EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) ON CYP1A GENE EXPRESSION AND ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) ACTIVITY IN PRIMARY CULTURED ADULT CHICKEN HEPATOCYTES

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Introduction

Recently, primary cultured hepatocytes have been widely employed to investigate in vitro toxic effects of bio-active componds, such as TCDD. Chicken embryo hepatocytes have been used for species-specific and chemical-specific studies as they are easy to isolate and culture. Few reports were published using primary adult chicken hepatocytes, although metabolic and biotransformation functions in adult liver are differ from those in embryo liver. Previously we developed a novel primary culture method for adult chicken hepatocytes in serum-free medium[2]. Adult chicken hepatocytes maintained liver–specific function involving biotransformation enzyme activities. Using this method we attempt to declair TCDD effects on chicken liver CYP1As. For this objective, hepatocytes were exposed to TCDD and CYP IAs mRNA and EROD activity were estimated. The CYP1A genes altered from those of mammalian, CYP1A4 and CYP1A5 were stimulated in chicken embryo liver with TCDD treatment[1]. We concerned with the different response of these genes between embryo and adult chicken liver.

Methods and Materials

Primary hepatocytes were prepared by modified method of Yamanaka[2]. Approximately two month old male PDL-1 chicken was euthanized and sequential in situ perfusion was carried out to isolate hepatocytes. Addition to previous methods, perfusate and media containing N^G-nitro-L-arginine methyl ester were employed through in situ perfusion and primary one day culture, to prevent inducible nitrogen oxide which causes cell damage by oxydative stress in the early stage of culture process[3].

Expression of CYP1As were estimate by RT-PCR. Nucleotide sequences of chicken CYP1A4 (X99453), CYP1A5 (X99454) and a house keeping gene GAPDH (K01458) were obtained from GenBank. Suitable upstream and downstream primers were designed to amplification of cDNA (Table 1). PCR thermal cycling profile was as followed, denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute and polymerisation at 77°C for 2 minutes. Cells were treated with 1μM 3-methylchlolanthrene for 24 h, and then mRNA was extracted. All the PCR products were directly sequenced and conformed for appropriate genes. The cycle number was determined when every template to reach the exponential phase of amplification with similar initial template concentrations (10 ng/μl of mRNA from 3-MC treated hepatocytes).

Effects of TCDD were determined by suitable amounts of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin stock solutions dessolved in DMSO adding to achieved final concentration of 100fM, 1.0,

10, 100 pM. Only DMSO was used as control. After 24 h of cell culture TCDD was added and incubated for 24 h. Then mRNA was collected and RT-PCR was performed.

Activities of ethoxyresorufin *O*-deethylase and protein contents of cells were determined by the modified method of Kennedy et al. (1996)[4]. Hepatocytes cultured onto the 24 well dishes were washed with PBS(-) and stored at -80°C until measurement. *O*-dealkylation reaction and fluorescamin labeling of cell protein were performed in the culture dish to prevent TCDD pollution. Fluorescent intensities for EROD activity and protein contents were measured by a spectrofluorophotometer (RF-5300PC, Shimadzu. Co. Japan).

Table 1. Oligonucleotide primers used for PCR analysis

Gene	5' primer	3' primer	Size (bp)
CYP 1A4	5'-gaaccattacagcacctttgataag-3'	5'-gcactgcttgatcttcatggtcag-3'	712
CYP 1A5	5'-acctctacagcttccgacacattac-3'	5'-gtcctttgggatatagtagccattc-3'	890
GAPDH	5'-tgtgacttcaatggtgaca-3'	5'-cagatcagtttctatcagc-3'	344

Results and Discussion

The effects of exposure to TCDD for CYP1A4 and CYP1A5 are shown in Fig 1. Amplification was either exponential to 24 - 28 cycles for CYP1As and GAPDH primers. Therefore the cycle number for PCR is determined 27 cycles for semi-quantification of chicken CYP1As expression in hepatocytes treated with TCDD. Transcripts of both CYP genes were not visible in control samples. GAPDH expression was similar in all samples. TCDD were cognitively induced both CYP1A4 and CYP1A5 transcripts. In compare to CYP1A4, CYP1A5 transcript seems to be lower in chicken hepatocytes. It is correnponded to the observation reported by Bosveld et al. using chicken embryo hepatocytes[1]. Similar to the CYP1A mRNAs, EROD activities were also significantly increased with TCDD in dose dependent manner (Fig. 2). Hence, CYP1A4 and CYP1A5 could be induced in chicken hepatocytes by very low TCDD amount such as 1.0 pmol level.

It is well known that CYP1A proteins catalyse EROD reaction. In adult chicken liver, small EROD activity was detected in nontreated hepatocytes (5.87+-5.55 pmoles/min/mg protein), although RT-PCR products were not visible. Mahajan et al. reported that CYP1A4 and CYP1A5 could apparantly observed constitutive expression in embro liver[1]. Both CYP1A genes expression in normal liver may decrease after hatching. In contrast to consitituteve activity, EROD induction extents were aproximately 2 times higher than those of embro study[5], demonstrating the difference of response against TCDD.

These results indicate that primary cultured adult chicken hepatocytes could be a useful method to investigate the effect of TCDD on chicken liver followed by a RT-PCR semi-quantitive method for CYP genes combined with EROD assay. This method has following advantage; 1. Various experimental conditions can be obtained form a chicken, 2. Secondary pollution is prevennted in isolated culture dishes, 3. The effects on adult chicken liver are reperesented.

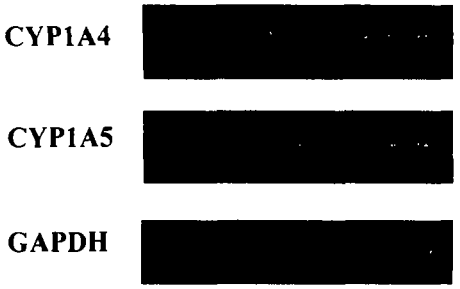

Fig. 1. CYP1A4 and CYP1A5 mRNA expression in adult chicken hepatocytes treated with TCDD

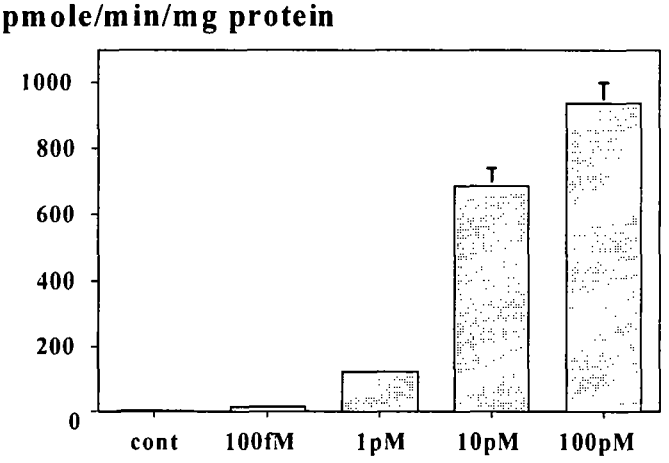

Fig. 2. EROD activity in adult chicken hepatocytes treated with TCDD

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