GLUCOCORTICOID MEDIATED EXPRESSION OF DIOXIN TARGET GENES IN RAT H4IIE CELLS BUT NOT IN HUMAN HEPG2 AND T47D CELLS

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Introduction

The effects of dioxins and dioxin-like compounds such as PCBs are mediated by the aryl hydrocarbon receptor (AhR) which can bind dioxin (TCDD) and dioxin-like compounds and subsequently induce the transcription of target genes. An example of a highly induced target gene is CYP1A1. In the past, the CYP1A1 promoter region responsible for the TCDD response was coupled to the firefly luciferase reporter gene and this DNA construct (pGudLuc1.1) was stably transfected in the recipient rat H4IIe hepatoma cell line[1]. Exposure of this so-called DR CALUX® cell line to TCDD results besides expression of endogenous CYP1A1 also in the expression of luciferase which can convert a substrate (luciferine) in a measurable light production. In this way, the light intensity is a quantitive measure for the TCDD exposure to the cells. Recently it was discovered that glucocorticoids like dexamethasone can have an additional positive effect on CYP1A1 induction and activity[2] and induction of the pGudLuc reporter construct in the DR CALUX bioassay[3]. The goal of this study is to determine the mechanism(s) by which glucocorticoids which do not bind the AhR, such as dexamethasone, have a (additional) dioxin-like activity. In addition, we have studied in detail how rodent and human cell lines deviate in their responses to TCDD and additive effects of glucocorticoids. This may provide insight in the known interactions of stress related pathways and biological effects of dioxin-like compounds.

Materials and Methods

Cell culture. Human T47D breast cancer cells, human HepG2 cells, mouse Hepa1 1c1c7 cells and rat H4IIe cells (all obtained from ATCC), were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supplemented with 7.5 % fetal calf serum. DR and GR CALUX cells were cultured in DF medium supplemented with 7.5% FCS and 200 µg/ml G418.

Transient transfections. For transient transfections, cells were plated in 24-well tissue culture plates. After culturing for one day, cells were transfected with 1 µg reporter plasmid (pGudLuc1.1 or pMMTVluc), 200 ng SV2-lacZ and 200 ng expression plasmids pSG5-neo-hGR, pSG5-rGR or empty vector DNA (pSG5-neo) using the calcium phosphate co-precipitation method. Luciferase activity was corrected for transfection efficiency by measuring LacZ expression as a result of SV2-lacZ co-transfection.

CALUX reporter gene assays. DR CALUX (rat H4IIe-pGudLuc1.1 and H4IIe-4xDREtataLuc stable cell lines) and GR CALUX (human U2OS-hGR-3xGREtataLuc stable cell line) bioassays were performed as described elsewhere[1,3,4].

CYP1A1 activity assay. 7-Ethoxyresorufin-O-deethylation (EROD) activity measuring the induced CYP1A1 enzyme activities in H4IIe, Hepa 1c1c7, HepG2 and T47D cells using a 96 well format, was described earlier[5].

Quantitative PCR. Total RNA from H4IIe, HepG2 and T47D cells was isolated using nucleospin RNAII columns (Macherey-Nagel, Düren, Germany). cDNA was prepared with the iScript™ cDNA synthese kit (Bio-Rad, Veenendaal, the Netherlands) from 1 µg of total RNA. Aliquots of 5 µl cDNAs (1:10 diluted) were used as templates for real time PCR using SYBR green (iQ supermix; Bio-Rad, Veenendaal, the Netherlands) in the MyIQ single color real time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). Gene and species specific primers were designed for CYP1A1, CYP1A2, CYP1B1 and RBPO (36B4; internal control) using Beacon designer 4.01 (PREMIER Biosoft International, Palo Alto, CA). Threshold C_T values for each cDNA were determined and relative gene expression analysis was performed using the Livak method[6]. First the C_T of the target gene was normalized to that of the reference gene (RBPO), for both the test sample (treated) and the calibrator sample (DMSO-treated): $\Delta C_{T(test)}=C_{T(target,\ test)}-C_{T(ref,\ test)}$ and $\Delta C_{T(calibrator)}=C_{T(target,\ calibrator)}-C_{T(ref,\ calibrator)}$. Second, the ΔC_T of the test sample was normalized to the ΔC_T of the calibrator: $\Delta\Delta C_T=\Delta C_{T(test)}-\Delta C_{T(calibrator)}$. Finally, the normalized expression ratio (fold induction) was calculated according to the equation: fold induction = $2^{-\Delta\Delta CT}$.

Data analysis. Luciferase activity per well was measured as relative light units (RLUs). Fold induction was calculated by dividing the mean value of light units from exposed and non-exposed (solvent control) wells. Luciferase induction as a percentage of maximal TCDD activity was calculated by setting the highest fold induction of TCDD at 100%. Data are represented as mean values ± SEM from at least three independent experiments with each experimental point performed in triplicate. Dose-response curves were fitted using the sigmoidal fit $y = a_0 + a_1/(1 + \exp(-(x-a_2)/a_3))$ in GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA), which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). EC50 values were calculated by determining the concentration by which 50 percent of maximum activity was reached using the sigmoidal fit equation.The relative transactivation activity (RTA) of each compound tested was calculated as the ratio of maximal luciferase reporter gene induction values of each compound and the maximal luciferase reporter gene induction value of reference compound TCDD. The transactivation activity of TCDD was arbitrarily set at 100.

Results and Discussion

We previously noticed that the original DR CALUX cell line (containing the pGudLuc reporter construct) besides responding to dioxins and dioxin-like compounds, also responds to glucocorticoids[3]. This is reflected in figure 1A where the glucocorticoid dexamethasone shows a typical dose-response curve, although not as active as TCDD. This activity of dexamethasone in the DR CALUX bioassay could be inhibited by the glucocorticoid receptor (GR) antagonist Ru486 suggesting the involvement of GR (Fig.1A). The response to glucocorticoids might be due to the used reporter construct, since the pGudLuc construct may contain additional responsive elements present in the CYP1A1 and MMTV promoter regions used (e.g. for the GR pathway). To demonstrate this, a second DR CALUX cell line was developed, only containing a minimal DRE construct coupled to luciferase (4xDREtataLuc)[3]. In this cell line, the response to dexamethasone was decreased substantially, confirming the presence of additional responsive elements in the pGudLuc reporter (Fig.1B).

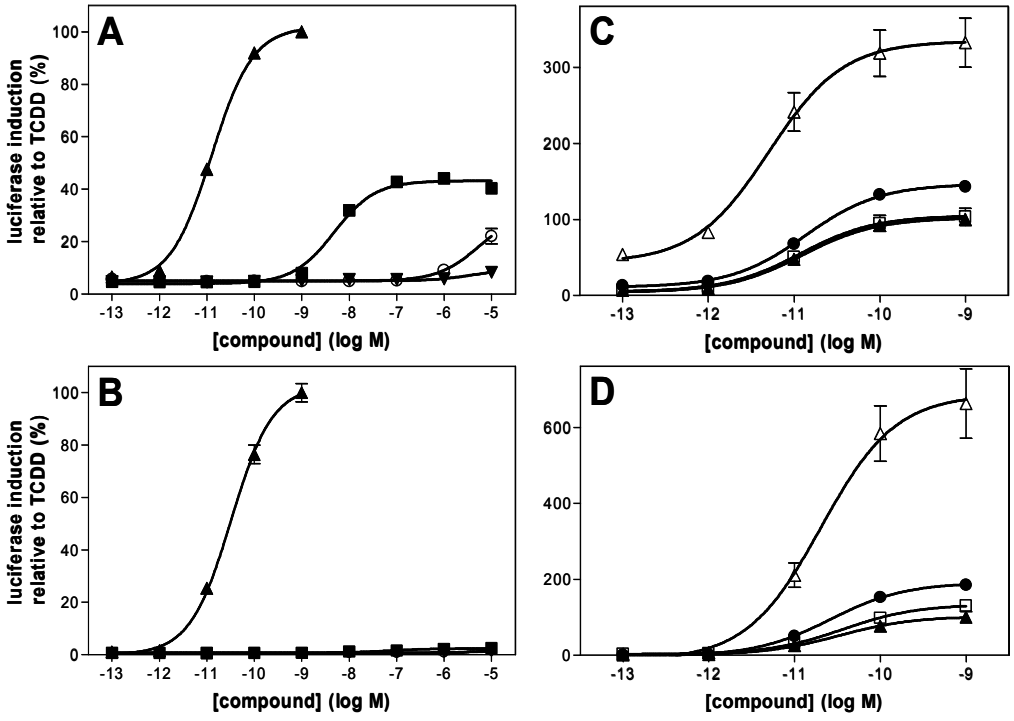

Figure 1. Activity of TCDD and dexamethasone in DR CALUX bioassays. H4IIe-pGudLuc cells (**A** and **C**) and H4IIe-4xDREtataLuc cells (**B** and **D**) were plated in 96-well plates with phenol red-free DF medium supplemented with 5% DCC-FCS. The next day cells were incubated with TCDD (▲), dex (■), Ru486 (▼), dex/ Ru486 (10^{-6}M) (○), TCDD/ dex (10^{-6}M) (Δ), TCDD/ dex (10^{-6}M)/Ru486 (10^{-6}M) (●), or TCDD/Ru486 (10^{-6}M) (□) for 24h. Transcriptional activity is represented as percentage of maximal induction by TCDD. Each data point is the average of at least 3 independent experiments ± S.E.M.

However, dexamethasone also had a synergistic effect on the dioxin response in both types of DR CALUX cell lines (so independent of the dioxin reporter construct used) and this effect could almost completely be inhibited by Ru486 (Fig.1C and 1D). Thus, the effect of dexamethasone itself in the DR CALUX bioassay seems to be caused by the used pGudLuc reporter construct mediated by GR, however the synergistic effects of dexamethasone on the dioxin response are mediated by GR although via another mechanism.

The additive effects of dexamethasone on the dioxin-response as determined in the DR CALUX assay were confirmed by transient transfection experiments using dioxin reporter constructs in H4IIe cells. In these experiments, the additive glucocorticoid effects on the dioxin response could also be inhibited by Ru486 (data not shown). Co-transfection of human as well as rat GR even resulted in enhanced dexamethasone effects, again showing the involvement of GR in this response. Similar results were obtained with mouse Hepa 1c1c7 cells, but not with human HepG2 and T47D cells (although both containing functional dioxin and glucocorticoid signaling) suggesting differences between rodent and human cell types. In addition, EROD experiments (measuring CYP1A1 enzymatic activity) with H4IIe, Hepa 1c1c7, HepG2 and T47D cells again confirmed the additive effects of glucocorticoids on TCDD signaling in rodent cells but not in human cells (data not shown).

Besides DR CALUX, transient transfection and EROD experiments, the additive effects of dexamethasone on the dioxin-response were also confirmed by real-time PCR on endogenous TCDD target genes (CYP1A1, CYP1A2 and CYP1B1), in wildtype rat H4IIe (Fig.2A). Interestingly, these effects were not observed in in dioxin-responsive human HepG2 (Fig.2B) and T47D cells (data not shown) again indicating differences between rodent and human cell lines.

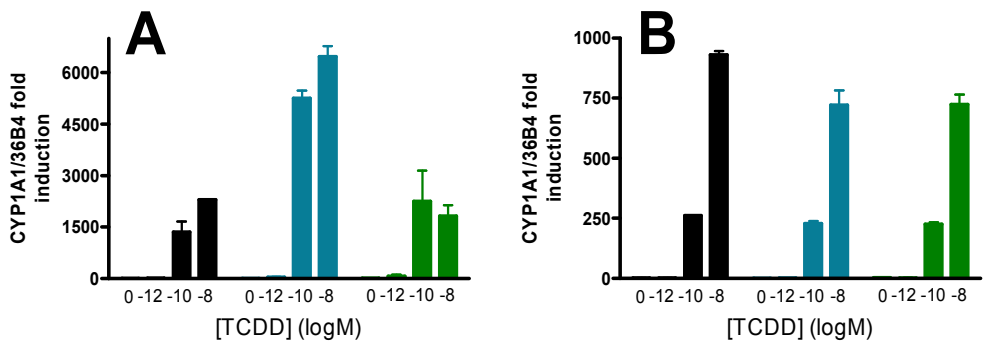

Figure 2. CYP1A1 mRNA induction by dioxin and increased expression of this gene by dexamethasone in rat H4IIe cells but not in human HepG2 cells. Rat H4IIe (**A**) and human HepG2 (**B**) cells were treated with increasing concentrations of TCDD (black bars), TCDD in combination with dexamethasone (10^{-6}M) (blue bars) or TCDD in combination with dexamethasone and Ru486 (both 10^{-6} M) (green bars). mRNA was isolated after 24h and cDNA was prepared by reversed transcription. Induction of CYP1A1 mRNA was determined by real-time PCR. Expression of the acidic ribosomal phosphoprotein P0 (36B4) was used for normalization. Each data point is the average of at least 3 independent experiments ± S.E.M.

In summary, the response of the glucocorticoid dexamethasone alone or in combination with TCDD in the DR CALUX bioassay is mediated by GR. This response of dexamethasone alone depends on the promoter context, while the additional dexamethasone effect on the TCDD response is independent of promoter

context. The observed effects of glucocorticoids on dioxin signal transduction seem to be mediated by GR in rodent cells (rat H4IIe cells and mouse Hepa1 1c1c7 cells), but not in human cells (HepG2 and T47D cells). The interactions of signal transduction pathways by dioxins and glucocorticoids may provide explanations for known interactions between stress-related pathways and dioxin effects. Although glucocorticoids were shown to be efficiently removed from matrices via clean-up procedures in the dioxin sample work-up in standard dioxin analysis with DR CALUX (as examined with the GR CALUX bioassay[4] measuring glucocorticoidal activity; data not shown), the interference of glucocorticoids in the DR CALUX bioassay argues the need to generate a human cell-based dioxin reporter cell line for specific applications (e.g in those in which non-standard work-up procedures are preferred).

References

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