QUANTITATION OF THE AHR-MEDIATED INDUCTION OF CYP1A1

Wall Richard, Bazzi Rana and Bell David

School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD

Introduction

The aryl hydrocarbon receptor (AhR) has a high affinity for certain dioxin-like compounds. Activation of the AhR increases the transcription of CYP1A1 which is involved in xenobiotic metabolism. TCDD is an agonist of AhR and activation of AhR by TCDD is believed to mediate the toxic response of TCDD. This paper will use CYP1A1 induction by TCDD as a measure for activation of the AhR. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent of a family of halogenated aromatic hydrocarbons (HAHs) and although they have varying potencies, they all undergo a similar pathway of toxicity by binding to the AhR, and causing ligand-dependent induction of a gene battery (Whitlock, 1993, Wu and Whitlock, 1993). Quantification of activation of the AhR requires a robust method of quantifying induction of an AhR-dependent gene, in this case CYP1A1. This involves developing a RT-PCR method which can be applied to several different AhR agonists.

Method and Materials

H4IIE Rat Liver cells were dosed with various concentrations of TCDD to measure CYP1A1 induction. Cells were processed in a 96-well plate producing small quantities of RNA. Examination of the RNA protocols allowed modification of the instructions to account for small cell volume. The probe and primer sequences for RT-PCR were obtained from Bell et al (2007) and the probe concentrations were optimised. PCR efficiency has a substantial effect on quantitation of RNA (Figure 1). The quantitation of RNA copy number used the ΔC_t method and statistical normalisation employed by qBase (Hellemans et al, 2007).

Expression of the CYP1A1 gene was achieved using RT-PCR. H4IIE Rat liver cells were treated with various concentrations of TCDD (10nM to 100fM and untreated) diluted in 24h old medium and 0.1% DMSO. The 'modification for small samples' protocol was used when purifying the RNA with a concentration of 1.0×10^6 cells with cDNA produced as per instructions. The AhR and β-Actin genes were also quantified to use as a control. A final 12.5µl buffer solution was used containing: 6.25µl master mix, 100-300nM probes and 200-600nM of the primer pairs with 2ng cDNA. All three probes were used in the same reaction (Bell et al, 2007) with PCR efficiencies between 98-115%. The control genes, AhR and β-Actin, were unaffected by TCDD treatment. RT-PCR was performed using the Stratagene MX4000. A program of 1 cycle at 95°C for 10 minutes followed by 40 cycles of: 95°C for 20 seconds and 58°C for 90 seconds, was used.

Results

The first aim of the research was to create a method of measuring AhR activation, and the induction of CYP1A1 RNA is one of the most sensitive measures of AhR activation. Measurement of RNA allows measurement at an early time-point, hence mitigating any effect of metabolism. It is necessary to determine the efficiency of PCR, as this has a substantial effect on estimation of RNA amount. Figure 1 shows the amount of RNA measured (Ct) as a function of input cDNA; this analysis shows a poor fit of the data to the line of best fit (Figure 1A), and efficiency of PCR is ~130-190%, an anomalous result. The initial quantity of input cDNA is shown on the x-axis as a relative amount, and the C_t for each dilution is shown on the Y-axis, for n=3 replicates. The fit of the data to the line was determined by r^2 analysis, and the efficiency of PCR was determined from the slope of the line. Figure 1B demonstrates the effect of a reduction in probe concentration from 200-600nM to 100-300nM, with improvement of both correlation coefficient and slope, with efficiency ~100-120%. Critical analysis of PCR efficiency is thus a prerequisite for accurate data.

Figure 2 shows RT-PCR results of RNA in cells treated with TCDD at various concentrations for 4hrs. The Y-axis shows the fluorescence at each Ct, for n=6 replicates (duplicates of 3 biological replicates). CYP1A1 (Figure 2A) was induced by 128-fold over control (10nM against untreated) with high induction indicating large

signal to noise ratio and accurate induction parameters. The input cDNA was first normalized using a quantitative assay thus giving 2ng of cDNA per sample. Figure 2B shows that one of the control genes, β-Actin, was unaffected by the TCDD treatment (AhR control gene was also unaffected but data not shown) and indicates that the cDNA used was of good quality and equal concentration. Qbase was used to analyse the data derived from RT-PCR analysis. Qbase normalizes the expression of CYP1A1 against the reference genes, AhR and b-Actin. The reference gene expression should be within two-fold, indicating accurate sample preparation and quantitation. Qbase derives a normalisation factor, which is a geometric mean of the reference genes (Hellenmans et al, 2007).

Two quality measures are calculated, firstly, the coefficient of variation of the normalized relative quantities, which for AhR and Actin, was 8.7% and 9.0% respectively indicating high stability. Secondly, the geNorm value which measures the stability of the genes: 0.26, confirming that they are stably expressed. The last phase was to produce a dose-response curve using the normalized relative quantities (NRQ) calculated by Qbase. Figure 3 shows the response of CYP1A1 against the dose. . The percentage of maximum response of CYP1A1 measured against log dose where n=3 ± S.D., EC_{50} = 188pM, 95% Confidence interval = 140-252pM, Cells exposed for 4hrs, in biological triplicates. Various concentrations between 10nM and 100fM were used against an untreated control. Maximal response was calculated using Qbase. An increase in the number of replicates for the larger concentrations could decrease the standard deviations. The dose/response relationship shows a classical curve with an EC_{50} of 188pM, and 95% confidence limits of 140-252pM, due to the use of multiple concentrations. Replicate EC_{50} estimates were similar.

Discussion

Quantitative analysis of the activation of AhR, through measurements of CYP1A1 RNA, is critically dependent on the methodology for RNA measurement. Several variables that affect accurate measurement of CYP1A1 RNA have been identified and optimised, yielding a methodology with considerable statistical power for the determination of the potency of an agonist for inducing CYP1A1 RNA. Statistical power is a prerequisite for detecting small differences in potency. This method for analysis of the dose-response can be used for other agonists of AhR in the future, such as polychlorinated biphenyls, dioxins and furans. Such quantitative measurement of induction potency enables the application of a variety of pharmacological tools to investigate the nature of agonism.

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References:

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Figure 1: PCR efficiency for CYP1A1, β-Actin and AhR, (A) 200-600nM Probe and (B) 100-300nM Probe.

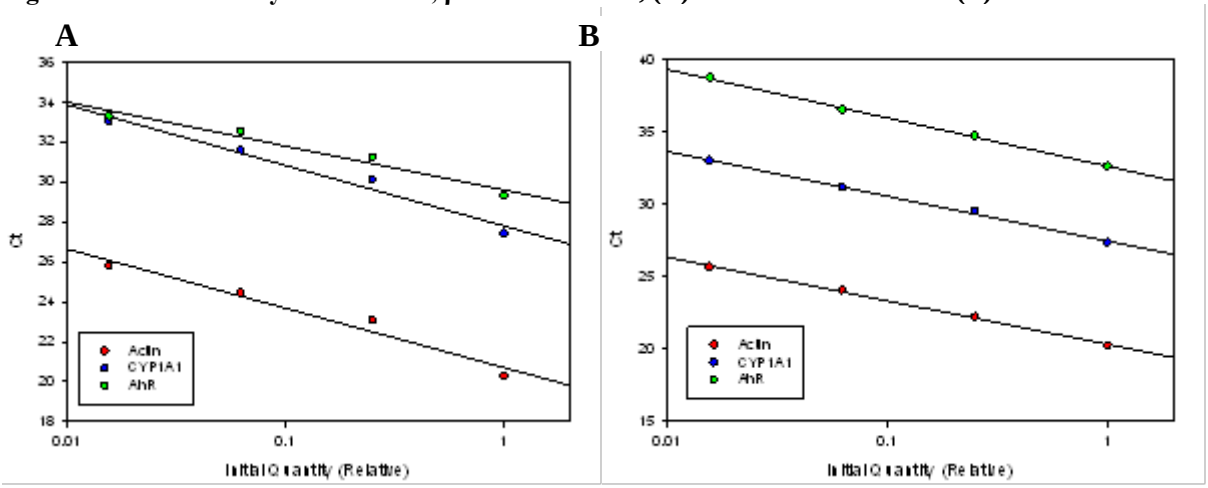

Figure 2: RT-PCR Amplification plots: (A) CYP1A1 and (B) β-Actin

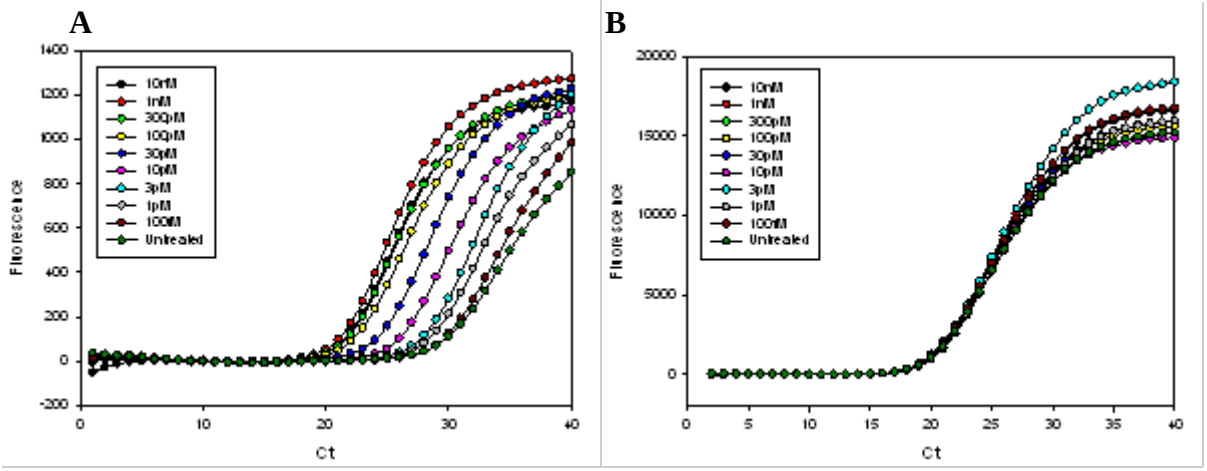

Figure 3: Induction of CYP1A1 RNA by TCDD