SPECIFIC INDUCTION OF CYP1A1 MRNA BY INDIRUBIN IN HEPG2 CELLS

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Introduction

It was found that a potent AhR ligand, indirubin was ~ 50 times more potent than TCDD as a ligand in the yeast assay, and present in human urine and fetal bovine serum in our previous work [1]. However there are many differences between yeasts and mammals with regard to metabolic systems and membrane permeability of ligands. Thus, we examined indirubin inductivity of CYP1A1 and CYP1A2 mRNA, biomarkers of AhR-mediated signaling, in the human hepatocarcinoma HepG2 cells. Moreover we investigated the indirubin inhibitory effect on CYP1A1 and catabolism by CYP1A1 to compare with those effects on exogenous AhR ligands such as TCDD and B[a]P.

Methods and Materials

Cell Culture and treatments

The human hepatocarcinoma HepG2 cells were grown at 37 ºC and 5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum. 4×10^6 cells were seeded into 10-cm dishes containing fresh media. 24 h later (70-80 % confluence), cells were exposed to the test chemicals. Solutions of the test chemicals dissolved in DMSO were added to the medium as a final DMSO concentration of 1.0 % (v/v).

RT-PCR

Total RNA was isolated from the HepG2 cells by using RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA synthesis and quantitative competitive PCR for CYP1A1, CYP1A2, CYP2C, CYP2D6 and CYP2E1 was carried out by using human cytochrome P450 competitive RT-PCR set (Takara, Shiga, Japan) and RNA PCR Kit (AMV) Ver.2.1(Takara, Shiga, Japan) as instructed. After PCR amplification, PCR products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining. Images were captured digitally and the bands were quantified using NIH image. All signals were normalized against GAPDH.

In Vitro Enzyme Inhibition Experiments with EROD assay

Microsomal 7-Ethoxyresolfin *O*-dealkylation activity was determined by a continuous spectrofluorometric method as described previously [2], but with minor modifications. The K_m, V_{max} and K_i values were determined according to the previously described procedure [2].

Indirubin Catabolic Analysis with Yeast Assay for AhR Ligand Activity

A 100 µl reaction mixture containing 15 pmol of recombinant CYP1A1 enzyme or control microsome, 100 nM indirubin, 4 mM NADPH and 3.3 mM magnesium chloride in 0.1 M sodium-potassium phosphate buffer (pH 7.4) was incubated at 37 ºC for 3 h. After incubation, 1 µl of reaction

mixture was applied to the yeast assay and indirubin equivalent concentration was determined. The yeast assay procedure was essentially as described in Miller *et al.* [3].

Results and discussion

CYP1A1 mRNA induction by indirubin in HepG2 cells were examined by competitive RT-PCR. When cells were incubated in fresh medium containing 10 % FBS, the CYP1A1 mRNA level 8 h after the medium change was 2500 copies/ ng total RNA (about 25 copies / cell). It decreased to 100 copies / ng total RNA (about 1 copies / cell) 36 h after the medium change. To lower the background level, the cells were plated 24 h before exposure and the DMSO solution of indirubin was added to the medium directly. Fig.1A shows that 1 pM of indirubin significantly induced CYP1A1 mRNA (9.4 folds) 8 h after exposure. CYP1A1 mRNA was induced by indirubin in a dose-dependent manner but the mRNA level was plateau from 1 pM to 1 nM at the level of 1000 – 2000 copies / ng total RNA (10-20 copies/ cell) and acutely elevated from 10 nM. 7900 copies / ng total RNA (79 copies/ cell) was observed when cells were exposed to 100 nM of indirubin.

Time-course of CYP1A1 mRNA induction by indirubin and the authentic AhR ligand B[a]P are shown in Fig. 1B. CYP1A1 mRNA level was increasing after indirubin addition with a maximum level at 8 h, whereas CYP1A1 mRNA level was increasing up to 24 h after B[a]P addition.

CYP1A2, CYP2C, CYP2D6, CYP2E1 and CYP2A6 mRNA induction by indirubin were also examined. The background level of CYP1A2 mRNA was 16 copies/ ng total RNA (0.16 copies/ cell). 1 pM of indirubin significantly induced CYP1A2 mRNA (0.67 copies/ cell), and the CYP1A2 mRNA was increased up to 1 nM (1.6 copies/ cell) , but decreased when 10 or 100 nM of indirubin was exposed (data not shown). We could not observe the significant induction of CYP2C, CYP2D6, CYP2E1 and CYP2A6 mRNA by indirubin (data not shown).

100 nM of indirubin was mixed with recombinant human CYP1A1 and NADPH at 37 °C for 3 h, and the AhR ligand activity was measured by the yeast strain YCM3. When indirubin was incubated with CYP1A1 and NADPH, AhR ligand activity was dramatically decreased and the NADPH was essential for this reaction (Fig.2). These results represent that indirubin was a substrate of the human CYP1A1 enzyme and the metabolite has no longer AhR binding activity.

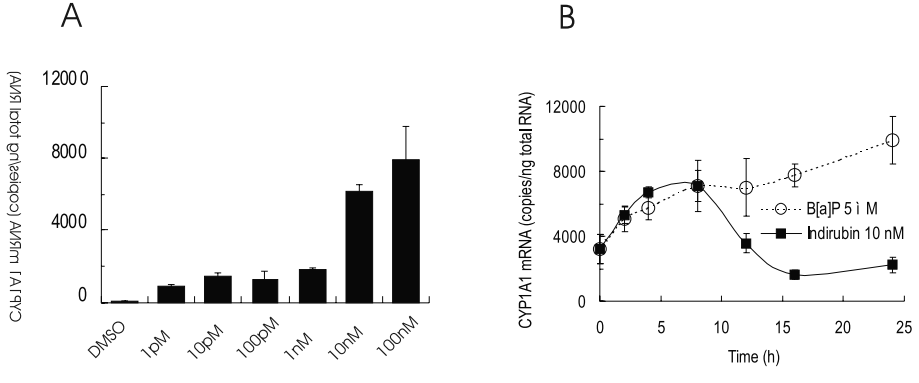

Figure 1. Effect of indirubin on CYP1A1 mRNA level in HepG2 cells.

To examine further nature of the interaction of indirubin with CYP1A1, we demonstrated the inhibitory effects of indirubin on EROD activity. Lineweaver-Burk plots of the enzyme kinetic data indicated that indirubin inhibited human recombinant CYP1A1 by a competitive inhibition. The K_i values of indirubin, B[a]P and indigo, which is also AhR ligand present in human urine [1], were 2.9 nM, 7.2 nM and 11.5 nM, respectively. The ratio of K_i/K_m, used to assess relative inhibition potency [2], were 0.0091, 0.0151 and 0.0213 for indirubin, B[a]P and indigo, respectively. TCDD also inhibits EROD activity competitively, the K_i value and the ratio of K_i/K_m are 200 nM and 2.0 respectively [4]. Thus, Indirubin was 1.7-, 2.3- and 200- fold more potent in inhibiting CYP1A1 than B[a]P, indigo and TCDD respectively.

In conclusion, indirubin is a potent inducer of CYP1A1 mRNA. Various data obtained from our experiments, strongly suggest that indirubin is rapidly catabolised by CYP1A1. We suppose that too much and continued induction of CYP1A1 by exogenous AhR ligands like TCDD causes a lack of indirubin and inhibits its physiological functions. This inhibition may be related with the toxicity of TCDD. Further study of indirubin may reveal the physiological role(s) of AhR and further our understanding of the toxic xenobiotics such as TCDD.

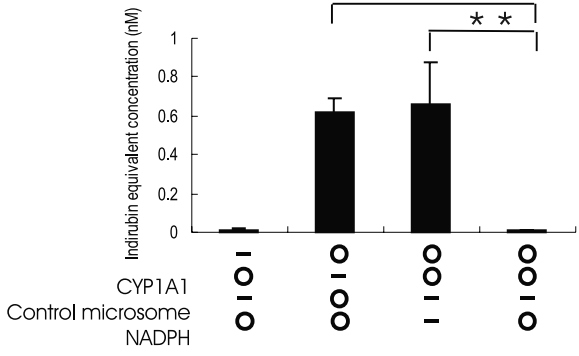

Figure 2. Indirubin inactivation by human recombinant CYP1A1

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