# IDENTIFICATION OF CYP1A1 GENE EXPRESSION AND ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY BY 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) IN PRIMARY CULTURED BOVINE HEPATOCYTES

Keerthi Siri Guruge[1], Noriko Yamanaka[1], Shigeru Miyazaki[1], Takayuki Kubota[2] and Yoshiko Motoi[1]

[1] Department of Safety Research, National Institute of Animal Health, Kannondai 3-1-5, Tsukuba, Japan
[2] Department of Immunology, National Institute of Animal Health, Kannondai 3-1-5, Tsukuba, Japan

## Introduction

Cytocrome P450 enzymes (CYPs) constitute a super gene family, which play a crucial role in metabolism of a wide variety of xenobiotics[1]. Contamination by members of TCDD-like chemicals in animals elicits a spectrum of biochemical and toxic effects including induction of CYP enzymes. To study such effects not only benefited the health and economic efficiency of farm animals but also provide insights on the health of human on a long-term basis. Cattle is commercially important livestock and have moderate risk of accumulating environmental pollutants in their bodies through various exposure routes. However, toxicological studies in such large animals certainly a difficult task. Therefore, it is with importance to develop a suitable method to identify toxic effects in large domestic animals. The present study was designed to develop an appropriate cell bioassay followed by identification of novel CYP1A1 gene of bovine and its induction effects by an in vitro exposure to lower doses of 2,3,7,8-TCDD.

## Methods and Materials

Primary cultured hepatocytes were prepared by modified method by Koide et al[2]. Hepatocytes were isolated by a sequential perfusion and seeded onto plastic dishes with positive charged surface at a density of 5 X $10^5$/mL in total volumes of 2 ml for 6 well dish and of 0.5 ml for 24 well dish. Cells were treated with 1μM 3-methylchlolanthrene (3-MC) for 24 h and mRNA were extracted. Upstream and downstream primers were designed for bovine CYP1A1 based on the conserved sequence between ovine, murine and human CYP1A1 mRNA cording sequences. After cDNA amplification by RT-PCR with 10pmol of primers, 550bp PCR products were cloned to pBluescriptII (Stratagene, USA) vector and transformed into *E. coli*.. Then plasmid DNA was sequenced by ABI Model 373 automated sequencer.

The semi-quantitative analysis of CYP1A1 (size: 382 bp) were performed with a house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (size: 564 bp) as a control. 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. The cycle number was determined when control and target template to reach the exponential phase of amplification with similar

initial template concentrations (10 ng/μl of mRNA from 3-MC treated hepatocytes). The primers for amplification are given in Table 1.

Table 1. Oligonucleotide primers used for PCR analysis

| Gene | 5' primer | 3' primer | Size (bp) |
|---|---|---|---|
| CYP 1A1 | 5'-gatgttggggaagaacccacac-3' | 5'-ccctgacatcagcacttggaac-3' | 382 |
| GAPDH | 5'-gctggtgctgagtatgatgtggagt-3' | 5'agtgtagcctagaatgcccttgagag-3' | 564 |

To determine the TCDD effects, appropriate amounts of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin stock solutions dissolved in DMSO were added to each well to achieved final concentration of 100fM, 1.0, 10, 100 pM. Only DMSO was used as control. After 24 h culture TCDD was added and incubated for 24 h. Then mRNA was collected and RT-PCR was performed.

Activities of ethoxyresorufin *O*-dealkylase (EROD) and protein contents of cells were determined by the modified method of Kennedy et al. (1996)[3]. 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).

**Results and Discussion**

Bovine hepatocytes displayed a multicellular structure and maintained liver-specific functions at least for 2 weeks compared with monolayer culture. The structure was similar to those in rat hepatocyte culture reported earlier[2].

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G GTC AGG ACC TGG CGG CCT CGG GTC CCT CAA GGC CTG AAG AGT CCC CCG
  V  R  T  W  R  P  R  V  P  Q  G  L  K  S  P  P
GAG CCC TGG GGC TGG CCC CTG CTC GGG CAC ATG CTG ATG TTG GGG AAG
 E  P  W  G  W  P  L  L  G  H  M  L  M  L  G  K
AAC CCA CAC GTG GTC CTG TCG CAG CTG AGC CAG CGC TAT GGG GAC GTG
 N  P  H  V  V  L  S  Q  L  S  Q  R  Y  G  D  V
CTG CAG ATC CGC ATT GGC TGC ACA CCC GTG CTG GTG CTC AGC GGC CTG
 L  Q  I  R  I  G  C  T  P  V  L  V  L  S  G  L
GAC ACC GTC CGG CAG GCC CTG GTG CGG CAG GGC GAT GAT TTC AAG GGC
 D  T  V  R  Q  A  L  V  R  Q  G  D  D  F  K  G
CGG CCC GAC CTC TAC AGC TTC ACC TTG ATC ACT AAC GGC CAG AGC ATG
 R  P  D  L  Y  S  F  T  L  I  T  N  G  Q  S  M
ACC TTC AAC CCA GAC TCT GGA CCG GTG TGG GCT GCC CGA CGA CGC CTG
 T  F  N  P  D  S  G  P  V  W  A  A  R  R  R  L
GCC CAG AAT GCT CTG AAG AGT TTC TCC ACT GCC TCA GAC CCG GCA TCC
 A  Q  N  A  L  K  S  F  S  T  A  S  D  P  A  S
TCA TCC TCT TGC TAT CTG GAA GAG CAT GTG AAC AAG GAG GCC AAG TAC
 S  S  S  C  Y  L  E  E  H  V  N  K  E  A  K  Y
CTC CTG GGG AAG TTC CAA GAG CTG ATG TCA GGG CCT GGG CGC TTT GAC
 L  L  G  K  F  Q  E  L  M  S  G  P  G  R  F  D
CCC TAC AGG TAT ATA GTG GTG TCA GTG GCC AAT GTC ATC TGT GCC ATA
 P  Y  R  Y  I  V  V  S  V  A  N  V  I  C  A  I
TGC TTT GGC CGG CGC TAT GAC
 C  F  G  R  R  Y  D
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Fig. 1. The nucleotide and amino acid sequences of bovine CYP 1A1

In this study we have successfully constructed 550 bp of nucleotide and the deduced 183 amino acid sequence of novel bovine CYP1A1. This amino acid sequence shares 80%, 82% and 93% similarity with mouse, human and sheep CYP1A1, respectively (Fig. 1). The nucleotide sequence data reported here was submitted to DDBJ (AB060696).

The cycle number for PCR is important to determine control and target templates to reach the exponential phase for quantification. Amplification was exponential to 24 - 28 cycles for both primers. Therefore 27 cycles were set up for semi-quantification of bovine CYP1A1 expression in hepatocytes treated with dioxin.

Although treatment with 2,3,7,8-TCDD in various experimental animals results in the induction of CYP1A gene, no such data has been reported for bovine CYP1A1. PCR products were not recognised in control samples suggesting that CYP1A1 may not be expressed in normal hepatocytes. However, dose dependent CYP1A1 induction in bovine hepatocytes was clearly observed by exposure to 100fM- 100 pM of TCDD (Fig. 2). Similarly EROD activities were increased. However, EROD activity was significant from 1 pM TCDD to higher doses than that was in the control (Fig. 3).

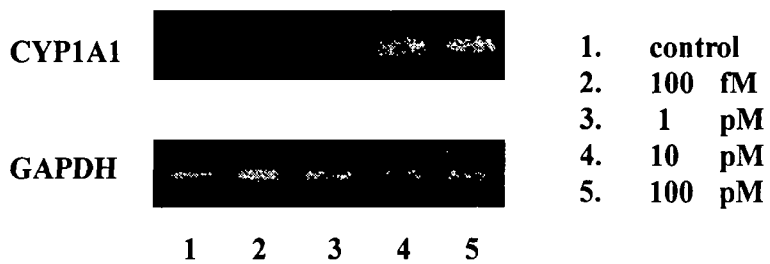


Fig. 2. CYP1A1 mRNA expression in bovine hepatocytes treated with TCDD

Collectively, spheroid cell culture of hepatocytes would particularly be suited to an alternative method for bovine research. These results clarified that bovine hepatocytes are very sensitive to very low doses of TCDD. Likewise, we have evidently presented that TCDD could be effected in $10^{-15}$ M levels to the animal cells. Similar experiment would be an economical for toxicological study in larger domestic animals like bovine.

**References**

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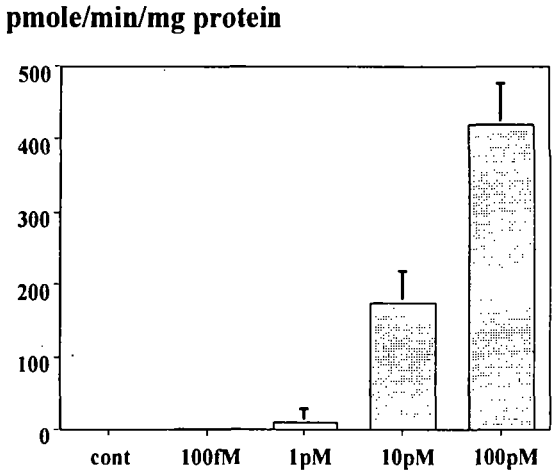


Fig. 3. EROD activity in bovine hepatocytes treated with TCDD