DEVELOPMENT OF IMPROVED DR-CALUX® BIOASSAY FOR SENSITIVE MEASUREMENT OF ARYL HYDROCARBON RECEPTOR ACTIVATING COMPOUNDS

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Introduction

Polyhalogenated aromatic hydrocarbons (PHAHs) including dioxins induce a wide variety of effects in mammals, birds and fish such as immunotoxicity, carcinogenicity and metabolic changes. The mechanism of action of PHAHs has been extensively studied over the past decades. These compounds bind to an intracellular receptor, known as the aryl hydrocarbon (Ah) receptor. Binding to the Ah receptor is followed by transportation of the PHAH-Ah receptor complex into the nucleus of the cell and subsequent binding to specific sequences in the DNA called dioxin responsive elements (DREs). Binding of the PHAH-Ah receptor to the DRE triggers the expression of DRE associated genes. By using this knowledge, a genetically modified cell-line (rat-hepatoma H4IIE) was designed containing part of the mouse CYP1A1 promoter including DREs coupled to part of the MMTV promoter and the firefly luciferase gene as a reporter for the presence of PHAHs[1] (pGudLuc1.1: Figure 1A). This cell-line contains the complete machinery that is involved in the mode of action of dioxins and dioxin-like compounds. The thus created stable cell-line expresses the firefly luciferase gene in addition to genes that are normally expressed in the parental cells upon exposure to dioxins or dioxin-like compounds. As a consequence, these cells emit light which can very easily be quantified. The amount of light-production is related to the amount of dioxins or dioxin-like compounds in the exposure mixture. This well-established assay is called the DR-CALUX® (Dioxin Responsive-Chemical Activated Luciferase gene eXpression) assay and has an advantage over traditional analysis of dioxins by HRGC-MS in terms of sensitivity, speed, sample clean-up/work-up, cost reduction, and biological relevance. Here we report on the development of an improved DR-CALUX® H4IIE cell line, containing a multimerized DRE in front of a minimal promoter coupled to the firefly luciferase gene, thus lacking the CYP1A1 and MMTV promoter regions. This has the advantage that regulation through promoter elements other than DREs is avoided and optimal selectivity for Ah-receptor interacting molecules is obtained. Combined with an optimized CALUX culture medium, a much-improved bioassay has been developed with a higher fold induction combined with a lower detection limit for TCDD.

Methods and Materials

DNA constructs

Gudluc1.1 reporter gene construction (figure 1A) has been described elsewhere[1]. The reporter gene pDREtataLuc was constructed as follows: 4 tandem repeats of a DRE oligo (based on the –985 to -979 DRE sequence of the rat CYP1A1 promoter) upstream of the minimal adenovirus E_1B TATA promoter sequence were inserted in the multiple cloning site of the enhanced luciferase reporter gene construct pGL3-basic (figure 1B). pSG5-neo was used as a selection construct[2].

Stable cell lines

Generation of the H4IIE-GudLuc1.1 cells has previously been described[1]. Rat hepatoma H4IIE cells were stably transfected with pDREtataLuc and pSG5-neo using calcium phosphate co-precipitation. A total of 96 G418-resistant clones were tested for their response to 1 nM TCDD of which 5 clones showed consequent high response. One of these clones called H4IIE-DREtataLuc51 responding to the lowest concentration of TCDD (1 pM) was selected for further comparison to the H4IIE-GudLuc1.1cell line.

DR-CALUX® assay

H4IIE cells stably transfected with pGudLuc1.1 or pDREtataLuc were cultured in DF medium supplemented with 7.5% FCS and 200 µg/ml G418. Cells were plated in 96 well plates with phenol red free DF medium supplemented with 5 % dextran coated charcoal stripped FCS (DCC-FCS) at a volume of 100 µl per well. About 24 h later, 100 ?l of DCC-FCS medium or an optimized CALUX culture medium, both containing TCDD (dissolved in DMSO, final dilution 1:250) was added directly to the medium. After 24 h the medium was removed, cells were lysed and measured for luciferase activity as described earlier[3] using the Luclite luciferase gene reporter assay kit and a Topcount scintillation counter (Packard).

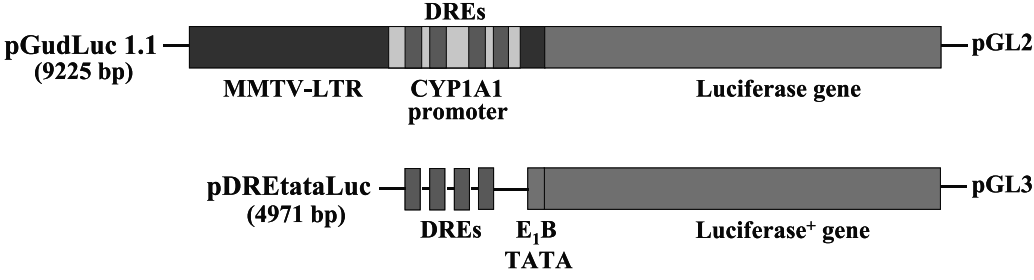

Figure 1. Schematic representation of the pGudluc 1.1 and pDREtataLuc expression vectors. pGudluc1.1 contains a 484-bp fragment from the upstream region of the mouse CYP1A1 gene, containing 4 dioxin response elements (DREs) inserted in the MMTV long terminal repeat (MMTV-LTR), inclusive of the viral promoter, but lacking known functional glucocorticoid responsive enhancers[1] inserted in the multiple cloning site of the luciferase vector pGL2-basic. pDREtataLuc contains four multimerized rat DREs upstream of a minimal adenovirus E1B TATA promoter inserted in the multiple cloning site of the enhanced luciferase (luc+) vector pGL3-basic.

Results and discussion

The sensitivity and responsiveness of the DR-CALUX® assay with the stably transfected H4IIE-GudLuc1.1 and newly developed H4IIE-DREtata-Luc cells was assessed by measuring the luciferase activity induced by TCDD compared to solvent control. Exposure of both cell lines to TCDD for 24 hours resulted in dose-response related luciferase induction (Figure 2). Using DF-medium with 5% DCC-FCS in a 96 well assay, the maximum fold induction for the H4IIE-GudLuc1.1 cells was 18 times (at 1nM TCDD), while the newly generated H4IIE-DREtataLuc cells showed 180 fold induction at this concentration of TCDD (Figure 2A). The EC_{50} values for both lines were 10 pM and 70 pM

respectively, while the minimal detectable concentration of TCDD was between 1 and 2 pM in both cell lines (Figure 2A inset). These results clearly demonstrate that the new developed H4IIE-DREtataLuc cell line showes an 10-fold increased response to TCDD at higher concentrations compared to the H4IIE-GudLuc cell line (from 10 pM to 1 nM TCDD), although at low concentrations (1 pM to 10 pM) no differences in sensitivity were observed in standard culture medium.

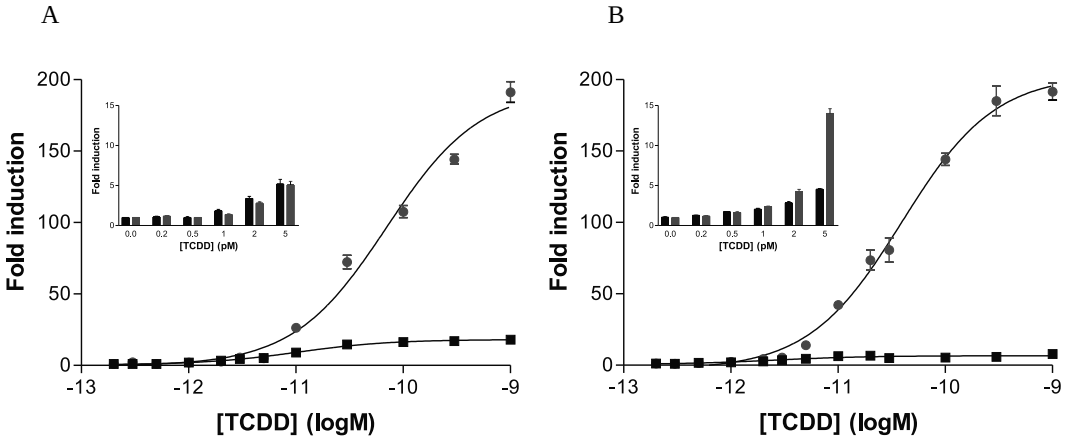

Figure 2. Luciferase induction by TCDD relative to solvent control in the DR-CALUX® assay. H4IIE cells were stably transfected with pGudLuc1.1[1] (■) or pDREtata-Luc (●), plated in 96 well plates and treated with TCDD for 24 hours, using DF medium containing 5 % DCC-FCS (A) or optimized culture medium (B). Each point represents the mean of three independent experiments ± SEM.

An optimized CALUX culture medium was developed to improve the performance of the H4IIE-DREtataLuc cell line. By using this medium, the sensitivity of the H4IIE-DREtataLuc cells towards TCDD was increased: the detection limit was lowered from 1 pM to 0.5 pM TCDD, while the fold induction in the 0.5 pM to 5 pM TCDD range was doubled (1.6-14 fold compared to 1-5.1 fold using the DF/5 % DCC-FCS medium: compare insets A with B of figure 2). Although the maximum fold induction (1 nM TCDD) was not altered, the EC_{50} value decreased to 39 pM using the optimized culture medium in combination with the H4IIE-DREtataLuc cells. Usage of this medium with the H4IIE-GudLuc1.1 cells did also result in a decreased detection limit (0.5 pM TCDD: 1.7 fold induction) and EC_{50} value (2.6 pM), but the response of the cells in the 0.5 pM to 5 pM TCDD concentration range was not altered. In addition, the maximum fold induction was decreased to 8 fold at 1nM TCDD resulting in a 24 times higher maximum fold induction of the H4IIE-DREtataLuc cells.

The above described results demonstrate that the newly developed CALUX culture medium in combination with the new generation DR-CALUX® cells (H4IIE-DREtataLuc) are very promising for improvement of the performance of the DR-CALUX® bioassay with optimal sensitivity and selectivity for Ah-receptor interacting molecules like TCDD.

References

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