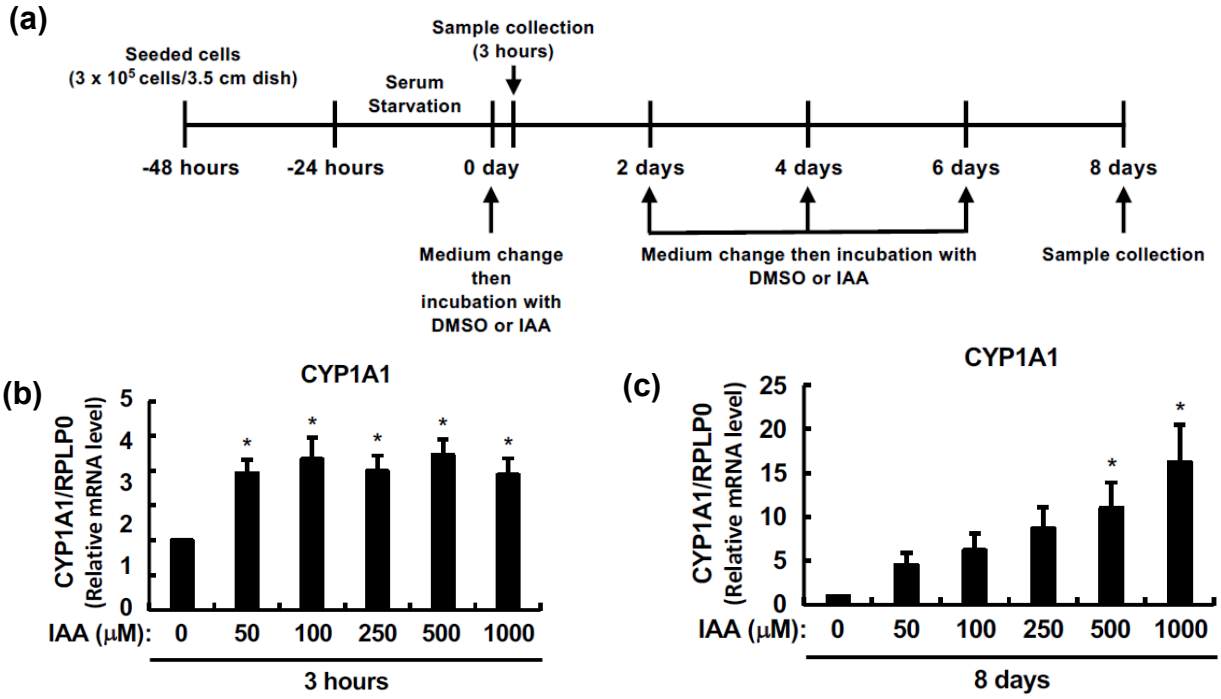


Figure 11. Dose-dependent changes in CYP1A1 mRNA expression increased by IAA in Caco-2 cells. (a) Experimental procedure. Samples were collected from Caco-2 cells incubated with 0.1% DMSO (vehicle) or IAA (0-1000 μM) for (b) 3 hours and (c) 2 and 8 days, then CYP1A1 mRNA expression was determined using real-time PCR. Medium was replenished every 2 days for 8 days. Data are expressed as means ± SE of four independent experiments each for (b) and (c). *P < 0.05 vs. vehicle only-treated cells.

3.2 Regulation of CYP1A1 expression involved TLR4 at 8 days, but not at 3 hours after IAA stimulation in Caco-2 cells

I examined whether TLR4 was involved in the CYP1A1 expression that remained increased from 3 hours to 8 days after stimulation with IAA (Fig. 10b and c). Figure 12a shows the experimental procedure. Figure 12a shows that the TLR4 antagonist TAK-242 did not affect the increased CYP1A1 expression induced by IAA at 3 hours. On the other hand, at 8 days after stimulation with TAK-242, the increased CYP1A1 expression induced by IAA was promoted (Fig.

12c). These results suggested that IAA regulated increased CYP1A1 expression by activating TLR4 at 8 days of incubation.

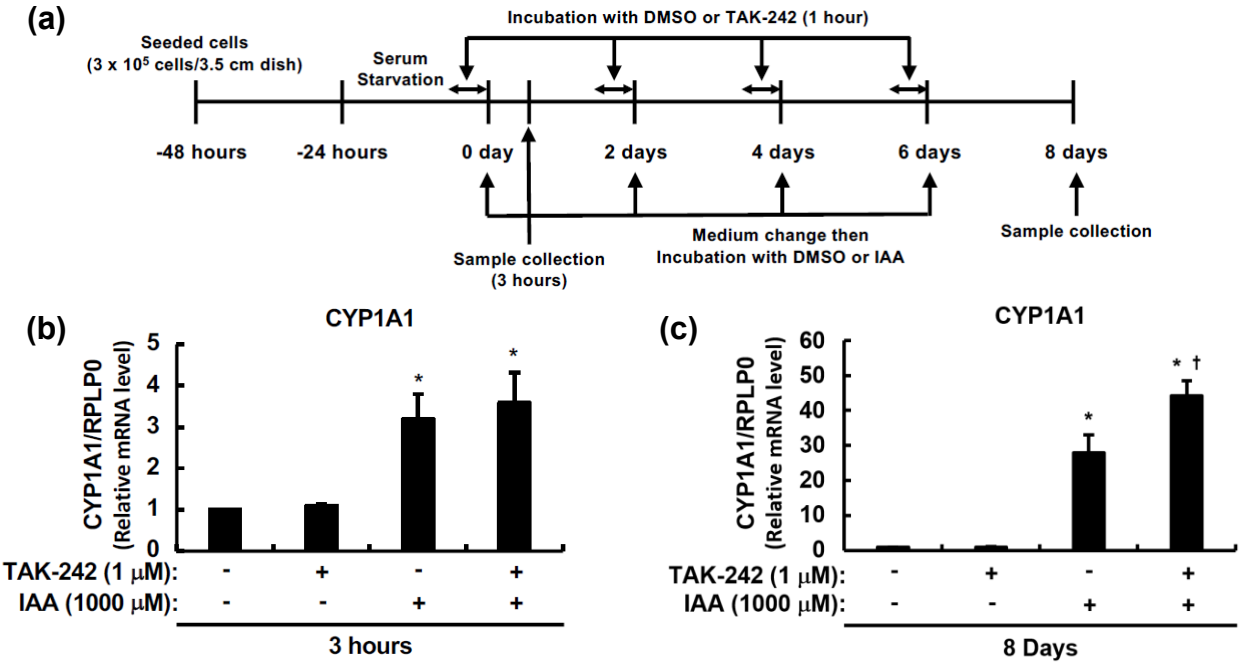


Figure 12. Effects of TAK-242 on CYP1A1 mRNA expression after incubation for 3 hours and 8 days with or without IAA. (a) Experimental procedure. (b) Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 1 μM TAK-242 (TLR4 antagonist) for 1 hour, followed by vehicle or 1000 μM IAA for 3 hours. Expression of CYP1A1 mRNA was measured using real-time PCR. (c) Caco-2 cells were incubated with vehicle or 1 μM TAK-242 for 1 hour, followed by vehicle or IAA (1000 μM) for 2 days. This procedure was repeated at 2, 4, and 6 days. Expression of CYP1A1 mRNA was measured at 8 days using real-time PCR. Data are expressed as means ± SE of five (b) and four (c) independent experiments. *$P < 0.05$ vs. vehicle only-treated cells; $^{\dagger}P < 0.05$ vs. IAA-treated cells.

3.3 Expression of AhRR and AhR in Caco-2 cells was not influenced by IAA and TAK-242

I investigated whether AhRR and AhR mRNA expression, and protein levels of AhR were altered to explain the increased CYP1A1 expression at 8 days after stimulation with TAK-242 and/or IAA. Figure 12a shows the experimental procedure. Figure 13 shows that AhRR mRNA expression did not change, indicating that the negative feedback regulation of AhR function by AhRR was not affected. Thus, I explored changes in AhR mRNA expression. However, AhR mRNA expression was also not affected at 8 days after stimulation with TAK-242 and/or IAA (Fig. 14a). I predicted that proteolytic AhR degradation was inhibited, and thus its protein

expression increased at 8 days after stimulation with TAK-242 and/or IAA. Figure 14b shows that AhR protein levels also remained unchanged. These results suggest that neither increased AhR mRNA and protein expression nor suppressed negative feedback regulation of AhR activity was involved in promoting the increase in expression CYP1A1 induced by TAK-242 at 8 days after stimulation with IAA.

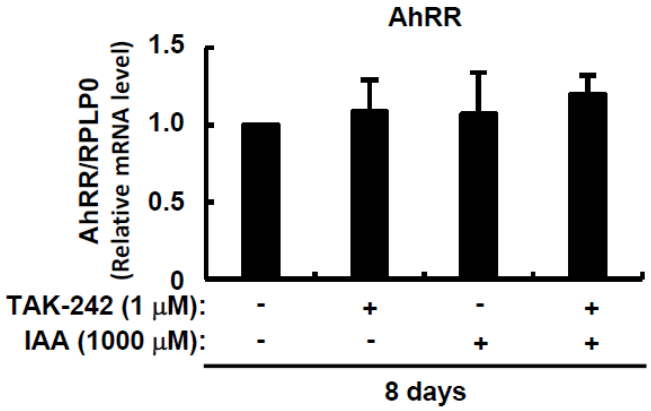


Figure 13. Effects of TAK-242 on AhRR mRNA expression at 8 days after incubation with or without IAA. Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 1 μM TAK-242 (TLR4 antagonist) for 1 hour, followed by vehicle or 1000 μM IAA for 2, 4, and 6 days. Expression of AhRR mRNA was measured at 8 days using real-time PCR. Data are expressed as means ± SE of four independent experiments.

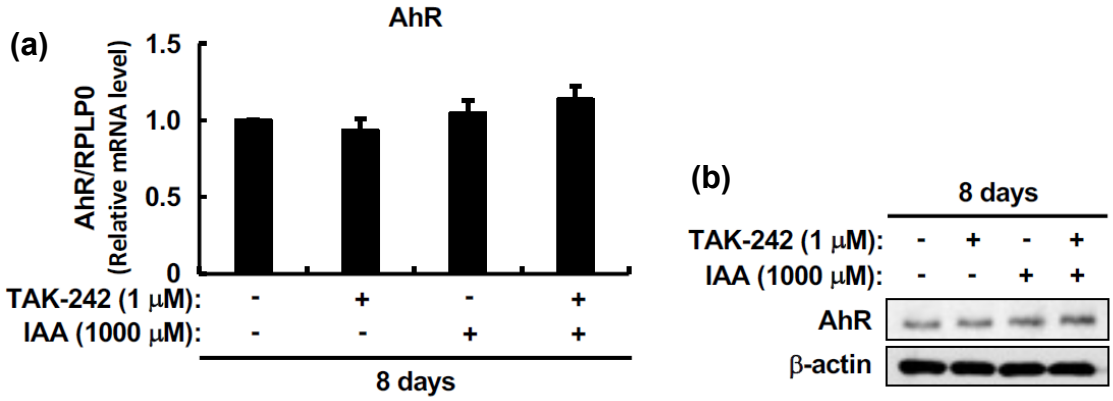


Figure 14. Effects of TAK-242 on AhR mRNA and protein expression at 8 days after incubation with or without IAA. (a) Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 1 μM TAK-242 (TLR4 antagonist) for 1 hour, followed by vehicle or 1000 μM IAA for 2, 4, and 6 days. Expression of AhR mRNA was measured by real-time PCR, and (b) cell lysates at 8 days were immunoblotted against anti-AhR and anti- β-actin antibodies. Data are expressed as means ± SE of four independent experiments for (a).

3.4 Activation of TLR4/JNK signaling pathway induced by IAA at 8 days after stimulation in Caco-2 cells attenuated increased CYP1A1 expression

Since JNK is activated downstream of TLR4 (Hambleton et al., 1996), the present study examined whether TAK-242 inhibited JNK activation in Caco-2 cells at 8 days after IAA stimulation and found that TAK-242 suppressed IAA-induced JNK activation (Fig. 15a). I then investigated the relationship between JNK activation and increased CYP1A1 expression at 8 days after stimulation with IAA. Figure 15b shows the experimental procedure. The JNK inhibitor SP600125 promoted the increased CYP1A1 expression induced by IAA at 8 days after stimulation (Fig. 15c). These results together indicated that activating the signaling pathway of TLR4/JNK inhibited by TAK-242 promoted increased CYP1A1 expression induced by IAA at 8 days after stimulation. That is, activation of TLR4/JNK signaling pathway induced by IAA may have partially attenuated increased CYP1A1 expression at 8 days after stimulation with IAA.

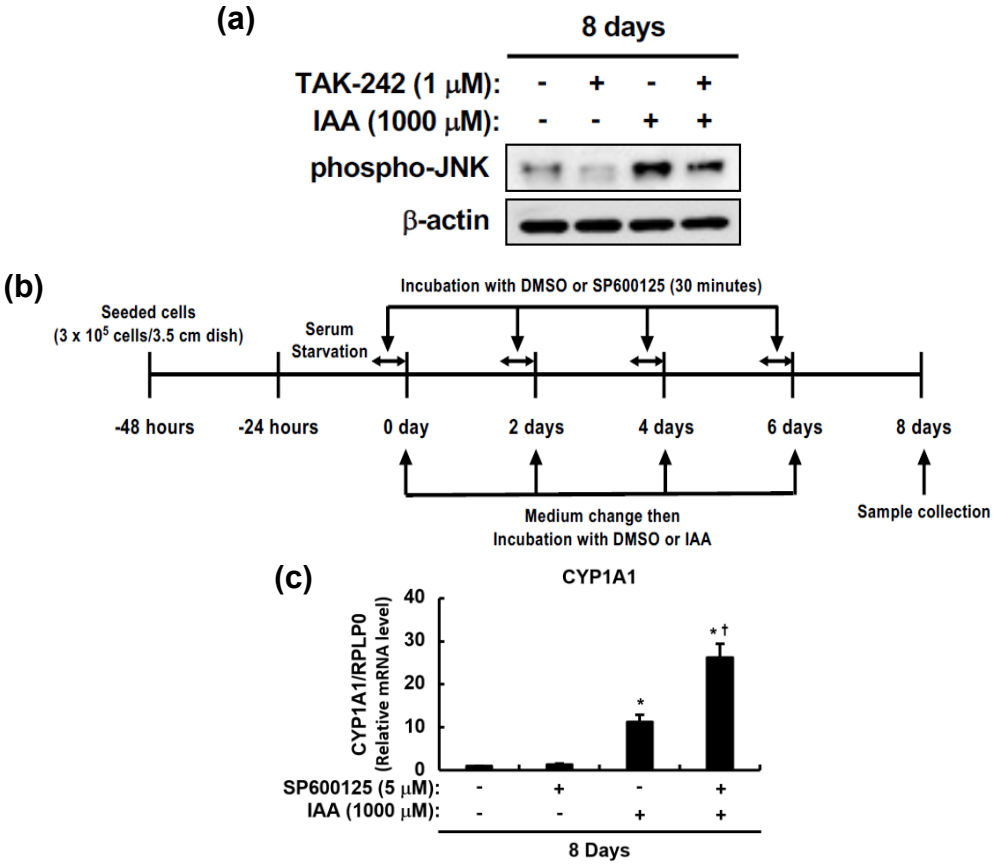


Figure 15. The validation of TLR4/JNK pathway activation on increased CYP1A1 expression at 8 days after stimulation with IAA. (a) Caco-2 cells were incubated with 0.1% DMSO (vehicle) or 1 μM TAK-242 (TLR4 antagonist) for 1 hour, followed by vehicle or 1000 μM IAA for 2, 4, and 6 days. Immunoblots of

cell lysates at 8 days using phospho-SAPK/JNK and anti- β-actin antibodies. (b) Experimental procedure. Caco-2 cells were incubated with vehicle or 5 μM SP600125 (JNK inhibitor) for 30 minutes, followed by vehicle or 1000 μM IAA for 2, 4, and 6 days. (c) Expression of CYP1A1 mRNA was measured at 8 days using real-time PCR. Data are expressed as means ± SE of four independent experiments for (c). *$P < 0.05$ vs. vehicle only-treated cells; †$P < 0.05$ vs. IAA-treated cells.

3.5 Suppression of TNFα expression is mediated by TLR4 at 2, but not at 8 days after stimulation with IAA in Caco-2 cells

Results from the Chapter 2 suggested that unknown receptors for IAA are involved in the suppression of TNFα expression at 2 and 8 days after stimulation. Based on the above findings, I predicted that TLR4 activation induced by IAA suppressed TNFα expression in Caco-2 cells. Since I identified a correlation between TNFα mRNA expression and protein levels induced by IAA (Chapter 2), I assessed the effects of TAK-242 on the suppression of TNFα expression induced by IAA using TNFα mRNA expression as an indicator. Figure 16a shows the experimental procedure. Figure 16b shows that TAK-242 alleviated the suppression of TNFα expression induced by IAA at 2, but not at 8 days after stimulation (Fig. 16c). These results indicate that the regulatory mechanism of TNFα expression through TLR4 activation induced by IAA differs depending on the culture stage of Caco-2 cells.

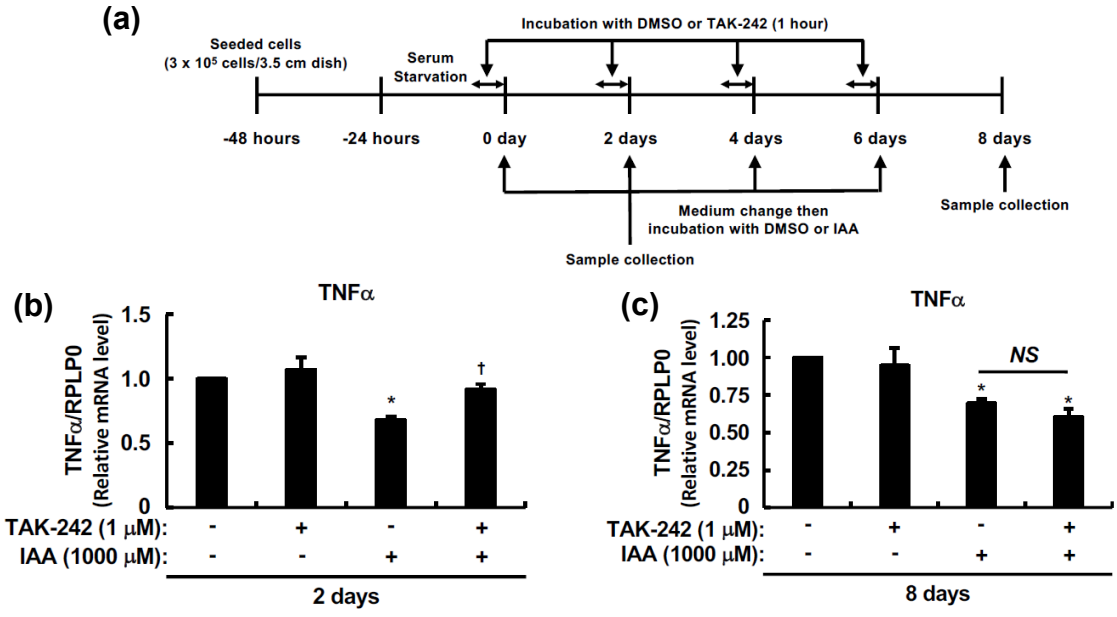


Figure 16. Effects of TAK-242 on suppressed TNFα expression at 2 and 8 days after stimulation with or without IAA. (a) Experimental procedure. Samples were collected at 2 and 8 days after stimulation with

0.1% DMSO (vehicle) or 1000 μM IAA. (b) Caco-2 cells were incubated with vehicle or 1 μM TAK-242 (specific TLR4 antagonist) for 1 hour, followed by vehicle or 1000 μM IAA for 2 days, then TNFα mRNA expression was measured using real-time PCR. (c) Caco-2 cells were incubated with vehicle or 1 μM TAK-242 for 1 hour, followed by vehicle or 1000 μM IAA for 2, 4, and 6 days, then TNFα mRNA expression at 8 days was measured using real-time PCR. Data are expressed as means ± SE of four (b) and three (c) independent experiments **P* < 0.05 vs. vehicle only-treated cells; †*P* < 0.05 vs. IAA-treated cells.

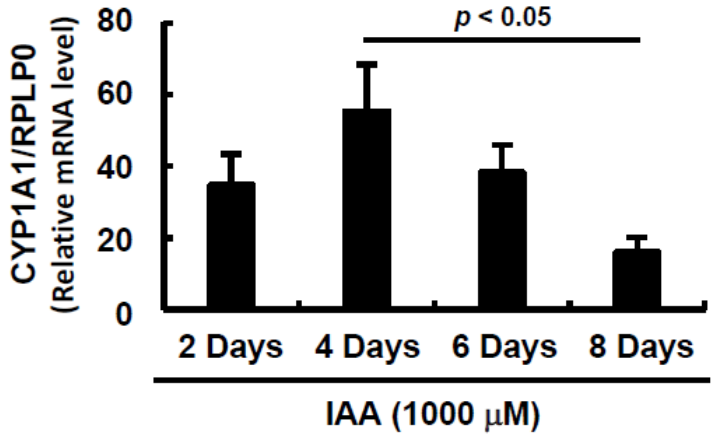


Figure 17. Time-dependent changes in CYP1A1 mRNA expression induced by IAA between 2 and 8 days. Caco-2 cells were incubated with 0.1% DMSO or 1000 μM IAA for 2 days, then the medium was replenished every 2 days for 8 days. Expression of CYP1A1 mRNA was determined at various times using real-time PCR. Data are expressed as means ± SE of four independent experiments.

4. Discussion

The novel findings of the present study are summarized in Figure 8 and as follows: 1) Increased CYP1A1 expression persisted from 3 hours until 8 days after stimulation with IAA; 2) The upregulated CYP1A1 expression was dose-dependently induced by IAA at 8 days, but not at 3 hours after stimulation; 3) TAK-242 did not affect the increased CYP1A1 expression induced by IAA at 3 hours after stimulation but promoted it by inhibiting JNK activation at 8 days after stimulation; 4) The IAA-induced decrease in TNFα expression was alleviated by TAK-242 at 2 days but not at 8 days after stimulation. Together with Chapter 2 indicating that AhR is a receptor for IAA but it is not involved in the IAA-induced suppression of TNFα expression, the present findings suggested that TLR4 is a receptor for IAA in addition to AhR, and that the mechanisms through which CYP1A and TNFα expression is regulated by IAA-activated TLR4 were suggested to change depending on the culture stage of Caco-2 cells. Furthermore, my findings at 8 days after stimulation with IAA, indicated that even more unidentified receptors for IAA regulate TNFα expression.

A substituted acetamide moiety attached to the pyrimidoindole ring system through a thioether linkage, a type of pyrimido[5,4-*b*]indole, is a synthetic ligand for TLR4 (Chan et al., 2013). A unique feature of this synthetic ligand is that the ligand does not require CD14 for binding to the TLR4/MD-2 complex although LPS is transported and binds to the TLR4/MD-2 complex by CD14 localized at the cell membrane (Chan et al., 2013). This synthetic ligand should also form a complex with TLR4/MD-2 and promote dimerization through a binding mechanism similar to that of LPS. Notably, predictions indicate that Glu439 and Phe440 of TLR4 and Ile124 of MD-2 bind to the indole ring within pyrimido[5,4-*b*]indole (Chan et al., 2013). That is, in addition to the possibility that IAA binds to TLR4 and MD-2 through a mechanism similar to that of pyrimido[5,4-*b*]indole and activates the TLR4 signaling pathway, IAA, like pyrimido[5,4-*b*]indole, may CD14-independently bind to the TLR4/MD-2 complex. Therefore, IAA may have a greater impact on various tissues than LPS through binding more readily than LPS to the TLR4/MD-2 complex, which is expressed in tissues with low CD14 expression. Furthermore, since LPS and palmitate cause increased TNFα expression through activating TLR4 (Shi et al., 2006), whereas IAA did not as I showed in Chapter 2 and herein, IAA may attenuate TLR4 signaling induced by LPS and palmitate via binding to TLR4. In fact, IAA significantly and dose-dependently reduces the increased TNFα expression induced by LPS+palmitate in mouse

macrophage RAW 264.7 cells (Krishnan et al., 2018). Although this mechanism is obscure, the present results indicate that it could be due to IAA competing with LPS+palmitate for TLR4. Thus, IAA may act as an agonist and an antagonist, depending on competition for TLR4.

The increased CYP1A1 expression induced by IAA was sustained for 8 days after stimulation. However, CYP1A1 expression induced by IAA peaked at 4 days after stimulation and was significantly reduced at 8 days (Figure 17). The reason for this may involve the activation of AhR signaling induced by IAA attenuated by TLR4/JNK signaling, because TAK-242 and SP600125 promoted increased CYP1A1 expression induced by IAA at 8 days after stimulation. However, since IAA also induces JNK activation at 10-30 minutes (unpublished data), activated JNK may not be directly involved in the pathway of IAA-induced increase in CYP1A1 expression. Therefore, genes with altered expression upon JNK activation may participate in promoting the IAA-induced increased CYP1A1 expression via TAK-242 at 8 days after stimulation. The present study showed that AhRR and AhR expression did not change at 8 days after incubation with or without IAA and/or TAK-242. In addition, other groups reported that transcriptional activity of AhR is enhanced by increasing its serine/threonine or tyrosine phosphorylation levels (Berghard et al., 1993; Gradin et al., 1994; Li and Dougherty, 1997; Long et al., 1998; Minsavage et al., 2003; Park et al., 2000). According to the previous studies on protein tyrosine phosphatases (PTPs) with other groups, PTPs, unlike kinases, may constantly act on their substrates (Hallé et al., 2009; Shimizu et al., 2010b, 2001). Taken together, I speculate that IAA-induced JNK activation inhibited by TAK-242 or SP600125 at 8 days caused decreased serine/threonine phosphatases and/or PTPs expression along with enhanced AhR transcriptional activity induced by increased phosphorylation levels, and subsequently promoted the upregulated CYP1A1 expression induced by IAA.

The expression of CYP1A1 was dose-dependently increased by IAA at 8 days, but not at 3 hours after stimulation. Had IAA been passively transported into the cells, CYP1A1 expression at 3 hours after stimulation with IAA would have dose-dependently increased. Therefore, the transporter may regulate the uptake of IAA regardless of the extracellular abundance of IAA. In line with this speculation, more IAA may have accumulated in cells incubated with TAK-242 than in control cells at 8 days, but not at 3 hours after stimulation. This may have been because free IAA that could not time-dependently bind to TLR4 via TAK242 that was gradually uptaken into cells after IAA stimulation. Thus, not only TLR4/JNK signaling pathway-mediated regulation but

also enhanced AhR transcriptional activity associated with increased intracellular IAA concentrations via transporters may promote the increase in CYP1A1 expression induced by IAA at 8 days of incubation with TAK-242.

The effect of TAK-242 on the suppression of TNFα expression induced by IAA also differed between 2 and 8 days. Thus, the regulatory mechanism of TNFα expression induced by IAA may differ during the 6-day interval. The results from chapter 2 showed that AhR activation is not involved in suppression of TNFα expression induced by IAA. Therefore, I consider that different mechanisms regulate TNFα and CYP1A1 expression induced by IAA. In addition, JNK activation induced by IAA at 8 days suppressed by TAK-242 did not significantly change TNFα expression in cells incubated with IAA alone, compared with TAK-242 plus IAA. These results indicated that activation of the JNK pathway is not involved in downregulated TNFα expression induced by IAA at 8 days. Furthermore, the suppressed TNFα expression induced by IAA was alleviated by TAK-242 at 2, but not at 8 days. This suggested that although TLR4 activation induced by IAA suppressed TNFα expression at 2 days, signal transduction molecules activated by IAA via unidentified receptors canceled the alleviative effect of TAK-242 on suppression of TNFα expression induced by IAA at 8 days. However, further study of unidentified receptors is required to elucidate details of the mechanism induced by IAA stimulation at 8 days.

PAHs such as Benzo[a]pyrene (BaP) are formed in red meat cooked at high temperatures (Kazerouni et al., 2001) and produce metabolites with carcinogenic effects in human colon cancer HT-29 cells via CYP1A1 and CYP1B1 (Myers et al., 2021). Furthermore, the oral ingestion of BaP leads to a sustained increase in colonic CYP1A1 and CYP1B1 expression in mouse models of adenomatous polyposis coli with multiple intestinal neoplasia. Both CYP1A1 and CYP1B1 then dose-dependently produced increased concentrations of BaP metabolites. These metabolites bind to DNA in the colon to form BaP-DNA adducts, concentrations of which closely correlate with the incidence of adenoma (Diggs et al., 2013). Thus, IAA derived from dietary proteins leads to increased intestinal CYP1A1 expression in the intestine and may induce the development and progression of colorectal cancer (CRC). In fact, consuming large amounts of meat, which is assumed to be involved in the increased intestinal IAA production, has been linked to the development and progression of CRC (Lippi et al., 2016). However, substantial amounts of detectable BaP-DNA adducts accumulate in the liver, intestine, spleen, and bone marrow of CYP1A1 global knockout mice fed with 12.5 mg/kg/day of oral BaP for 18 days (Uno et al., 2004).

In addition, 12.5 mg/kg/day of BaP is associated with the development of an immunoglobulin-secreting adenocarcinoma in the intestinal epithelium of CYP1A1 global knockout mice. Because the oral ingestion of BaP does not lead to evidence of BaP-DNA adducts or malignancies in wild-type mice, inducible CYP1A1 is crucial for detoxification and protection against oral BaP toxicity (Shi et al., 2010). Furthermore, CYP1A1 may metabolize and detoxify not only BaP but also (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), the ultimate carcinogenic intermediate of BaP, which could explain why levels of detectable BaP-DNA adducts are substantial in organs of CYP1A1 global knockout mice (Shiizaki et al., 2017). Hepatocyte-specific CYP1A1 knockout mice orally administered with BaP remained as healthy as wild-type mice, whereas intestinal epithelial cell-specific CYP1A1 knockout mice died of immunosuppression after about 30 days like CYP1A1 global knockout mice (Shiizaki et al., 2017). The most important issue revealed by these findings is that the intestine plays the most important role in CYP1A1-mediated metabolism of orally ingested compounds, which in turn affects various organs and tissues. However, a mutation in exon 7 of CYP1A1 from Ile to Val (nucleotides A to G) at codon 462 associated with increased its activity is also associated with the development and progression of CRC (Jin et al., 2011). Thus, not only phase I enzymes such as CYP1A1 but also phase II enzymes act as conjugates with metabolites of phase I enzymes, rendering them water-soluble. This prevents them from enhancing carcinogenesis; thus, facilitating their excretion may be important. That is, enhancing phase II, and phase I enzymes before BaP exposure could rapidly detoxify carcinogens and activated procarcinogens, and efficiently avoid tumor development and progression. For instance, indole-3-carbinol (I3C) is an indole derivative that has antitumor activity, not only increases phase II enzyme expression (Bonnesen et al., 2001), but also reduces the amounts of intermediates produced by CYP1A1 in Caco-2 cells (Ebert et al., 2005). Furthermore, although the focus is on gastric tumor formation, dietary I3C suppressed BaP-induced neoplasia of the forestomach in ICR/Ha mice (Wattenberg and Loub, 1978). Therefore, if IAA can increase the expression of phase II enzymes as well as CYP1A1 in the intestine like I3C, it may inhibit the development and progression of CRC mediated by genetic mutations caused by BaP by detoxifying carcinogenic intermediate metabolites of BaPs. However, whether IAA leads to increased phase II enzyme expression and less carcinogenic detoxified BaP metabolites is unclear. At least for now, consuming foods that lead to the induction of phase II enzyme expression along

with proteins that generate IAA may prevent the development and progression of CRC induced by increased CYP1A1 expression.

5. Conclusion

Since a low intake of dietary protein has been related to a loss of body muscle mass (Houston et al., 2008), and protein intake has been oppositely associated with frailty (Bartali et al., 2006; Beasley et al., 2010), a high-protein diet has recently been recommended to prevent sarcopenia, specially in the elderly (Kobayashi et al., 2013). On the other hand, abundant intake of meat is involved in the development and progression of CRC worldwide (Lippi et al., 2016). High dietary protein and meat intake results in abundant intestinal production of IAA, which may persistently affect intestinal epithelial cells. The consumed amount and timing of TLR4 inhibitory components and CYP1A1 substrates in foods may make a difference between health promotion and organ dysfunction when consuming a high-protein diet. Therefore, the present study indicates the potential for elucidating the molecular mechanisms involved in the health benefits conferred by high-protein diets. My findings also provide important clues for understanding the mechanisms underlying the actions of indole derivatives such as IAA produced by intestinal microbiota from tryptophan in dietary proteins that affect hosts.

Chapter-4

General Conclusion

General conclusion

The present study may add to my understanding of the importance of indole derivatives on the pathophysiology of the intestinal epithelium. Numerous indole derivatives produced from various pathways, such as dietary consumption, by host commensal microorganisms have been postulated as physiological ligands for aryl hydrocarbon receptor (AhR), through which most of the biological responses occur. The current study features the novel effects of indole-3-acetic acid (IAA) generated from the dietary tryptophan by intestinal microbiota on colorectal cancer (CRC) cells. CRC, one of the most commonly diagnosed cancer, affecting millions around the world and considered a life-threatening disease due to its immense recurrence rates, and both lifestyle practices and genetic factors play a critical role in the development and progression of CRC. Many therapies for CRC are available which are not free from adverse effects. The new findings of the present research delineate a precise mechanism, where IAA may activate the AhR as well as toll-like receptor 4 (TLR4) and regulate the mRNA expression levels of cytochrome P450 1A1 (CYP1A1), a phase I metabolizing enzyme, and tumor necrosis factor α (TNFα), one of the inflammatory cytokines, differently depending on the culture stage of Caco-2 cells and also indicates the involvement of more unidentified receptors for IAA. This study manifests my advancements in understanding the impact of IAA as a regulator of TNFα, which is involved in the tumor growth and development of metastasis in patients with CRC. Since the amount of IAA is directly proportional to the amount of protein intake, these novel findings may offer new strategies for CRC management. However, based on the current results that IAA may also bind with other receptors rather than AhR and TLR4, further studies are required to elucidate the detailed mechanism of IAA-induced CYP1A1 and TNFα mRNA expressions in Caco-2 cells.

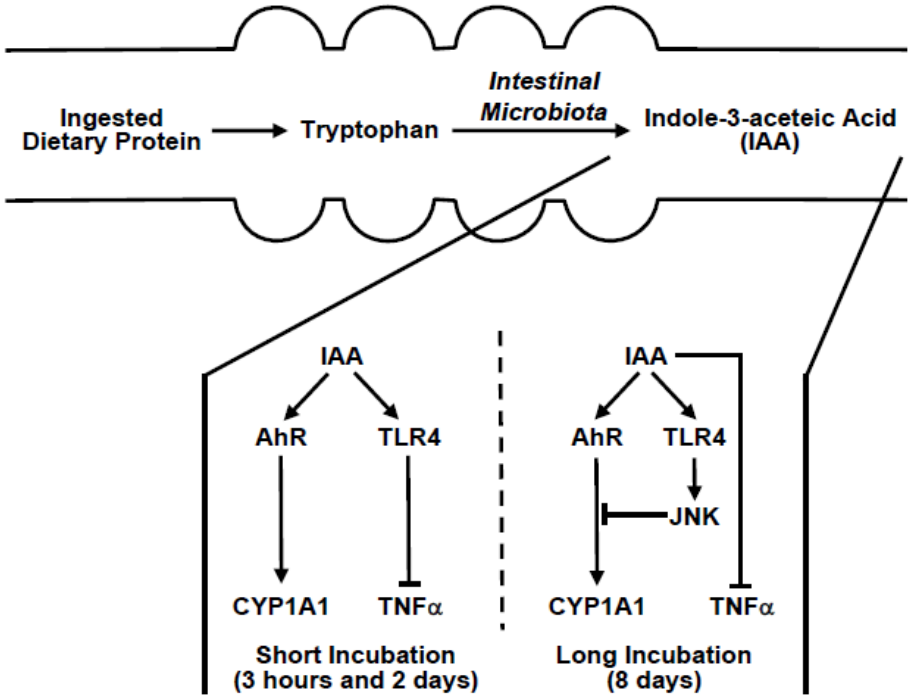


Figure 18. Indole-3-acetic acid may bind to TLR4 as well as AhR that regulates CYP1A1 and TNFα expression depending on the culture stage of Caco-2 cells.

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List of publications related to the Ph.D. thesis

Chapter 1

Chowdhury, M.M.I., Kurata, K., Yuasa K., Koto, Y., Nishimura, K., Shimizu, H. Suppression of TNFα expression induced by indole-3-acetic acid is not mediated by AhR activation in Caco-2 cells. Bioscience, Biotechnology, and Biochemistry. 85 (4):902-906, 2021. (DOI: 10.1093/bbb/zbaa101)

Chapter 2

Chowdhury, M.M.I., Tomii, A., Ishii, K., Tahara, M., Hitsuda, Y., Koto, Y., Kurata, K., Yuasa, K., Nishimura, K. and Shimizu, H. TLR4 may be a novel indole-3-acetic acid receptor that is implicated in the regulation of CYP1A1 and TNFα expression depending on the culture stage of Caco-2 cells. Bioscience, Biotechnology, and Biochemistry, accepted on June 20, 2021.

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Summary (English)

Colorectal cancer (CRC) has gained much attention recently because it is one of the most common cancer worldwide, and about 1.9 million new cases are diagnosed with CRC globally. In CRC tissues, elevated expression of tumor necrosis factor α (TNFα), an inflammatory cytokine, is positively correlated with the growth of CRC as well as involved in the induction of the epithelial-mesenchymal transition in promoting CRC invasion and metastasis. Moreover, increased TNFα expression is associated with tumor recurrence in patients with metastases of CRC. Furthermore, TNFα expression is increased in serum from patients with CRC. On the other hand, cytochrome P450 1A1 (CYP1A1), one of P450 inducible phase I metabolizing enzyme, is involved in the process of metabolic activation of the xenobiotics like polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) in the carcinogenesis. Furthermore, researchers found a relationship between the progression of CRC and the increased CYP1A1 activity by single nucleotide polymorphisms in its gene previously. However, other researchers showed a loss of metabolic clearance of carcinogenic DNA-adduct in CYP1A1 global knockout mice. Therefore, the present study attempted to unveil the effects of indole-3-acetic acid (IAA) derived from dietary tryptophan produced by the intestinal microbiota and the mechanism of IAA involved in the regulation of TNFα and CYP1A1 expressions.

Initially, I studied the influence of treating human epithelial colorectal adenocarcinoma (Caco-2) cells with IAA on the expression of CYP1A1, a typical target gene for aryl hydrocarbon receptor (AhR), and TNFα. Since the moisture content of normal feces is 60%-80%, and the maximum fecal concentration of skatole, an IAA metabolite synthesized by intestinal microbiota, is 1000-1200 μM, the maximum fecal IAA concentration may be similar. Therefore, I treated the Caco-2 cells with 1000 μM IAA. I found significant effects of IAA on the Caco-2 cells, as evident with the substantial increase in the mRNA levels of CYP1A1 expression for 1-8 days, suggesting that IAA activates AhR. Conversely, I also found that IAA downregulated the mRNA expression of TNFα for 2-8 days. Besides, the present immunoblotting study also showed a decreased TNFα protein levels after incubation of Caco-2 cells for 8 days with 1000 μM IAA. Since most studies of indole derivatives such as IAA have been conducted based on the premise that such compounds attenuate the inflammatory response through activating AhR, I conducted the tests with CH223191 (10 μM), an AhR antagonist, to check if AhR

regulates CYP1A1 and TNFα expression levels or not. Results from my antagonist experiments demonstrated that CH223191 significantly repressed the IAA-induced expression levels of CYP1A1 mRNA at 2 and 8 days after stimulation, whereas CH223191 had no antagonistic action on the IAA-induced suppression of TNFα expression in Caco-2 cells on any day. These results suggested that AhR is not involved in suppressing TNFα expression induced by IAA in Caco-2 cells rather than via AhR-independent pathways. These findings also indicate that the IAA also binds to receptors other than AhR. Thereafter, I intended to identify a new receptor for IAA. To do that, I checked the CYP1A1 expression by dose- and time-dependently. I found that the mRNA expression of CYP1A1 started to increase significantly from 3 hours after stimulation with 1000 μM IAA, and gradually increased up to 4 days and then started decreasing from 4 days, but continued for 3 hours-8 days. Moreover, the current results also indicated that the abundance of CYP1A1 expression was similar at 3 hours after stimulation with 50-1000 μM IAA, while expression of CYP1A1 at 8 days after stimulation with IAA increased dose-dependently. Next, I planned to examine whether Toll-like receptor 4 (TLR4) is involved in the regulation of CYP1A1 expression from 3 hours to 8 days after stimulation with IAA. I got that after treating the cells with TAK242 (1 μM), a TLR4 antagonist, it did not affect the increased CYP1A1 expression induced by IAA at 3 hours, although, on 8 days, it promoted the IAA-induced CYP1A1 expression. Based on the results of 8 days, I examined whether any changes of AhR and AhR repressor (AhRR) mRNA expression levels at 8 days in TAK242 treated Caco-2 cells and found no significant changes for both the genes. The AhR protein level also remains unchanged, indicating that neither increased AhR mRNA nor protein expression was involved in promoting the increase in expression CYP1A1 induced by TAK-242 at 8 days after stimulation with IAA. Since researchers reported the c-Jun-N-terminal kinase (JNK) activation in the TLR4 pathway, the present study confirmed by the immunoblotting results that the TAK-242 suppressed IAA-induced JNK activation. Furthermore, I used SP600125 (5 μM), a JNK inhibitor, and found that after treating the Caco-2 cells with SP600125 followed by IAA resulted in the promotion of IAA-induced increased CYP1A1 expression levels at 8 days after stimulation. These two data suggest that activation of TLR4/JNK signaling pathway induced by IAA may have partially attenuated increased CYP1A1 expression at 8 days after stimulation with IAA. Finally, I assessed the effects

of TAK-242 on the suppression of TNFα expression induced by IAA and found that TAK-242 alleviated the suppression of TNFα expression induced by IAA at 2 days, but not at 8 days after stimulation. Thus, the present findings suggest that TLR4 is a receptor for IAA in addition to AhR and IAA-activated TLR4 regulates CYP1A1 and TNFα expression depending on the culture stage of Caco-2 cells. Furthermore, my findings at 8 days after stimulation with IAA indicated that even more unidentified receptors for IAA regulate TNFα expression. Therefore, more extensive studies are required for the identification of new receptor(s) involving in the regulation of TNFα expression by IAA in CRC cells.

Thus, the results of my current study may be useful to understand the benefits of IAA derived from dietary tryptophan produced by the intestinal microbiota on the pathophysiology of gastrointestinal cells. Moreover, these results can help to offer new approaches in CRC management. On the other hand, increased CYP1A1 expression has been shown in the production of toxic metabolites, though others relate this with metabolic clearance of carcinogenic compounds. Besides, there is no such information about the impacts of IAA on phase II metabolizing enzymes, which are involving in the conjugation reactions to make the metabolites more hydrophilic and helps them out from the body. Therefore, the balance between a high amount of protein intake and substrates for the CYP1A1 may provide a better alternative for the protective and delayed effects on the initiation and progression of CRC. In conclusion, my studies provide novel findings to understand how indole derivative IAA produced by intestinal flora affects the host physiology.

Summary (Japanese)

大腸癌は、世界的に最も多いがんの一つであり、全世界で約 190 万人が新たに大腸癌と診断されていることから、近年注目を集めている。大腸癌組織では、炎症性サイトカインである Tumor necrosis factor α（TNFα）の発現レベルが大腸癌の増殖レベルと正の相関を示すのに加え、大腸癌の浸潤や転移を促進する上皮間葉転換の誘導にも関与している。さらに、TNFαの発現レベルの上昇は大腸癌の転移を有する患者の腫瘍再発と関連しており、また大腸癌患者では血清 TNFαの発現レベルが上昇している。一方、P450 誘導型第 I 相代謝酵素の一つである Cytochrome P450 1A1（CYP1A1）は、発癌における benzo[a]pyrene（BaP）などの多環芳香族炭化水素（Polycyclic aromatic hydrocarbons：PAH）のような異種化合物の代謝活性化過程に関わっている。また、一塩基多型による CYP1A1 の活性上昇と大腸癌の進行との間に関連があることが明らかとなっている。しかし、CYP1A1 の全身性ノックマウスでは、発癌性 DNA 付加物の代謝によるクリアランスが失われていることが示されている。そこで本研究では、腸内細菌が産生する摂取トリプトファン由来代謝産物であるインドール酢酸（Indole-3-acetic acid: IAA）の効果と、TNFαや CYP1A1 の発現調節に IAA が関与するメカニズムを明らかにすることを試みた。

まず、ヒト大腸癌由来細胞株（Caco-2 細胞）を IAA で処理することで、Aryl hydrocarbon receptor（AhR）の代表的な標的遺伝子である CYP1A1 と TNFαの発現レベルに与える影響を調べた。正常な便の水分量は 60～80％であり、腸内細菌によって生成される IAA の代謝産物であるスカトールの最大便中濃度は 1000～1200 μM であることから、IAA の最大便中濃度も同様であると推測される。

そこで、Caco-2 細胞を 1000 μM の IAA で処理したところ、CYP1A1 の mRNA レベルが 1〜8 日間にわたって大幅に上昇していたことから、IAA が AhR を活性化することが示唆された。逆に、IAA 刺激 2〜8 日後、TNFαの mRNA レベルの低下が導かれていた。加えて、1000 μM の IAA 刺激 8 日後における TNFαのタンパク質レベルも同様に低下していた。IAA のようなインドール誘導体の研究の多くは、AhR の活性化を介して炎症反応を減衰させることが前提に行われているため、IAA による AhR の活性化が CYP1A1 や TNFαの発現レベルを制御しているのか、AhR に対する特異的なアンタゴニストである CH223191（10 μM）を用いて検証を行った。CH223191 は、IAA による CYP1A1 の mRNA レベルを刺激後 2 日目と 8 日目で有意に低下させた一方、TNFαの発現抑制に対しては、いずれの日においても効果を示さなかった。これらの結果から、IAA によって誘導される TNFαの発現抑制には AhR は関与せず、むしろ AhR 非依存的な経路の存在が示唆された。つまり、IAA は AhR 以外の受容体にも結合していることが示された。このため、IAA の新たな受容体の同定を試みることにした。まず、CYP1A1 の発現増加に対する IAA 濃度および処理時間について調べた。CYP1A1 の mRNA レベルは、1000 μM の IAA で刺激した 3 時間後から有意に上昇し始め、4 日目までは徐々に増加し、4 日目からは減少し始めたが、刺激 3 時間後〜8 日目までの期間、一貫してその発現上昇は持続していた。さらに、50 μM〜1000 μM の濃度で IAA 処理を行ったところ、刺激 3 時間後での CYP1A1 の発現上昇レベルはそれぞれの濃度で同程度であったが、IAA 刺激 8 日後の CYP1A1 の発現レベルは濃度依存的に増加していた。次に、IAA 刺激 3 時間後と 8 日目での CYP1A1 の発現制御に Toll-like receptor 4（TLR4）が関与しているのか調べ

た。TLR4 に対する特異的なアンタゴニストである TAK242（1 μM）を細胞に処理したところ、IAA 刺激 3 時間後に誘導された CYP1A1 発現増加には影響を与えなかったが、8 日後では IAA 刺激によって上昇した CYP1A1 の発現レベルを促進させていた。この結果を踏まえて、 IAA 刺激 8 日後における AhR および AhR reporessor（AhRR）の mRNA レベルを調べたところ、どちらの遺伝子においても有意な変化は認められなかった。加えて、AhR のタンパク質レベルも同様に変化していなかった。よって、IAA 刺激 8 日後の TAK-242 による CYP1A1 の発現亢進には、AhR や AhRR の発現増加は関与していないと考えられた。TLR4 経路に関しては、c-Jun-N-terminal kinase（JNK）の活性化が報告されている。本研究でも、TAK-242 が IAA による JNK の活性化を抑制したことから、TLR4 の下流に JNK が存在することが確かめられた。さらに、JNK に対する特異的阻害剤である SP600125（5 μM）を用いたところ、IAA 刺激 8 日後に上昇した CYP1A1 の発現レベルが促進した。これら 2 つの結果は、IAA によって惹起される TLR4/JNK シグナル経路の活性化が、IAA 刺激 8 日後での CYP1A1 発現レベルの上昇を一部抑制させる可能性を示している。最後に、IAA によって誘導される TNFαの発現抑制に対する TAK-242 の効果を検討した。TAK-242 は IAA 刺激 2 日後に誘導される TNFαの発現抑制を緩和したが、刺激後 8 日目では効果はなかった。これらの結果から、TLR4 が AhR に加えて IAA の受容体であり、IAA で活性化された TLR4 は Caco-2 細胞の培養段階に応じて CYP1A1 や TNFαの発現制御に関わっていることが明らかとなった。さらに、IAA 刺激 8 日後で得られた結果から、未同定の IAA に対する受容体が TNFαの発現制御に関わっていることが示された。したがって、IAA による大腸癌細胞の TNFα発現

制御に関与する新たな受容体を同定するために、今後もより広範な研究が必要である。

以上から、本研究結果は、トリプトファン由来の腸内細菌代謝産物 IAA が消化器系細胞の病態生理に与える影響を理解するのに役立つと期待される。加えて、大腸癌の治療に対する新たなアプローチの提供に寄与する可能性がある。一方、CYP1A1 の発現増加は、毒性代謝産物の産生に関与しているとされているが、発癌性化合物の代謝クリアランスとも関連付けられている。また、IAA が、CYP1A1 の代謝産物を親水性にし体外へ排出するための抱合反応に関与する第 II 相代謝酵素に与える影響に関する報告はない。したがって、タンパク質の多量摂取と CYP1A1 の基質とのバランスをとることは、大腸癌の発症を防ぎ、進展を遅延させるためのより良い選択肢となり得ると考えられる。結論として、本研究は、腸内細菌が産生するインドール誘導体である IAA が宿主の生理機能に与える影響を理解する上での新たな知見を提供するものである。