PROGRESSIVE SILENCING OF CYP1A1 INDUCTION IN RAT KERATINOCYTES

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In mammals, P4501A1 is expressed in a wide variety of extrahepatic tissues, including skin, kidney, and lung. Expression of the CYP1A1 gene is normally extremely low but is highly inducible upon exposure to PAHs, TCDD, and related compounds. Regulation of CYP1A1 expression is under complex transcriptional control, with *trans*-acting factors interacting with separate response elements to regulate constitutive expression, tissue- and differentiation-specific control, and PAH induction [1]. A variety of the PAHs that induce P4501A1 are also metabolized by this enzyme to products that are highly reactive and form adducts with cellular components, including DNA. Some PAHs have been identified as skin carcinogens in both humans and rodents[2]. Since the epidermis is the primary barrier to toxic agents, understanding the molecular responses of both normal and transformed keratinocytes to PAHs and TCDD is important in evaluating possible toxic outcomes of dermal exposure to these and related compounds. Primary cultures of rat keratinocytes are known to induce expression of CYP1A1 and increase aryl hydrocarbon hydroxylase activity in response to treatment with TCDD or benzo(a)pyrene[3]. It has also been previously reported that after repeated passaging in culture, rat epidermal cells lose responsiveness to TCDD and B(a)P. In the above studies, the inducibility of CYP1A1 was determined by measuring mRNA levels at a single time point (24 hr) after treatment. However, we have found that these cells do not always exhibit an "all or none" response when treated with TCDD. Here, we examine additional patterns of response to TCDD treatment in rat keratinocytes in which CYP1A1 mRNA expression can be transient or biphasic.

Materials and Methods

Cell Culture. Rat epidermal keratinocyte cultures were grown with irradiated 3T3 feeder layers and supplements in a 3:1 mixture of Dulbecco Modified Eagle's Medium and Ham's F-12 media as described[4]. TCDD was added to cultures to a final concentration of 10 nM, and cycloheximide (CYH) was used at 10 μg/ml.

Northern Analysis. Medium was removed, cells were washed with PBS, dissolved in 1 ml Trizol and total RNA was isolated as recommended by the manufacturer. Total cellular RNA (15 μg/lane) was electrophoresed through a 1% agarose gel containing 0.66 M formaldehyde and transferred to a nylon membrane. RNA was fixed to the membrane by UV-crosslinking followed by baking in vacuum oven at 80°C for 1 hr. The membrane was pre-hybridized at 65°C as described[5]. The CYP1A1 probe used was the full-length rat CYP1A1 cDNA. The rat glyceraldehyde-phosphate dehydrogenase (GAPDH) probe has been described previously[6]. cDNA probes were labeled with [α-^{32}P]dCTP, denatured, and added to the hybridization mixture at a final concentration of approximately 10^6 cpm/ml. After 24 hr at 65°C, the membrane was washed three times, 30 min each at 65°C, with 3% SDS, 40 mM Na_2PO_4 (pH 7.2), and 1 mM *EDTA*[5]. Radioactivity on blots was quantitated by phosphorimaging and resulting values for CYP1A1 were normalized to those for GAPDH.

Plasmid Reporter Constructs. pGudLuc5.0 consists of -1317 to +256 bp of the mouse CYP1A1 gene inserted into the HindIII site of the luciferase reporter vector pGL2-basic (Promega)[7]. This construct therefore contains the CYP1A1 promoter, negative regulatory region, and four DREs controlling expression of the downstream luciferase gene. The promoter of the pGudLuc1.1 vector[8] was constructed previously by splicing a 480-bp region (-1317 to -818 bp) of the mouse cytochrome P4501A1 promoter, which contains four DREs, into a fragment of the mouse mammary tumor virus (MMTV) promoter. This new promoter was then subcloned into pGL2-basic. Expression of the luciferase reporter enzyme from this construct is strongly induced by ligands for the Ah receptor.

Transfections and Luciferase Assays. Cells were transiently transfected by calcium phosphate co-precipitation[9]. For each 6-cm dish, 10 μg reporter plasmid DNA, 10 μg pGL3 DNA (Promega), and 0.2 μg pRL-CMV DNA (Promega) were precipitated with 2 M calcium phosphate and 2 X Hepes-buffered saline for at least 30 minutes and added to the cultures. Forty-eight hours later, cells were treated with 10 nM TCDD or equivalent volume of DMSO for 24 hr. Cells were harvested using Promega's Dual Luciferase Assay Kit, and firefly and renilla luciferase activities of the cleared lysates were measured in a Turner luminometer. Firefly values were normalized to renilla values.

Results and Discussion

We have found that as rat keratinocytes are serially passaged, the time-course of CYP1A1 mRNA expression in response to TCDD treatment can change dramatically. Primary cultures of rat keratinocytes show a prolonged, maximal induction of CYP1A1 mRNA in response to a single TCDD treatment (Fig 1A), and this pattern of induction is usually observed in these cells for at least 4-5 passages. After this point, most strains of rat keratinocytes go through periods in which they express CYP1A1 mRNA in either a biphasic or transient fashion in response to TCDD. The biphasic response (Fig 1B) consists of the normal rapid increase in mRNA levels in response to TCDD, followed by an equally rapid decline in mRNA levels, after which the levels increase again and remain at maximum levels for at least 24 hrs. This biphasic response appears to be a "transition" period for these cells, as this pattern of expression is usually seen only for a few passages, after which the cells either induce CYP1A1 mRNA levels transiently or not at all. In the transient response (Fig 1C), the mRNA increases rapidly in response to TCDD, remains at maximal levels for 2-4 hrs, and then decreases to undetectable levels by 8-10 hrs. This pattern of response has been maintained by some strains of rat keratinocytes for over 50 passages. Finally, some stains progress to the non-inducible response (Fig 1D) in which no increase in CYP1A1 mRNA is observed at any time point after TCDD exposure.

To examine the mechanism by which rat keratinocytes regulate the inducibility of CYP1A1, cells displaying the various patterns of CYP1A1 mRNA induction were transiently transfected with the luciferase reporter construct GudLuc1.1, which contains four DREs from the murine CYP1A1 gene. This construct was induced for over 48 hrs after TCDD treatment in all cells. This indicated that the Ah receptor pathway was activated and functional, even in cells that showed no increase in CYP1A1 mRNA. Cells were also transfected with GudLuc5.0, which contains 1.3 kb of the CYP1A1 5'-flanking region. This construct was also inducible in all cells, suggesting that the observed negative regulation of the CYP1A1 gene in the transiently inducible and non-inducible cells was not due to a regulatory element within this region. To examine this phenomenon further, a luciferase reporter construct containing 1.3 kb of the rat CYP1A1 5'-

flanking region was stably transfected into non-inducible cells. Though the mRNA levels of the endogenous CYP1A1 gene were completely uninducible in these cells, the stably integrated reporter construct showed strong induction in response to TCDD.

To determine if synthesis of protein is required for the down-regulation seen in the transiently inducible and non-inducible cells, the protein synthesis inhibitor cycloheximide (CYH) was added to these cells with or without TCDD. In the non-inducible cells, no CYP1A1 mRNA was detectable when cells were treated with either CYH or TCDD alone (Fig 2B). However, a dramatic, sustained increase in CYP1A1 mRNA levels was seen in these cells treated with TCDD and CYH together. To determine if continuous inhibition of protein synthesis was required to maintain CYP1A1 mRNA induction, the medium was removed at 4 hrs, cells were washed once with medium, and new medium was added containing 10 nM TCDD with or without CYH (Fig 2A). When CYH was removed from the cells, mRNA levels quickly returned to control levels, indicating that protein synthesis inhibition was required not only for the initial increase of CYP1A1 mRNA but also for maintenance of these induced levels. In transiently inducible cells, treatment with CYH along with TCDD resulted in a prolonged induction without the decrease in mRNA levels normally seen after 4-6 hrs with TCDD treatment alone (data not shown).

We have therefore shown that in rat keratinocytes the response of CYP1A1 mRNA levels to TCDD treatment can change dramatically as the cells are serially passaged. This response can include biphasic or transient induction of the CYP1A1 mRNA. The changes in inducbility do not appear to occur due to changes in the Ah receptor pathway and could not be attributed to response elements in the first 1.3 kb of the 5'-flanking region. In addition, the ability of transiently inducible and non-inducible cells to down-regulate CYP1A1 mRNA levels can be blocked by inhibition of protein synthesis, suggesting the requirement of a labile repressor protein. Further study into these mechanisms of regulation will aid in understanding how the expression of this important metabolic enzyme is regulated in normal and immortalized cells.

Acknowledgements

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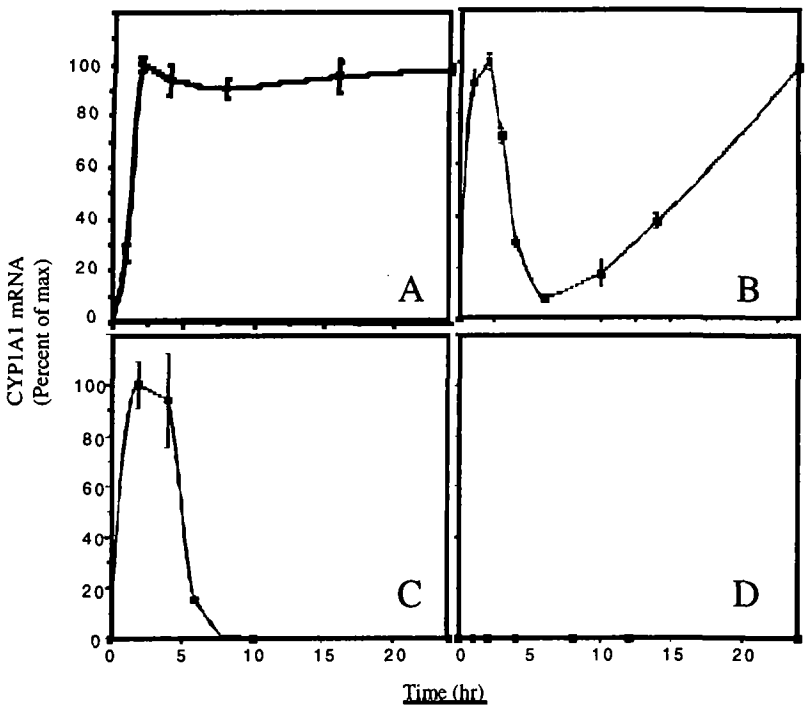
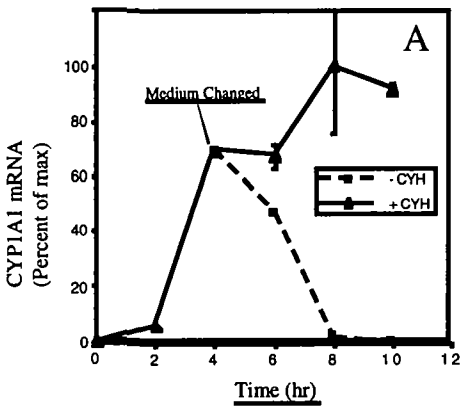


Fig 1 – Effects of TCDD Treatment on CYP1A1 mRNA Levels in Rat Keratinocytes. *Panel A*- Prolonged Induction; *Panel B* – Biphasic Induction; *Panel C* – Transient Induction; *Panel D* – Non-Inducible



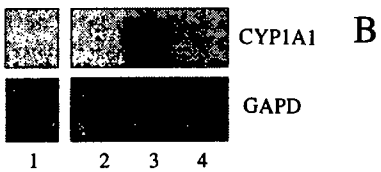


Fig 2 – Effects of CYH and TCDD on CYP1A1 mRNA In Non-Inducible Cells – *Panel A* – CYH and TCDD added to all cultures at 0 hr. Medium changed at 4 hr, TCDD was added to all cultures, with or without CYH. *Panel B* – Northern blot of non-inducible cells. *lane 1* – Control, no treatment; *lane 2* –CYH, 8 hr; *lane 3* – CYH + TCDD, 8 hr; *lane 4* – TCDD, 8hr