EFFECTS OF LUTEOLIN ON TCDD- AND *tert*-BUTYLHYDROQUINONE-INDUCED DRUG-METABOLIZING ENZYMES AND NUCLEAR FACTOR-ERYTHROID-2-RELATED FACTOR 2

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Introduction

Drug-metabolizing system is consisting of phase I and phase II drug-metabolizing enzymes and phase III transporters[1]. Phase I enzymes such as cytochrome P450s (CYPs) converts hydrophobic compounds to the hydrophilic ones. Among CYPs, CYP1A subfamily plays a critical role in the metabolism of many drugs and xenobiotics including polycyclic aromatic hydrocarbons (PAHs). However, during this process, reactive intermediates and metabolites are often formed. Their highly reactivity and an ability to bind covalently to DNA are deeply involved in the carcinogenesis of PAHs[2]. Phase II enzymes, such as glutathione-*S*-transferases (GSTs), and NAD(P)H:quinone oxidoreductase-1 (NQO1) change the reactive intermediates and metabolites into less toxic or inactive metabolites[1,3]. The phase III transporters are involved in the exclusion of xenobiotics and their metabolites.

An aryl hydrocarbon receptor (AhR), which is a ligand-dependent transcription factor after forming a heterodimer with the aryl hydrocarbon nuclear translocator (Arnt) in nucleus, regulates expression of certain drug-metabolizing enzymes[1-3]. This AhR/Arnt complex binds to a specific DNA sequence called the dioxin or xenobiotic responsive element (DRE or XRE), resulting in the transcriptional activation of adjacent genes encoding drug-metabolizing enzymes such as CYP1A1 and NQO1 (reviewed in reference 1). AhR also regulates expression of nuclear factor-erythroid-2-related factor 2 (Nrf2)[4,5]. Upon oxidative stress, Nrf2 escapes kelch-like ECH-associated protein 1 (Keap1)-mediated proteosomal degradation and travels into the nucleus[6]. In the nucleus, Nrf2 forms a heterodimer with small Maf proteins and binds to antioxidant response elements (AREs) to induce a battery of Nrf2-dependent drug-metabolizing genes including NQO1, GSTs, and aldo-keto reductases (AKRs). Therefore, the AhR/DRE pathway regulates expression of many drug-metabolizing enzymes directly or indirectly through the activation of Nrf2/ARE pathway.

It is well known that halogenated aromatic hydrocarbons (HAHs) including dioxins and PAHs such as benzo(a)pyrene and 3-methylcholanthrene, bind to the AhR as 'classical exogenous ligands'. Of these, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent chemical, exerting variety of toxicological and biological effects including gene expression[7,8]. In contrast to the 'classical exogenous ligands', certain natural flavonoids may bind to AhR and activate and/or inactivate AhR-dependent signaling pathways[1,9]. In the case of Nrf2, *tert*-butylhydroquinone (tBHQ) is a well-known inducer for the Nrf2/ARE pathway. As the natural inducers, isothiocyanates and a flavonol quercetin induce Nrf2-dependent genes including NQO1, GSTA2, UGTs and so on[10-12]. From these results, flavonoids can modulate the expression of drug-metabolizing enzymes.

A flavone luteolin, which appears in fruits, vegetables, and medicinal herbs, possesses various health beneficial functions, such as prevention of cardiovascular diseases, anti-inflammatory activity and anti-cancer activity[13-15]. The effects of luteolin on drug-metabolizing enzyme expression have not fully understood yet, although previous studies have shown that luteolin inhibits AhR transformation[16] and CYP1A1 expression[17], while it induces heme oxygenase-1 expression[18]. In this study, we investigated the effects of luteolin on the expression of drug-metabolizing enzymes through the AhR/DRE and Nrf2/ARE pathways.

Materials and methods

Cells culture and treatment: Human hepatoma HepG2 cells were cultured in DMEM containing 10% (v/v) FBS and antibiotics. The cells (90% confluent) were pre-treated with luteolin for 10 min and then treated with TCDD or tBHQ for another 24 h. To inhibit AhR, HepG2 cells were pre-treated with AhR inhibitor CH-223191 for 1 h before treatment with TCDD and tBHQ for 24 h. To knockdown Nrf2 expression, siRNA for Nrf2 was transfected into the cells. Twenty-four hours after transfection, the cells were treated with chemical inducers for another 24 h.

Estimation for the gene and protein expression levels of drug-metabolizing enzymes: For estimation of gene expression of drug-metabolizing enzymes, RNA was isolated from HepG2 cells and purified as previously described[19]. After cDNA was synthesized from purified RNA with a commercially available kit, real-time PCR was carried out using suitable forward and reverse primers for *CYP1A1, Nrf2, NQO1, GSTP1, AKR1B10, AKR1C1* and *β-actin* according to our previous report[19]. Relative gene expression level was calculated by the comparative cycle threshold (CT) method using the expression of the *β-actin* gene as an internal control.

As to estimation of protein expression of drug-metabolizing enzymes, cell lysate was prepared from HepG2 cells and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis[19]. Separated proteins were transferred onto a PVDF membrane and then immuno blotting was performed using the commercially available anti-bodies against CYP1A1, Nrf2, NQO1, GSTP1, AKR1B10, AKR1C1 and β-actin. After imuno-complex was detected, the density of the specific band was determined using ImageJ image analysis software and normalized the density of β-actin protein.

Statistical analysis: Determination of the significance of differences was analyzed by a Dunnett's test. The level of statistical significance was set to $p<0.05$.

Results and discussion

Expression of drug-metabolizing enzymes and Nrf2 in AhR-inhibited or Nrf2-deficient HepG2 cells

TCDD expectedly induced expression of CYP1A1, NQO1, GSTP1 and Nrf2 at both mRNA and protein levels. To investigate the effect of AhR on TCDD-induced expression of drug-metabolizing enzymes, CH-223191, an AhR inhibitor was introduced. This AhR inhibitor significantly suppresseed protein expression of CYP1A1, NQO1 and GSTP1 (Table 1). However, the inhibitor did not decrease TCDD-induced mRNA expression of *NQO1*, although it significantly suppressed mRNA expression of *CYP1A1* and *GSTP1*. These results indicate that the expression of CYP1A1 and GSTP1 but not NQO1 was mainly controlled by the AhR/DRE pathway. In the case of Nrf2 expression, TCDD-induced both protein and mRNA levels of Nrf2 were canceled by the AhR inhibitor. Thus, Nrf2 expression is also mainy regulated by the AhR/DRE pathway.

Table 1. Effects of an AhR inhibitor and siRNA for Nrf2 on expression of drug-metabolizing enzymes and Nrf2

Target enzyme	AhR/DRE pathway				Nrf2/ARE pathway			
	mRNA expression		Protein expression		mRNA expression		Protein expression	
	TCDD	TCDD+ AhR inh.	TCDD	TCDD+ AhR inh.	tBHQ	tBHQ+ siNrf2	tBHQ	tBHQ+ siNrf2
CYP1A1	Up	Down	Up	Down	Up	±	±	±
NQO1	Up	±	Up	Down	Up	Down	Up	Down
GSTP1	Up	Down	Up	Down	±	±	Up	Down
Nrf2	Up	Down	Up	Down	–	–	–	–

AhR inh. ; AhR inhibitor, siNrf2; siRNA for Nrf2, Up; up-regulation, Down; down-regulation; ±; no change; and –; not determined.

When the cells were treaed with tBHQ, this chemical increased the expression of NQO1 and GSTP1 but not CYP1A1 at the protein level. Moreover, siRNA for Nrf2 canceled tBHQ-induced protein expression of NQO1 and GSTP1 without alteration of CYP1A1 expression as expectedly. At the mRNA levels, expression of *NQO1* showed the same tendency as the protein expression level, but tBHQ slightly enhanced the mRNA expression of *CYP1A1* and this slight increase did not changed by siRNA for Nrf2. It was noteworthy that mRNA expression of GSTP1 was entirely different from its protein expression level: tBHQ did not induce *GSTP1* mRNA. These results indicate that expression of NQO1 is mainly regulated by the Nrf2/ARE pathway and pertially by the AhR/DRE pahway.

Effect of luteolin on TCDD-induced expression of drug-metabolizing enzymes and Nrf2 in HepG2 cells

As shown in Table 2, TCDD not only induced both mRNA and protein expression of phase I drug-metabolizing enzyme CYP1A1 but also phase II drug-metabolizing enzymes NQO1 and GSTP1 in human HepG2 cells. It was found that luteolin significantly inhibited TCDD-induced protein and mRNA expression of CYP1A1, NQO1 and GSTP1 in HepG2 cells compared with the cells treated with TCDD alone. Luteolin itself did not affect expression of these enzymes (data not shown). To confirm the inhibitory effects of luteolin on the

expression of these drug-metabolizing enzymes, we introduced mouse Hepa1c1c7 and rat RL-34 cells and found that luteolin showed similar effects (data not shown). Under our experimental conditions, luteolin did not show any cytotoxicity against used hepatic cells (data not shown). These results strongly suggest that the inhibitory effect of luteolin against the expression of drug-metabolizing enzymes is universal effect among animal species. It is known that CYP1A1 expression is regulated by AhR transformation[1]. Our previous studies showed that luteolin inhibited a TCDD-caused transformation of AhR in a cell-free system[20], Hepa1c1c7 cells[16] and Caco-2 cells[21].

We further investigated expression of Nrf2 by TCDD. As a result, TCDD significantly induced both mRNA and protein expression of Nrf2, although we did not adress the effect of luteolin on TCDD-induced Nrf2 expression. Nrf2 regulated many phase II drug-metabolizing enzymes after binding to ARE. Indeed our results clearly indicate that TCDD induced phase II drug-metabolizing enzymes, NQO1 and GSTP1, in addition to Nrf2. Taken these results together, luteolin is an effective compound to modulate expression of drug-metabolizing enzymes through the AhR/DRE-dependent pathway.

Table 2. Effects of luteolin on expression of drug-metabolizing enzymes and Nrf2

Target enzyme	AhR/DRE pathway				Nrf2/ARE pathway			
	mRNA expression		Protein expression		mRNA expression		Protein expression	
	TCDD	TCDD+ Luteolin	TCDD	TCDD+ Luteolin	tBHQ	tBHQ+ Luteolin	tBHQ	tBHQ+ Luteolin
CYP1A1	Up	Down	Up	Down	–	–	–	–
NQO1	Up	Down	Up	Down	Up	Down	Up	Down
GSTP1	Up	Down	Up	Down	±	±	Up	Down
Nrf2	Up	–	Up	–	±	±	Up	Down
AKR1B10	–	–	–	–	Up	Down	Up	Down
AKR1C1	–	–	–	–	Up	Down	Up	Down

Up; up-regulation, Down; down-regulation; ±; no change; and –; not determined.

Effect of luteolin on tBHQ-induced expression of Nrf2 and drug-metabolizing enzymes in HepG2 cells

We, next, investigated the effect of luteolin on tBHQ-induced protein expression of Nrf2 and phase II drug-metabolizing enzymes. In this experiment, we introduced AKR1B10 and AKR1C1 as other molecular targets for Nrf2 pathway. Luteolin inhibited tBHQ-induced expression of Nrf2 protein, resulting in the down-expression of NQO1, GSTP1, AKR1B10, and AKR1C1 at the protein levels in HepG2 cells compared with the cells treated with tBHQ alone (Table 2). Luteolin itself did not change the expression of these proteins. At the mRNA expression level, tBHQ did not increase Nrf2, NQO1 and GSTP1, while it significantly increased AKR1B10 and AKR1C1. Luteolin tended to suppress their mRNA expression, but significant suppression was observed in mRNA level of *NQO1*, *AKR1B10* and *AKR1C1*. These results strongly suggest that tBHQ-induced phase II drug-metabolizing enzymes are depending on the stability of Nrf2 protein, and luteolin affects its stability. To confirm this issue, we measured nuclear translocation of Nrf2 protein and found that tBHQ significantly promoted nuclear translocation of Nrf2 protein, which is one of the markers for stability of Nrf2 protein, and luteolin signigficantly suppressed this translocation (data not shown). This indicates that tBHQ increases stability of Nrf2 and certain drug-metabolizing enzymes such as NQO1 and GSTP1, because tBHQ fails to increase mRNA of these proteins. As the putative molecular mechanism of luteolin, this flavonoid decreases stability of Nrf2 protein. In the case of AKRs, tBHQ might increase the activity of Nrf2 and luteolin inhibited this activity, since tBHQ induced expression of AKR1B10 and AKR1C1 at both mRNA and protein levels and luteolin significantly suppressed these induction. From these results, luteolin inhibited tBHQ-induced drug-metabolizing enzymes through Nrf2/ARE pathway indepdendent of its inhibitory effect on AhR/DRE-driven induction of the drug-metabolizing enzymes.

Concluding remarks

In this study, we investigated the effects of luteolin on TCDD- and tBHQ-induced drug-metabolizing enzymes in hepatic cells. We found that luteolin suppressed TCDD-induced protein and mRNA expression of phase I enzyme CYP1A1 and phase II enzymes NQO1 and GSTP1 in HepG2 cells. From the results using an AhR inhibitor CH-223191, TCDD-induced expression of CYP1A1, GSTP1 and Nrf2 is mainly dominated by AhR/DRE pathway. Up-regulation of Nrf2 was due to an increase in its stability estimated by its translocation

into nucleus. On the other hand, TCDD- and tBHQ-induced NQO1 expression was mainly dominated by the Nrf2/ARE pathway and tBHQ-induced expression of AKR1B10 and AKR1C1 was also dominated by the Nrf2 activity. Luteolin inhibited Nrf2-dependent expression of phase II drug-metabolizing enzymes through decreasing stability of Nrf2, because luteolin cancelled tBHQ-promoted nuclear translocation of Nrf2. These results indicate that luteolin inhibits expression of phase I and phase II drug-metabolizing enzymes through the AhR/DRE and Nrf2/ARE pathways in hepatic cells. Based on these results, we conclude that luteolin is an effective compound to modulate the expression of drug-metabolizing enzymes.

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