Molecular mechanisms of human CYP1B1 gene regulation

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学位の種類	博士（薬学）
学位記番号	博甲第807号
学位授与の日付	平成18年3月22日
学位授与の要件	課程博士（学位規則第4条第1項）
学位授与の題目	Molecular Mechanisms of Human *CYP1B1* Gene Regulation （ヒト *CYP1B1* 遺伝子の発現調節機構）
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Human CYP1B1 is one of the members of the cytochrome P450 isoforms, and plays a role in the metabolic activation of chemically diverse procarcinogens and hydroxylation of 17β-estradiol (E2). In this study, the mechanisms regulating the human *CYP1B1* gene were investigated. First, it was found that the mutual interaction of two XREs (–853 and –834) to which AhR/ARNT heterodimer binds is important for transcriptional regulation, and that the Sp1 binding to the Sp1-like motif (-824) enhances both the constitutive and inducible transcriptional activities of the human *CYP1B1* gene. Second, it was demonstrated that the human CYP1B1 is induced by E2 via the binding of estrogen receptor to the *CYP1B1* promoter region (–63 to –49). Third, the involvement of SF-1, CREB, and PKA in the transcription of human *CYP1B1* gene was demonstrated. Forth, it was clarified that the human CYP1B1 is posttranscriptionally regulated by microRNA, miR-27b, at the 3'-untranslated region (+4358 to +4381). The unusual expression of CYP1B1 might affect the development of cancer as well as estrogen homeostasis and/or estrogen-dependent cancers. Thus, the findings in this thesis are useful to consider the clinical significance of CYP1B1 for the tumorigenesis, estrogen homeostasis, and potential target of anticancer drugs.

Human CYP1B1 is one of the members of the cytochrome P450 isoforms. It plays a role in the metabolic activation of chemically diverse procarcinogens including polycyclic aromatic hydrocarbons (PAHs), and catalyzes the hydroxylation of 17β-estradiol (E2). CYP1B1 is mainly expressed in extrahepatic tissues, such as ovary, uterus, breast, testis, prostate, and adrenal gland. The expression level is high in tumor tissues. The unusual expression of CYP1B1 might affect the development of cancer as well as estrogen homeostasis and/or estrogen-dependent cancers. Therefore, it is important to clarify the mechanisms of CYP1B1 regulation. The purpose of this thesis is to investigate the mechanisms regulating the human *CYP1B1* gene.

1. Mutual Role of AhR/ARNT and Sp1 on Constitutive- and TCDD-inducible Expression of CYP1B1

CYP1B1 is induced by PAHs as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). It is well-known that the induction of drug metabolizing enzymes by TCDD is mediated by aryl hydrocarbon receptor (AhR)/AhR nuclear translocator (ARNT) heterodimer interacted with xenobiotic responsive element (XRE). There are eight XRE core sequences on the 5'-flanking region of the human *CYP1B1* gene up to –2299 bp. However, the binding of the AhR/ARNT to the XREs in the human *CYP1B1* gene has never been directly proven. In order to identify the *cis*-element(s) involved in the constitutive and TCDD-inducible transcription of *CYP1B1*, luciferase reporter plasmids containing a series of deletions of the XRE core sequence in the 5'-flanking region of the human *CYP1B1* gene were constructed. Luciferase reporter assays showed the potential enhancer elements for TCDD-induction were located from –1022 to –852 of human *CYP1B1* gene in human hepatoblastoma HepG2 cells. Gel shift analyses revealed the binding of the AhR/ARNT heterodimer to XRE2 at –834 and XRE3 at –853. In addition, the binding of a nuclear transcriptional factor, Sp1, near XRE2 and XRE3 was observed. It was found that mutual interaction of XRE2 and XRE3 is important for transcriptional regulation, and that the Sp1 binding to the Sp1-like motif (–824) enhances both the constitutive and inducible transcriptional activities of the human *CYP1B1* gene[1].

2. Induction of CYP1B1 by Estradiol via Estrogen Receptor

CYP1B1 is a key enzyme in the metabolism of E2. The high expression level of CYP1B1 in estrogen-related tissues suggests that CYP1B1 is important in the localized metabolic control of estrogen homeostasis. Many CYP enzymes are likely to be induced by the substrates themselves. In addition, computer-assisted homology search identified a potential estrogen receptor (ER)-binding site on the *CYP1B1* promoter. These backgrounds prompted me to investigate whether the human *CYP1B1* gene is a target of E2. Real-time RT-PCR analysis revealed that E2 treatment induced CYP1B1 mRNA expression in ER-positive MCF-7 cells. Luciferase reporter assays using MCF-7 cells showed a significant transactivation by E2 with a reporter plasmid containing a region from -152 to +25 of the human *CYP1B1* gene. Specific binding of ERα to the putative estrogen responsive element (ERE), which is between -63 and -49 in the *CYP1B1* promoter region, was demonstrated by chromatin immunoprecipitation assays and gel shift analyses. With reporter plasmids containing the wild or mutated putative ERE on the *CYP1B1* gene and the wild or mutated ERα expression vectors, luciferase assays using the human endometrial adenocarcinoma Ishikawa cells demonstrated that the putative ERE and ERα are essential for the transactivation by E2. Since endometrial tissue is highly regulated by estrogens, the expression pattern of CYP1B1 protein in human endometrial specimens was examined by immunohistochemistry. The staining of CYP1B1 was stronger in glandular epithelial cells during a proliferative phase than those during a secretory phase, consistent with the pattern of estrogen secretion. These findings clearly indicated that the human CYP1B1 is regulated by estrogen via ERα[2]. Since 4-hydroxylestradiol produced by CYP1B1 is estrogenically inactive but toxicologically active, the estrogen-regulated CYP1B1 expression indicates a clinical significance for the homeostasis of estrogens as well as estrogen-dependent carcinogenesis.

3. Involvement of Steroidogenic Factor-1 and Protein Kinase A in CYP1B1 Regulation

CYP1B1 is expressed in steroid-related tissues including ovary, testis, and adrenal gland. In these tissues, the steroidogenic CYPs such as CYP19 and CYP17 are generally expressed, which are known to be transcriptionally regulated by steroidogenic factor-1 (SF-1) and cAMP response element (CRE) binding protein (CREB). In addition, the transcriptional regulation of rat *CYP1B1* gene has been reported to be regulated by SF-1 and CREB. In this study, the

possibility that the human *CYP1B1* gene might be regulated by SF-1 and CREB was examined. Gel shift analyses revealed that *in vitro* translated SF-1 can bind to the putative SF-1 binding sites, SF-1a (at -1722) and SF-1b (at -2474), on the human *CYP1B1* gene. *In vitro* translated CREB barely bound to the putative SF-1 binding sites. In order to investigate the involvement of SF-1 in the transcriptional regulation, luciferase reporter assays were performed with various reporter plasmids containing the 5'-flanking region of *CYP1B1* gene in human ovarian granulose-like tumor KGN cells. A reporter plasmid pGL3 (-2623/+25) containing the SF-1a and SF-1b elements was transactivated by the concomitant co-expression of SF-1 and protein kinase A (PKA). Although the transcriptional activity was induced by PKA alone, it was not affected by the co-expression of SF-1. The mutation in the SF-1a and SF-1b elements did not affect the luciferase activity. Thus, the binding of SF-1 to the putative SF-1 binding sites of the human *CYP1B1* gene might not be essential for the transcriptional regulation. Interestingly, deletion analyses revealed that the PKA-dependent transactivation occurred in the region -910 to -732 in the 5'-flanking region, which includes XRE2 and XRE3. Mutations in two functional XREs located at -853 and -834 abolished the PKA-dependent transactivation. These results indicate that the PKA signaling pathway would be involved in the XRE-mediated transactivation of the human *CYP1B1* gene[3].

4. Posttranscriptional Regulation of CYP1B1 by MicroRNA

MicroRNAs (miRNAs) are widespread small non-coding RNAs and regulate the gene expressions, resulting in the alterations of various biological functions and development of cancers. MiRNAs incorporated into the RNA-induced silencing complex (RISC) recognize the 3'-UTR of the target mRNA and cause the translational repression or mRNA cleavage. Recently, a number of studies have reported that the expression profiles of miRNAs were associated with various human tumors development. On the other hand, the causal factor for the higher expression of CYP1B1 protein in tumor than in normal tissues has been uncleared. In contrast to the protein levels, no apparent difference in CYP1B1 mRNA level was observed between the tumor and normal tissues. Interestingly, a near-perfect matching sequence with microRNA-27b (miR-27b) was identified in the 3'-untranslated region of human CYP1B1. These evidences made me envisage the possibility that the human CYP1B1

might be regulated by miRNA. In this study, it was investigated whether miRNA might be involved in the regulation of human CYP1B1 or not. RNase protection assays indicated miR-27b was expressed in MCF-7 cells, but not in human T-cell leukemia Jurkat cells. Luciferase assays revealed that the reporter activity was decreased with the miR-27b recognition element in human CYP1B1 (1B1MRE27b) (+4358 to +4381) in MCF-7 cells, but not in Jurkat cells. The exogenously expressed miR-27b in Jurkat cells could decrease the luciferase activity of reporter plasmid containing 1B1MRE27b. In MCF-7 cells, antisense oligoribonucleotide (AsO) for miR-27b recovered the luciferase activity of reporter plasmid containing the 1B1MRE27b. Furthermore, the AsO could increase the protein level and enzymatic activity of CYP1B1 in MCF-7 cells. These results suggested that the human CYP1B1 would be regulated by miR-27b through the 1B1MRE27b. The expression level of miR-27b is conceivably decreased in tumor tissues like other reported miRNA. Thus, the posttranscriptional regulation by the miR-27b might be one of causes of the high expression of CYP1B1 protein in tumor tissues. This is a first study to demonstrate that miRNAs would regulate not only the essential genes for the physiological event but also drug metabolism enzymes[4].

Conclusion

In this thesis, the transcriptional regulation by AhR, Sp1, ER, and SF-1 and posttranscriptional regulations by and miRNA of the human CYP1B1 were clarified. CYP1B1 plays a role in metabolic activation of variety of procarcinogens and metabolism of estrogens. Furthermore, *in vitro* studies revealed that CYP1B1 could inactivate some anticancer agents such as docetaxel and tamoxifen. Therefore, high expression of CYP1B1 in tumors might be one of causes of resistance to anticancer drug in cancer cells. It is important to understand the mechanisms regulating the CYP1B1 expression. I could throw new insights into the molecular mechanism regulating human CYP1B1 expression from various viewpoints. This information is useful to consider the clinical significance of CYP1B1 for the tumorigenesis, estrogen homeostasis, and potential target of anticancer drugs.

References:

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学位論文審査結果の要旨

ヒトチトクロムP450の一分子種であるCYP1B1は主にステロイドホルモン関連臓器に高く発現しており、環境中の化学発癌物質の代謝的活性化やエストロゲンの代謝を担っている。本論文ではヒトCYP1B1遺伝子の発現調節機構について多方面より検討している。第一に、ヒトCYP1B1遺伝子5'-上流にarylhydrocarbon receptor（AhR）/AhR nuclear translocator（ARNT）のヘテロダイマーが結合する2つのxenobiotic responsive element（XRE；－853と－834）が存在し、相互に作用することでCYP1B1の転写に重要な役割を果たしていること、また上流－824に存在するSp1様配列へのSp1の結合を見出し、常在的および誘導的な転写活性化を促進することを明らかにした。第二に、CYP1B1はエストロゲンにより誘導され、上流－49に存在するestrogen response element（ERE）にestrogen receptorが結合することで*CYP1B1*遺伝子の転写が促進されることを明らかにした。また、ヒト子宮内膜におけるCYP1B1の発現量が月経周期に依存して変動することを示した。第三に、steroidogenic factor- 1（SF-1）cAMP responseelement binding protein（CREB）およびprotein kinase A（PKA）の影響について検討し、SF-1およびCREBは転写活性に寄与しなかったが、PKAにより－853と－834のXREを介して活性化されることが示された。第四に、ヒトCYP1B1 mRNAの3'－非翻訳領域の+4358/+4381にmicroRNA（miR-27b）の認識配列を見出し、CYP1B1のタンパク発現および酵素活性がmiR-27bにより転写後調節されていることを明らかにした。CYP1B1の発現量の変動は癌の発生やエストロゲン代謝能に影響を及ぼすことが示唆される。従って、本研究成果は発癌作用およびエストロゲンのホメオスタシスの臨床的な重要性を考慮するうえで有益な情報を提供するものと考えられる。以上より博士（薬学）として評価できるものと判定した。