ENHANCING CALUX AND CAFLUX CELL BIOASSAY RESPONSES THROUGH INTRACELLULAR SIGNALING PATHWAYS AND INCUBATION TEMPERATURE

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Introduction

Proper epidemiological, risk assessment and exposure analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related dioxin-like halogenated aromatic hydrocarbons (HAHs) requires accurate measurements of these chemicals both in the species of interest and in various exposure matrices (i.e. biological, environmental, food and feed). While high-resolution instrumental analysis techniques are established for these chemicals, these procedures are impractical for large scale screening analysis. Accordingly, numerous in vitro and cell-based bioanalytical methods have been developed over the past 10 years for the detection of these chemicals in extracts from a variety of matrices and the majority of these systems take advantage of the ability of these chemicals to activate one or more aspects of the AhR-dependent mechanism of action.[1] The most sensitive cell-based bioassay systems developed to date are the so-called CALUX (Chemically Activated Luciferase Expression) and CAFLUX (Chemically Activated Fluorescent Expression) bioassays, which utilize recombinant cell lines that contain stably transfected dioxin (AhR)-responsive firefly luciferase or enhanced green fluorescent protein (EGFP) reporter genes, respectively. Treatment of these cells with TCDD and related HAHs, other AhR ligands and extracts containing AhR agonists, results in induction of these reporter gene expression in a time-, dose-, AhR-, and chemical-specific manner and the level of reporter gene expression correlates with the total concentration of TCDD-like AhR inducers (agonists) present in the sample.[1,2,3] Although these systems use the same AhR-dependent mechanism to induce reporter gene expression, differences in the characteristics of the respective reporter gene products result in bioassay systems with distinct advantages and disadvantages.[1,4] While the firefly luciferase reporter gene in the CALUX bioassay system has a very high degree of sensitivity and response, primarily due to enzymatic signal amplification, it also has limitations with respect to repeated measurement, cost and rapidity for high-throughput screening analysis. In contrast, the EGFP reporter gene is more rapid, cost effective and is amenable to high throughput and repeated analysis and the induction response can be measured in "real time". However, since the EGFP output signal is directly proportional to the number of EGFP molecules (i.e. it is not an amplified response like luciferase) the reporter signal develops significantly slower than that of luciferase, but because EGFP is extremely stable ($T_{1/2}$ >23 h), much higher reporter gene signal output is obtained with extended time of incubation. While the current CALUX and CAFLUX bioassays are very sensitive, increasing their lower limit of sensitivity as well as magnitude of response and dynamic range for chemical detection would greatly increase their utility, particularly with samples that contain low levels of dioxin-like HAHs. Recent reports of enhancement of AhR and AhR-dependent signal transduction by chemical treatment as well as increased luciferase and EGFP reporter gene activity by modulating cell culture conditions provide several avenues in which to improve the sensitivity, signal-to-noise ratio, detection limits and responsiveness of our current CALUX and CAFLUX bioassays.[3,5-8] Here we described studies examining the effect of modulators of cell signaling pathways (i.e. dexamethasone (Dex) and phorbol esters) as well as alterations in cell culture incubation temperature on the inducibility and responsiveness of the CALUX and CAFLUX cell bioassays.

Materials and Methods

Chemicals: TCDD was from Dr Steven Safe (Texas A&M University, USA), Dex from Sigma Chemical Company (St. Louis, MO, USA), phorbol-12-myristate-13-acetate from Calbiochem (LaJolla, CA USA).

CALUX and CAFLUX cell cultures and reporter gene activity: Determination of the ability of various chemicals to stimulate AhR-dependent gene expression was carried out using recombinant mouse and rat hepatoma and guinea pig intestinal cell lines that contains the AhR-responsive firefly luciferase reporter plasmid pGudLuc1.1 (H1L1.1c2, H4L1.1c4 and G16L1.1c8 cells, respectively) or pGudLuc6.1 (H1L6.1c2 cells) and rat hepatoma cells containing the AhR-responsive EGFP reporter plasmid pGreen1.1 (H1G1.1c3 cells). These cells were grown as previously described and each recombinant cell line responds to TCDD and related AhR agonists with the induction of luciferase/EGFP reporter gene activity in a time-, dose-, ligand-dependent manner as previously described.[1-4]

Results and Discussion

Dexamethasone (Dex). The ability of glucorticoid receptor (GR) agonists like Dex to enhance AhR-dependent induction in rat, human and pig cell lines and in a rat hepatoma CALUX (pGudLuc1.1) cell line has been reported.[5,6] Accordingly, we considered that the addition of Dex to the standard CALUX/CAFLUX bioassay incubation might improve these assays by increasing their sensitivity and magnitude of response. While exposure of the rat hepatoma CALUX and CAFLUX cell bioassays to Dex resulted in an increase in the magnitude of TCDD induced luciferase and EGFP activity, Dex had no effect on TCDD-dependent luciferase gene induction in the mouse or guinea pig CALUX cell lines. Interestingly, Dex alone induced luciferase/EGFP activity in the rat CALUX/CAFLUX cell lines and this induction was inhibited by the GR antagonist RU486, demonstrating a role for the GR in this response. Additionally, the ability of RU486 to inhibit Dex enhancement of TCDD induction but not affect TCDD induction in the absence of Dex in the rat cell lines indicates that the enhancement by Dex was independent of the AhR. The inability of Dex to affect TCDD induction in the mouse liver cell lines, which are known to contain GRs, demonstrates the species specificity of the response. The ability of Dex to both induce expression and enhance AhR-dependent reporter gene expression in the CALUX/CAFLUX rat hepatoma cell lines not only complicates its usefulness as a supplement to improve the response in the rat cell bioassay, but the presence of Dex-like agonists or activators of the glucocorticoid receptor in a sample extract will result in inaccurate bioassay-TEQ estimates or false positive results.

Phorbol-12-myristate-13-acetate (PMA). The AhR and ARNT have been shown to be phosphoproteins and the role of phosphorylation in the regulation of AhR signaling and species- and tissue-specific responsiveness to TCDD and other ligands is currently being examined. Of particular interest is the role of protein kinase C (PKC) in the modulation of AhR responsiveness since activation of PKC by PMA has been reported to augment TCDD-inducible AhR- and DRE-dependent gene expression by 2- to 3-fold.[7,8] Thus, we tested whether the addition of PMA to the standard CALUX/CAFLUX bioassay might improve these bioassays. In contrast to the Dex experiments, exposure of the mouse or rat hepatoma CALUX cell bioassays had no effect alone, but when added along with TCDD or BNF it significantly enhanced dose-dependent luciferase gene induction by 3-4 fold. While these results demonstrate that the addition of PMA to the inducing solution can increase the magnitude of the induction response, comparisons TCDD and BNF dose response curves in the absence and presence of PMA reveals that this compound does not increase the limit of detection of the assay, but it does result in a greater magnitude of reporter gene response at lower doses. In addition, the PMA enhancement of AhR signaling is transient, occurring within eight hours after PMA treatment, and decreasing thereafter as PKC is feedback inhibited. The transient nature of this enhancement may limit the window of time that PMA could be useful in improving detection and quantitation of dioxin-like chemicals by the CALUX bioassay.

Tissue culture induction temperature. In previous studies using the mouse hepatoma CAFLUX bioassay we demonstrated that the tissue culture incubation temperature during the induction period was critical in maximizing the overall EGFP activity obtained by a defined amount of AhR agonist (TCDD), with 33^{o}C resulting in several fold

greater EGFP activity than cells grown at 37°C (optimal for the mammalian cell growth).[3] Although the firefly luciferase used in reporter gene studies has been reported to be very thermolabile at 37°C, its relative activity in cells grown at other temperatures has not been determined.[9] To determine the effect of incubation temperature on the overall fluorescence of the EGFP reporter gene product or the luminescent enzymatic activity of the firefly luciferase reporter gene product, DMSO- and TCDD-treated mouse hepatoma CALUX and CAFLUX cell lines (H1G1.1c3 and H1L6.1c2 cells, respectively) were incubated for 24 hours at 26ºC, 30ºC, 33ºC or 37ºC followed by analysis of reporter gene activity. The results of dose response studies for each reporter gene at different temperatures are shown in figure 1. As is readily apparent, cells incubated at 33°C exhibited significantly more EGFP activity than those at 37°C, 30°C or 26°C. Not only was the fluorescence of EGFP at 33°C 2-3 fold greater than that observed in cells incubated at 37°C at all TCDD concentrations, but it improved detection of reporter gene activity at the minimal detection limit of 1 pM (compare 323.4 ± 30.5 relative fluorescent units (RFUs) at 33ºC to 126.3 ± 42.3 RFUs at 37ºC). Similarly, a 5-10 fold greater amount of luciferase activity was obtained from cells that had been incubated for 24 hours at 30-33°C than that obtained from cells incubated for 24 hours at 37°C or 26°C (figure 1, lower panel). Luciferase activity in cells treated with 1 pM TCDD for 24 hours at 30-33°C significantly higher that that observed from cells incubated at 37ºC. PCR analysis not only confirmed that the variability in reporter gene activity was not due to differences in the level of reporter gene mRNA expression at the different temperatures, but that expression of an endogenous AhR-responsive mammalian gene (CYP1A1) was also unaffected (data not shown). These and other results indicate that the induction response in the CALUX and CAFLUX cell lines was unaffected by the lower incubation temperature and suggest that the temperature-dependence of reporter gene activity is due to an effect on the reporter gene itself, rather than an effect on the AhR signaling pathway. Although the underlying cause of the temperature dependence remains to be established, given previous studies on GFP and luciferase reporter genes, we suspect that it is likely due to effects on the folding and/or stability of these proteins. These results demonstrate that the current CALUX and CAFLUX bioassays are being carried out at suboptimal temperatures necessary for production of maximal reporter gene activity. In those situations where optimal reporter gene activity in transfected mammalian cells is desired, cells should be incubated at lower temperatures (i.e. 33°C) during the gene expression period. This simple modification of gene induction protocols can lead to substantial increases in measurable reporter gene activity.

Acknowledgements

This work was supported by grants from the National Institutes of Environmental Health Sciences (ES07685, ES05707 and a Superfund Basic Research Grant (ES04699)) and the California Agriculture Experiment Station.

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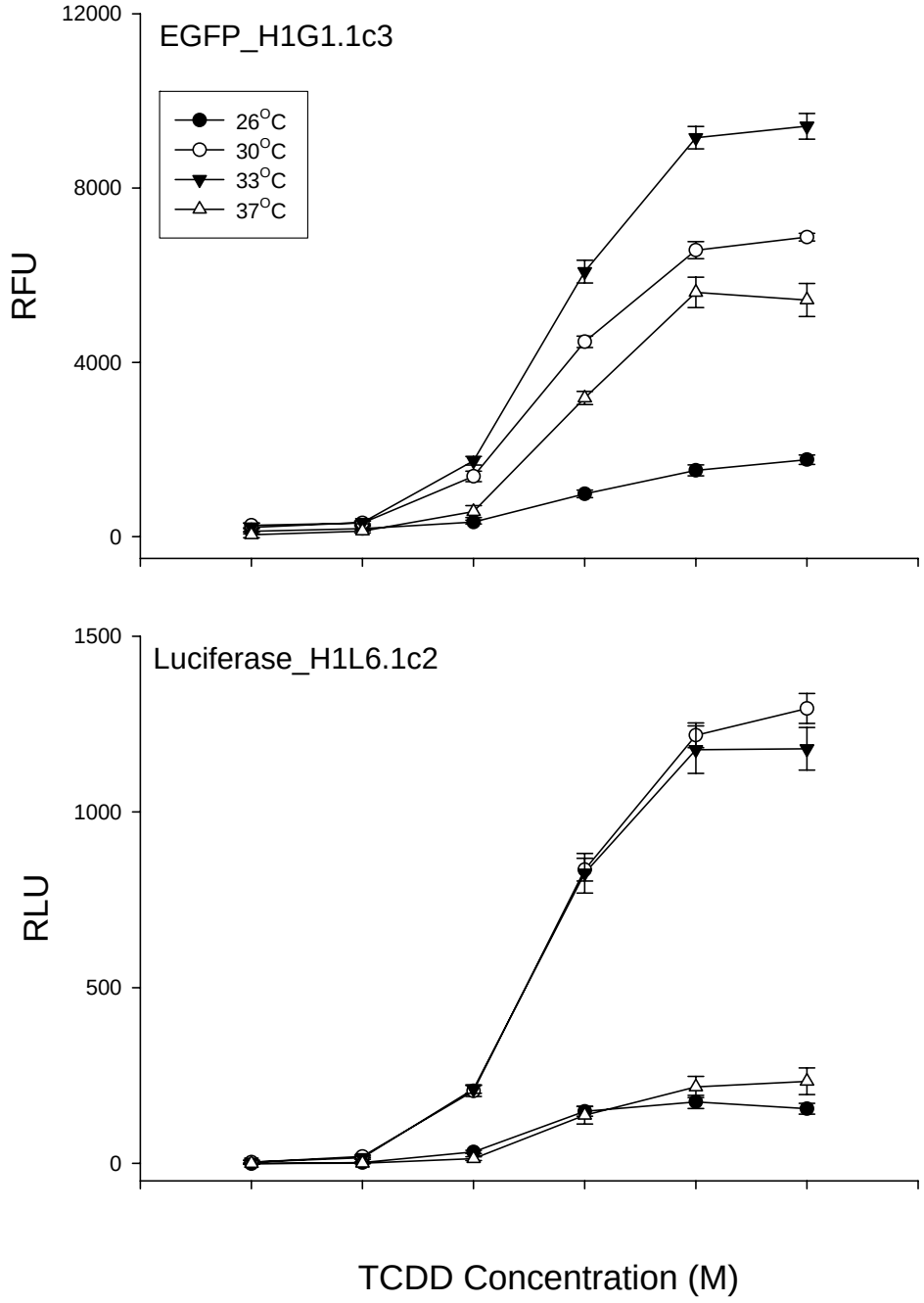

Figure 1. Temperature-dependent reporter gene expression in mouse CAFLUX (H1G1.1c3) and CALUX (H1L6.1c2) cell lines. H1G1.1c3 and H1L6.1c2 cells were incubated at the indicated temperatures with increasing concentrations of TCDD for 24 hours and EGFP or Luciferase activity determined. Values are expressed as relatively fluorescence or Light units (RFUs or RLUs) and represent the mean ± SD of triplicate determinations.