Glucocorticoid-enhanced expression of dioxin target genes through regulation of the rat aryl hydrocarbon receptor
Sonneveld, E.; Jonas, A.J.G.; Meijer, O.C.; Brouwer, A.; van der Burg, B.

published in
Toxicological Sciences
2007

DOI (link to publisher)
10.1093/toxsci/kfm176

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl
Glucocorticoid-Enhanced Expression of Dioxin Target Genes through Regulation of the Rat Aryl Hydrocarbon Receptor

Edwin Sonneveld,*,1 Arjen Jonas,*, Onno C. Meijer,† Abraham Brouwer,* and Bart van der Burg*

*BioDetection Systems B.V., Amsterdam, and †Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research and Leiden University Medical Center, the Netherlands

Received May 2, 2007; accepted July 3, 2007

The aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR) are ligand-activated transcription factors and members of the basic helix–loop–helix Period-aryl hydrocarbon nuclear translocator-single minded and nuclear hormone receptor superfamilies, respectively. Besides their individual role as activators of specific gene transcription, also interplay between both transcription factors can be an important mechanism of regulation. In this study, we report that GR can strongly activate AhR-mediated transcription and consequent gene expression in rat H4IIE cells. Reporter gene assays showed an enhanced effect of dexamethasone on the dioxin response mediated by GR in rat H4IIE cells and mouse Hepa 1c1c7 cells, but not in human HepG2 cells and human T47D cells. These deviations between the rodent and human cell lines were confirmed by CYP1A1 enzyme activities. In addition, quantitative reverse transcription–PCR showed enhanced GR-mediated effects of dexamethasone on endogenous 2,3,7,8-tetrachlorodibenzo- p-dioxin target genes as well in rat H4IIE cells, but not in human HepG2 and human T47D cells. Surprisingly, AhR itself was upregulated by combined dioxin/glucocorticoid exposure in rat H4IIE cells but not in the human cells which could be explained by the presence of two putative glucocorticoid response elements in the rat AhR promoter, but not in the human AhR promoter. This GR-mediated expression of dioxin target genes through upregulation of the AhR in rat but not in human cells opens the possibility that dioxin responses in rodent-based models for toxicity differ from humans and provides new insight into the interactions of stress-related pathways, biological effects of dioxin-like compounds and may possibly have implications for risk assessment.

Key Words: aryl hydrocarbon receptor; CALUX; dioxin; glucocorticoid receptor; glucocorticoids.

Polyhalogenated aromatic hydrocarbons (PHAHs) including dioxins and dioxin-like polychlorinated biphenyls (PCBs) induce a wide variety of effects in mammals, birds, and fish such as immunotoxicity, carcinogenicity, and metabolic changes. These compounds bind to an intracellular receptor, known as the aryl hydrocarbon receptor (AhR). AhR is a member of the basic helix–loop–helix Period-ARNT (aryl hydrocarbon nuclear translocator)–single minded family of transcription factors that control a variety of developmental and physiological events, including neurogenesis, toxin metabolism, circadian rhythms, hypoxia response, and steroid hormone receptor function (Gu et al., 2000). Ligand binding to the AhR is presumed to produce conformational changes in the AhR protein followed by translocation of the PHAH–AhR complex from the cytoplasm into the nucleus of the cell (Pollenz et al., 1994) and dimerization with its nuclear partner ARNT (Safe, 1995). The AhR–ARNT heterodimer forms an active transcription factor which binds with high affinity to defined DNA sequences called xenobiotic responsive elements (XREs) (Denison et al., 1988). Binding of the AhR–ARNT dimer to the XRE triggers the transcription of XRE-associated genes, such as the drug metabolizing monoxygenases cytochrome P4501A1 (CYP1A1), CYP1A2, and CYP1B1 (enzymes participating in biotransformation phase I) (Hankinson, 1995) as well as phase II enzymes like glutathione-S-transferase (GST) and nicotinamide adenine dinucleotide phosphate, reduced (NADPH) quinone oxiireductase (Rushmore and Kong, 2002). Although its physiological role is unknown, the AhR mediates most, if not all, of the toxic effects of 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD) (Lahvis and Bradfield, 1998).

Glucocorticoids are steroid hormones which are important regulators of gluconeogenesis, cellular proliferation and differentiation, and inhibition of inflammation. The effects of glucocorticoids in target cells are mediated by the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily including receptors for steroids, retinoids, and thyroid hormones (McKenna and O’ Malley, 2002). GR is a ligand-dependent transcription factor which acts as a homodimer that regulates specific gene expression by binding to specific glucocorticoid response elements (GREs) within the regulatory DNA sequences of many glucocorticoid-responsive genes, referred to as type 1 mechanism of GR action (Bamberger et al., 1996). Besides direct transcriptional regulation of specific target genes by GR, also protein–protein interactions with transcription factors of distinct families (in the absence of

1 To whom correspondence should be addressed at BioDetection Systems B.V., Kruislaan 406, 1098 SM Amsterdam, the Netherlands. Fax: +31-20-43-50-757. E-mail: edwin.sonneveld@bds.nl.

© The Author 2007. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org
specific DNA binding; type 2 mechanism of GR action) have been described such as activating protein-1, nuclear factor κB (NF-κB), estrogen receptor (ER), and thyroid hormone receptor (Bamberger et al., 1996). Usually, this type 2 GR action results in inhibition rather than activation of target genes and is especially relevant for the tissue specific anti-inflammatory and immunosuppressive effects of glucocorticoids (Dumont et al., 1998).

Besides the effect of AhR as an activator of gene transcription, crosstalk has been observed among AhR and other nuclear receptors, which has been most well studied with respect to ER signaling (Matthews and Gustafsson, 2006). Activated AhR inhibits ER activity through a number of different mechanisms (Harper et al., 1994; Kharat and Saatcioglu, 1996; Rogers and Denison, 2002), whereas ERs has been reported to have a positive role in AhR signaling (Ohtake et al., 2003). In addition, the progesterone receptor negatively interferes with AhR-mediated transcription (Kuil et al., 1998), while mutual interference between the AhR and the androgen receptor (Jana et al., 1999), but also between the AhR and NF-kB has been reported to be positive role in AhR signaling (Ohtake et al., 2003). Crosstalk between glucocorticoids and the dioxin pathway also exists, reported as dexamethasone-mediated potentiation of CYP1A1 induction and 7-ethoxyresorufine-O-deethylase (EROD) activities in rat hepatoma H4IIE cells (Lai et al., 2004; Wiebel and Cikryt, 1990), fetal rat hepatocytes (Mathis et al., 1989; Sherratt et al., 1990), adult rat hepatocytes (Pinaire et al., 2004; Xiao et al., 1995), and interaction between TCDD and glucocorticoids in embryonic palate (Abott, 1995) and livers of adrenalectomized- or neonatal rat models (Linder et al., 1999). So far, the mechanism behind this crosstalk between dioxins and glucocorticoids is very unclear.

Recently, it was shown that glucocorticoids have a positive response in the dioxin-responsive Chemically Activated Luciferase eXpression (DR CALUX) bioassay, and were able to enhance the response obtained with TCDD (Hoogenboom et al., 1999). The goal of this study is to determine to what extent glucocorticoids interfere with dioxin signaling and what the mechanism(s) of glucocorticoid-enhanced dioxin signaling is. Furthermore, large differences in TCDD susceptibility within and between species exist (Hengstler et al., 1999; Karchner et al., 2006; Poland et al., 1994) believed to be partly due to ligand affinity differences between polymorphic AhRs. Therefore, we have studied in detail how rodent and human cell lines deviate in their responses to TCDD and how the interaction of glucocorticoids influences this. This may provide additional insight in the known interactions of stress-related pathways and biological effects of dioxin-like compounds and the known inter- and intraspecies differences in dioxin signaling.

MATERIALS AND METHODS

Chemicals. Dexamethasone (dex), cortisol, cycloheximide, and mifepristone (RU486) were obtained from Sigma-Aldrich ( Zwijndrecht, The Netherlands). TCDD was purchased from Cambridge Isotope Laboratories (Anover, MA). All chemicals were diluted in either ethanol or dimethylsulfoxide (DMSO; Acros, Geel, Belgium) and stored at –20°C, except for TCDD which was stored at room temperature. Neomycin (G418) was purchased from Life Technologies (Breda, The Netherlands).

DNA constructs. Full-length complementary DNA (cDNA) for human GR (ATCC, Manassas, VA) was inserted in the multiple cloning site (MCS) of the expression vector pSG5-neo to obtain pSG5-neo-GR (Sonneveld et al., 2005). The GudLuc1.1 dioxin reporter gene construct has been described elsewhere (Garrison et al., 1996). The dioxin reporter gene pDREtataLuc was constructed as follows: four tandem repeats of a DRE (dioxin-responsive element) oligo (based on the –985 to –979 DRE sequence of the rat CYP1A1 promoter) upstream of the minimal adenovirus E1B TATA promoter sequence was inserted in the MCS of the enhanced luciferase reporter gene construct pGL3-basic. The glucocorticoid reporter construct pMMTVLuc was described earlier (Sonneveld et al., 2005).

Cell culture. Human T47D breast cancer cells, human HepG2 cells, mouse Hep1 1clc7 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 (FCS), DR 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 cell culture plates. After culturing for one day, cells were transfected with 1 μg reporter plasmid (pGudLuc 1.1 or pMMTVLuc), 200 ng SV2-lacz, and 200 ng expression plasmid pSG5-neo-lgCR or empty vector DNA (pSG5-neo) using the calcium phosphate coprecipitation method. Luciferase activity was corrected for transfection efficiency by measuring β-galactosidase activity as a result of SV2-lacz cotransfection.

Establishment of stable DR CALUX cell lines. Generation of the H4IIe-pGudLuc cell line has previously been described (Garrison et al., 1996). Stable H4IIe-pDREtataLuc cells were generated as follows: H4IIe cells were transfected with pDREtataLuc and pSG5-neo using calcium phosphate coprecipitation to generate DR CALUX cells. Neomycin-resistant clones were tested for their response to TCDD and five clones showed consequent high response. One of these responding to the lowest concentration of TCDD (10pM) was selected for further investigation.

CALUX bioassays. DR CALUX cells (H4IIe-pGudLuc and H4IIe-pDREtataLuc stable cell lines) were plated in 96-well cell culture plates with phenol red-free DF medium supplemented with 5% dextran coated charcoal stripped FCS (DCS-FCS; Sonneveld et al., 2005) at a volume of 200 μl per well. The next day, the medium was refreshed and cells were incubated with the compounds to be tested (dissolved in ethanol or DMSO) in triplicate at a 1:1000 dilution. After 24 h the medium was removed, cells were lysed in 30 μl of Triton-lysis buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min per well.

EROD bioassay. The Micro-EROD bioassay was performed as described earlier (Behnisch et al., 2002) with minor modifications. Briefly, cells were seeded into 96-well cell culture plates with phenol red-free DF medium supplemented with 5% DCC-FCS. The next day, the medium was refreshed and cells were incubated with the compounds to be tested (dissolved in ethanol or DMSO) in triplicate at a 1:1000 dilution. After 24 h the medium was removed, cells were washed twice with 0.5× PBS and lysed in 30 μl of water. Plates were frozen at –80°C for minimally 20 min. After thawing, 30 μl of dicumarol buffer (40μM dicumarol in Tris buffer pH 8; Sigma-Aldrich) and 25 μl of 7-ethoxyresorufin (20μg in dicumarol buffer; Sigma-Aldrich) were added to the wells. After incubation at 37°C for 20 min, 25 μl of NADPH (1mM in dicumarol buffer) was added and mixed. Plates were incubated at 37°C for 1 h and resorufin-associated fluorescence was measured at 530-nm excitation and 590-nm emission using a fluorescence plate reader (Packard fluorocount, model BF10001). Measured values were corrected for the protein content per well.
**Quantitative reverse transcription–PCR.** Total RNA was isolated using nucleospin RNAII columns (Macherey-Nagel, Düren, Germany). cDNA was prepared with the iScript cDNA synthesis 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 AhR, CYP1A1, CYP1A2, CYP1B1, NMOR, GST-A1, HFH1, SGK, FKBP51, luciferase (from the pGL2 vector), and RBPO (36B4; internal control) using Beacon designer 4.01 (PREMIER Biosoft International, Palo Alto, CA). Table 1 shows the sequences of the sense and antisense primers, amplicon lengths, and accession numbers of the analyzed genes. Threshold Ct values for each cDNA were determined and real-time gene expression analysis was performed using the Livak method (Livak and Schmittgen, 2001). First the Ct of the target gene was normalized to that of the reference gene (RBPO), for both the test sample and the calibrator sample (DMSO treated): \( \Delta \text{C}_{\text{T(target, test)}} = \text{C}_{\text{T(target, test)}} - \text{C}_{\text{T(test, calibrator)}} \) and \( \Delta \text{C}_{\text{T(calibrator)}} = \text{C}_{\text{T(target, calibrator)}} - \text{C}_{\text{T(test, calibrator)}} \). Second, the \( \Delta \text{CT} \) of the test sample was normalized to the \( \Delta \text{CT} \) of the calibrator: \( \Delta \text{ACT} = \Delta \text{C}_{\text{T(target, test)}} - \Delta \text{C}_{\text{T(calibrator)}} \). Finally, the normalized expression ratio (fold induction) was calculated according to the following equation: fold induction = \( 2^{-\Delta \text{ACT}} \).

**Identification of GREs and XREs in orthologous Ahr genes.** Ten kilobases of the regions upstream of the first exons, and introns 1 and 2 of rat, mouse, and human Ahr genes were downloaded from ensembl (http://www.ensembl.org) GRE sequences can show significant deviation from the canonical AGACA-nnn-TGTTCT palindromic consensus, particularly in the 5' half site (Nelson et al., 1999). To find potential GREs that are conserved between species we took a very liberal definition based on the anchoring nucleotides at positions 2 and 5 of each half site that are present in most genes used in this study including gene accession numbers and amplicon lengths (bp).

**Results**

**Glucocorticoids Enhance the TCDD Response in Stable DR CALUX Reporter Cell Lines**

Glucocorticoid-mediated potentiation of CYP1A1 messenger RNA (mRNA) induction (Lai et al., 2004) and CYP1A1 enzymatic activity (Lai et al., 2004; Wiebel and Cikryt, 1990)

**TABLE 1**

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene*</th>
<th>Length (bp)</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_012540</td>
<td>rCyp1A1</td>
<td>124</td>
<td>GCTTCACACTTATCGCTAATG</td>
<td>GTGGGTCTCAGAGCTATGG</td>
</tr>
<tr>
<td>NM_012541</td>
<td>rCyp1A2</td>
<td>207</td>
<td>TGTTCTTCTACCATCCCC</td>
<td>GCACATCATTTCTACCTAG</td>
</tr>
<tr>
<td>NM_012940</td>
<td>rCyp1B1</td>
<td>183</td>
<td>TGGCTGGCATTTAGAGACATC</td>
<td>CGATGTGAAATGGTTGTCGACAC</td>
</tr>
<tr>
<td>NM_013149</td>
<td>rAhr</td>
<td>179</td>
<td>AAACAAACACGAGGTTAAACCT</td>
<td>GTGGCCTCTGAACTGCTAG</td>
</tr>
<tr>
<td>K00136</td>
<td>rGstYa</td>
<td>177</td>
<td>ATGCTGCTGCTGCTGCTGCTG</td>
<td>AGGCGATCTCTACAGGCTACAG</td>
</tr>
<tr>
<td>AF153193</td>
<td>rHfB1</td>
<td>152</td>
<td>CGAGATTCACTCAGGGGAAGG</td>
<td>AGCATGCTGGATGAGCAG</td>
</tr>
<tr>
<td>J0608</td>
<td>rNmor</td>
<td>170</td>
<td>GTATGCACACTTGTAGCACAGAAG</td>
<td>AGACCTGAGAACACAGAAAG</td>
</tr>
<tr>
<td>NM_019232</td>
<td>rSgk</td>
<td>139</td>
<td>TGAAGTATCCTGCTGAGTTC</td>
<td>GCTGATATCCTCAGGCTACAG</td>
</tr>
<tr>
<td>NM_001012174</td>
<td>rFkbp5</td>
<td>208</td>
<td>GAGCAGAAAGAAGCAGGAG</td>
<td>GCTGAGGATCAGAGGACAG</td>
</tr>
<tr>
<td>X15906</td>
<td>rRplp0</td>
<td>163</td>
<td>CATCCTCCCCCTGGGTAGG</td>
<td>GCTTCCAGCAATCACAGAG</td>
</tr>
<tr>
<td>NM_000499</td>
<td>bCyp1A1</td>
<td>171</td>
<td>CTGGCATGCTGCAAAGATG</td>
<td>ATTACTGAGGTTAGGGAAG</td>
</tr>
<tr>
<td>NM_000761</td>
<td>bCyp1A2</td>
<td>169</td>
<td>TCAGGTTGTGTTCTGAGTGG</td>
<td>AGGGAGTCTAGGAGAAG</td>
</tr>
<tr>
<td>NM_000104</td>
<td>bCyp1B1</td>
<td>193</td>
<td>AACCGAACATCTGCAAC</td>
<td>CTGGAGTCTAGTATGAGT</td>
</tr>
<tr>
<td>NM_001621</td>
<td>bAhr</td>
<td>163</td>
<td>CCAATCACACTCTAGGCAGT</td>
<td>ATACCTGTTGGAAAAGCAGCAG</td>
</tr>
<tr>
<td>NM_145740</td>
<td>bGstA1</td>
<td>174</td>
<td>ATCTTCTTCTGCGCTGTGTCG</td>
<td>AGTTCTCTACAGGTTAGCAG</td>
</tr>
<tr>
<td>AF225950</td>
<td>bhf1</td>
<td>150</td>
<td>AGTACCTTACCTGGAGCTT</td>
<td>AGTTCTCTACAGGTTAGCAG</td>
</tr>
<tr>
<td>J03954</td>
<td>bNmor</td>
<td>195</td>
<td>ACCCTCGGAACTTCTGTAGTC</td>
<td>AGGCAAATCTTCTATGACAG</td>
</tr>
<tr>
<td>NM_005627</td>
<td>hSgk</td>
<td>119</td>
<td>AGGAGAAATGGAAACAACAGC</td>
<td>TCCACAGGGCAAGACAG</td>
</tr>
<tr>
<td>U71321</td>
<td>hFkbp5</td>
<td>225</td>
<td>TTGAGAAGAGGTCCTGAGAATC</td>
<td>CATCTGCTGACTGCTGTC</td>
</tr>
<tr>
<td>NM_001002</td>
<td>hRplp0</td>
<td>198</td>
<td>GGCAGCATCACCACCTGGTAGT</td>
<td>AGTTGAGAAATGGTAAG</td>
</tr>
</tbody>
</table>

Notes. Oligonucleotide primers (5'–3') of genes used in this study including gene names and amplicon lengths (bp).

*Abbreviations: r, rat; h, human; Cyp1A1, cytochrome P450 1A1; Cyp1A2, cytochrome P450 1A2; Cyp1B1, cytochrome P450 1B1; GstA1, glutathione-S-transferase Ya; GstA1, glutathione-S-transferase A1; HfB1, winged helix/forkhead transcription factor-1; Nmor, NAD(P)H:menadione oxidoreductase; Fkbp5, FK506 binding protein 5; Fkbp5, FK506 binding protein 51; Rplp0, acidic ribosomal phosphoprotein P0.
in TCDD or BaP-treated rat H4IIe hepatoma cells has been demonstrated. This phenomenon was also noticed by using the DR CALUX reporter gene assay (rat H4IIe cells containing the dioxin-responsive pGudLuc reporter construct), which besides responding to dioxins and dioxin-like compounds, was reported to respond to glucocorticoids (Hoogenboom et al., 1999). To study the mechanism of glucocorticoid/dioxin interaction in more detail, two DR CALUX cell lines were used (Fig. 1): the original DR CALUX cell line stably containing the pGudLuc construct (Fig. 1A), which besides the DREs may contain additional responsive elements from the CYP1A1 and/or MMTV-long terminal repeat (LTR) sequences (e.g., for the GR pathway), and a second H4IIe cell line only containing a minimal promoter construct with four multimerized DREs in front of the TATA promoter coupled to luciferase (pDREtataLuc) (Fig. 1A). Such a minimal promoter has the advantage that regulation through promoter elements other than DREs (such as GREs) is avoided and optimal selectivity for AhR interacting molecules is obtained. Typical TCDD dose–response curves for both CALUX cell lines are shown in Figure 1B, with EC50 values of 11 and 44 pM for H4IIe-pGudLuc and H4IIe-pDREtataLuc, respectively. Although the EC50 values differ around four times between both CALUX cell lines, the response to TCDD in the lower picomolar range is more similar for both lines. The glucocorticoid response of the H4IIe-pGudLuc cell line is reflected in Figure 2A where dexamethasone itself (and cortisol, data not shown) shows a dose–response curve, although not as active as TCDD (EC50 of 5 nM, reaching around 40% of maximal TCDD activity at 100 nM) raising the possibility that dexamethasone can transactivate the AhR. However, the activity of dexamethasone in the DR CALUX reporter gene assay occurs at physiological GR-activating levels, and can be inhibited by the GR antagonist Ru486 suggesting the involvement of GR instead of AhR (Fig. 2A). The response to glucocorticoids might be due to GRE-like sequences within the pGudLuc reporter construct. Using the H4IIe-pDREtataLuc cell line, containing a minimal reporter construct, the response to dexamethasone (and cortisol, data not shown) was decreased substantially (Fig. 2B). Interestingly, besides the activity of dexamethasone itself on the pGudLuc reporter construct, dexamethasone also had an enhanced effect on the dioxin response in both types of DR CALUX reporter gene cell lines and this effect could almost completely be inhibited by Ru486.

**FIG. 1.** Schematic representation of dioxin reporter constructs present in DR CALUX reporter cell lines their response to TCDD. (A) The pGudLuc 1.1 construct contains a 484-bp fragment from the upstream region of the mouse CYP1A1 gene, including four DREs, within the MMTV-LTR inserted in the MCS of the luciferase vector pGL2. The pDREtataLuc construct contains four multimerized rat DREs upstream of a minimal adenovirus E1B TATA promoter inserted in the MCS of the luciferase vector pGL3. (B) H4IIe-pGudLuc cells (open circle) and H4IIe-pDREtataLuc cells (closed square) 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 for 24 h. Transcriptional activity is represented as percentage of maximal induction by TCDD. Each data point is the average of at least three independent experiments ± SEM.
The effect of dexamethasone on the dioxin response observed in H4IIe cells with DR CALUX reporter gene assays suggests the involvement of GR although via a mechanism independent of the reporter construct used.

Species-Specific Enhancement of Dioxin Signaling by Glucocorticoids

To study if the enhancing effects of glucocorticoids on the dioxin response as determined with DR CALUX reporter cell lines are a general phenomenon, we performed transient transfection experiments with a dioxin reporter construct in rat H4IIe cells and mouse Hepa 1c1c7 cells (Fig. 3). In H4IIe cells, TCDD could induce the dioxin reporter construct pGudLuc in a dose-dependent fashion (fourfold induction at 10pM TCDD and 12-fold induction at 1nM TCDD), while cotreatment with 10nM dexamethasone resulted in highly increased induction levels (63-fold induction at 10pM TCDD and 84-fold induction at 1nM TCDD; Fig. 3A, left panel), comparable with earlier CALUX results. Again, dexamethasone itself (10nM) could also induce the pGudLuc reporter construct (eightfold). Cotransfection of human GR (Fig. 3A; right panel) as well as rat GR (data not shown) even resulted in enhanced dexamethasone effects, again showing the involvement of GR in this response. The interference was not mutual, as TCDD did not influence glucocorticoid-induced promoter activity on a pMMTVluc reporter construct (data not shown). Similar results were obtained with mouse Hepa 1c1c7 cells, which contain a functional dioxin signaling pathway (Miller et al., 1983) (Fig. 3B). In all these experiments with H4IIe cells and Hepa 1c1c7 cells, the enhanced glucocorticoid effects on the dioxin response could also be inhibited by Ru486 (data not shown), suggesting that GR is responsible for these effects. To test if the observed effects in rodent cell lines can be extended to human cells, we also tested a panel of human cell lines for the effect of dexamethasone on the dioxin response. Human HepG2 cells (Fig. 3C) and T47D cells (Fig. 3D) showed a dose-dependent response to TCDD, although they were not as sensitive as the rodent cell lines (reviewed by Harper et al., 2006), and showed hardly induction with 10pM TCDD. Surprisingly, cotreatment with dexamethasone (10nM) did not result in an enhanced TCDD response, although all human cell lines tested contain functional dioxin (Figs. 3A–D) and glucocorticoid signaling pathways (Figs. 4A–D). Cotransfection with human or rat GR also did not result in the dexamethasone effects observed with H4IIe cells and Hepa 1c1c7 cells, showing that GR was not limiting in these human cell lines and the property to enhance dioxin signaling was not depending on the presence of a rodent GR (Figs. 3C and 3D).
right panels). As a control for functional glucocorticoid signaling, we studied the effect of dexamethasone on the GR reporter construct MMTV-luc. In all four cell types this reporter construct could be induced by moderate concentrations of dexamethasone (although with differences in fold induction), and these inductions could be inhibited by Ru486 indicating GR-mediated effects (Fig. 4).

To confirm the functional significance of the enhanced reporter gene induction upon cotreatment with glucocorticoids, induction of endogenous CYP1A1 enzyme activity was determined. EROD experiments were performed with wildtype rat H4IIe cells, measuring CYP1A1 enzyme activity (Fig. 5). A clear dose-dependent response to TCDD (by means of increased CYP1A1 enzyme activity) was observed for H4IIe cells (Fig. 5A) comparable with earlier reported results (Behnisch et al., 2002). Dexamethasone could enhance this TCDD response as reporter earlier (Lai et al., 2004), which could be inhibited completely by Ru486 treatment. Furthermore, dexamethasone itself was not able to increase CYP1A1 enzyme activity (Fig. 5B). In the human HepG2 cells (Fig. 5C) and human T47D cells (Fig. 5D), dexamethasone could not enhance the TCDD-induced CYP1A1 enzyme activity, confirming earlier results obtained with transient transfection experiments (Fig. 3). Again, both human cell types showed a 10 times decreased response to TCDD compared to the rodent cell lines. This decreased response was also observed in the transient transfection experiments (Fig. 3). In summary, these EROD experiments confirmed the enhancing effects of glucocorticoids on TCDD signaling in rodent cells but not in human cells. The results obtained with the EROD bioassay are comparable with the results obtained with stable DR CALUX cell lines and transient transfection experiments.

Enhancement of TCDD-Induced AhR Target Genes by Dexamethasone in Rat Cells, but not in Human Cells

To study the mechanism of glucocorticoid/dioxin interaction in H4IIe cells in more detail, the effects of dexamethasone on the dioxin-response were studied by quantitative reverse
transcription–PCR (RT-PCR) analysis on endogenous AhR target genes. In addition, to test if the observed effects in the rat H4IIe cell line can be extended to human, we also tested a panel of human cell lines (HepG2 cells and T47D cells) for the effect of dexamethasone on the dioxin response. The genes tested were well known AhR regulated genes (CYP1A1, CYP1A2, CYP1B1, GST1A, NMOR; Table 1), but also the HFH1 gene, identified as a high TCDD-responding gene in a DNA array experiment using TCDD exposed H4IIe cells (data not shown) (Table 2). Figure 6 shows an example of the regulation of CYP1A1 by TCDD in all three cell lines. A clear dose-dependent induction of the CYP1A1 gene by TCDD is observed in H4IIe cells (Fig. 6A), even at concentrations as low as 1pM (Table 2). Dexamethasone could strongly enhance the CYP1A1 induction by TCDD in these cells (from 1.4 thousand times induction with 0.1nM TCDD alone up to 5.3 thousand times induction with a combination of 0.1nM TCDD and 10nM dexamethasone; Table 2), even without TCDD (four times induction over solvent control; Table 2). These effects were mediated by GR, since antagonizing GR with Ru486 (1μM) resulted in decreased CYP1A1 expression resembling expression levels as for TCDD alone (Fig. 6A). Besides CYP1A1, these effects in H4IIe cells were also observed for CYP1A2, CYP1B1, GST1Ya, NMOR, and HFH1 (Table 2). Interestingly, these effects were not observed in dioxin-responsive human HepG2 cells (Fig. 6B) and human T47D cells (Fig. 6C) although the tested genes responded to TCDD alone (except for GST1A and HFH1). These results indicate that dexamethasone increases TCDD-induction of endogenous AhR target genes via a GR-dependent mechanism in rat cells, but not in human cells. As a control for functional GR, we tested the regulation of the GR target genes serum/glucocorticoid regulated kinase (SGK) and FK506 binding protein 51 (rat FKBP5 and human FKBP51) in H4IIe cells, HepG2 cells, and T47D cells. In H4IIe cells and T47D cells, the SGK gene was inducible by dexamethasone in a dose-dependent fashion (Figs. 6A and 6C, right panels), while TCDD had no effect on GSK expression (not by itself, nor in addition to dexamethasone; Table 2). The GR antagonist Ru486 could completely block the dexamethasone-induced expression of SGK, indicating the involvement of GR. In HepG2 this gene was not induced by dexamethasone (Fig. 6B and Table 2) although RNA transcripts were present (data not shown). The FKBP51 gene was induced by dexamethasone in both HepG2 cells and T47D cells, which could be blocked by Ru486 cotreatment (Table 2). This gene was not inducible by dexamethasone in rat H4IIe cells (Table 2). These data show that in all cell lines tested functional GR signaling is present and that the absence

![Figure 4](https://example.com/figure4.png)

**FIG. 4.** Functional GR signaling in rat, mouse, and human cells. H4IIe cells (A), Hepa1 1c1c7 cells (B), HepG2 cells (C), and T47D cells (D) were transiently transfected with pMMTVLuc reporter construct and hGR expression construct and treated with dexamethasone (open bars) or dexamethasone in combination with Ru486 (1μM) (black bars) for 24 h. Fold induction indicates reporter activity in cells treated over solvent treated cells. Each bar represents the mean of three independent experiments ± SEM.
potentiating effect of dexamethasone on the dioxin response in the human cell lines is not due to absent GR.

Mechanism of Glucocorticoid Enhancement of TCDD Signaling: Upregulation of AhR in Rat Cells but not in Human Cells

Since the rat CYP1A1 promoter contains GREs in the first intron (Linder et al., 1999; Xiao et al., 1995), this might explain the enhancing effect of dexamethasone on this TCDD target gene. However, since dexamethasone enhanced the expression of all tested TCDD target genes in H4IIE cells but not in human cells (Table 2), GRE dependency seems questionable. Upregulation of a master gene upstream all these TCDD target genes seem more likely. One candidate gene might be AhR itself. To test this hypothesis, we studied the expression of AhR in rat H4IIE cells by quantitative RT-PCR (Fig. 7). Expression of this gene was induced by TCDD (threelfold), while dexamethasone enhanced this TCDD-induced expression up to 19-fold. Note that dexamethasone itself was also able to induce AhR (threelfold; Fig. 7A and Table 2). This TCDD-induced expression of AhR by dexamethasone was GR dependent, since Ru486 could inhibit this enhancement. The human AhR was not upregulated by TCDD and TCDD/dexamethasone treatments in HepG2 cells (Fig. 7B) and T47D cells (Fig. 7C), explaining the observed differences between the rat cells and the human cells obtained within all previous assays.

A recent study (Lai et al., 2004) reported that the enhancing effects of dexamethasone on TCDD-induced CYP1A1 expression in rat H4IIE cells depend on a posttranscriptional process (requirement of protein synthesis), although the observed effects were not very clear. We used stable H4IIE-pGudLuc cells to study not only the effects of protein synthesis inhibition by cycloheximide on endogenous CYP1A1 and AhR expression, but also on the exogenous luciferase gene. For this reason an early time point of exposure was required. Figure 8A (left panel) shows that even at 6 h exposure, a clear dose-dependent induction of the CYP1A1 gene by TCDD is observed in H4IIE-pGudLuc cells, even at concentrations as low as 1pM (fourfold induction) as observed for wildtype H4IIE cells at 24 h of treatment (Table 2). Also at 6-h exposure, dexamethasone could enhance the CYP1A1 induction by TCDD in these cells: from 2.8 thousand times induction with 0.1nM TCDD alone up

FIG. 5. Dexamethasone enhances the TCDD-induced EROD response in rat and mouse cells but not in human cells. H4IIE cells (A), Hepa1 1c1c7 cells (B), HepG2 cells (C), and T47D cells (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 (closed square), TCDD and dex (10nM) (closed inverted triangle), TCDD and dex (10nM) and Ru486 (1µM) (open square), and dex (closed triangle) for 24 h. EROD activity is represented as percentage of maximal EROD activity by TCDD. Each data point is the average of at least three independent experiments ± SEM.
to 4.8 thousand times induction with a combination of 0.1nM TCDD and 10nM dexamethasone. These effects were mediated by GR, since antagonizing GR with Ru486 (1 nM) resulted in decreased CYP1A1 expression resembling expression levels as for TCDD alone (data not shown). Similar results were obtained for the exogenous luciferase gene which is under control of a 484-bp fragment from the upstream region of the mouse CYP1A1 gene, including four DREs, although levels of induction were not as high as for the endogenous rat CYP1A1 gene (Fig. 8B; left panel). Cotreatment with cycloheximide resulted in over 10-fold higher induction levels of CYP1A1 and luciferase genes due to mRNA accumulation, as observed earlier for CYP1A1 (Lai et al., 2004). Furthermore, the enhanced TCDD-induced expression of both CYP1A1 and luciferase by dexamethasone was not observed after cycloheximide treatment (Figs. 8A and 8B; right panels), indicating that preceding protein synthesis (such as AhR protein synthesis) is essential for the enhancing effects of dexamethasone indicating that preceding protein synthesis (such as AhR protein synthesis) is essential for the enhancing effects of dexamethasone.
induced twofold by both TCDD and dexamethasone individually (Fig. 8C; left panel). Combined exposure of TCDD and dexamethasone did not enhance the expression of AhR after 6 h, in contrast to 24 h of exposure (see Fig. 7A). Cotreatment with cycloheximide did not result in reduction of the enhanced effect of dexamethasone as observed for TCDD-induced CYP1A1 and luciferase (Fig. 8C; right panel); it even resulted in enhanced AhR induction. This enhancement could be blocked completely by RU486, suggesting the involvement of GR (data not shown). These results indicate that synthesis of a protein such as AhR is essential for the dexamethasone-mediated enhancement of TCDD-induced genes.

FIG. 6. Dexamethasone enhances the expression of the dioxin target gene CYP1A1 in rat cells but not in human cells. H4IIE cells (A), HepG2 cells (B), and T47D cells (C) were treated with increasing concentrations of TCDD (open bars, left panels), TCDD in combination with dexamethasone (10 nM) (black bars, left panels), TCDD in combination with dexamethasone (10 nM) and RU486 (1 μM) (hatched bars, left panels), or increasing concentrations of dexamethasone (black bars, right panels). Total RNA was isolated after 24 h and cDNA was prepared by reversed transcription. Induction of CYP1A1 (left panels) and SGK (right panels) mRNA was determined by real-time quantitative PCR. Expression of the acidic ribosomal phosphoprotein P0 (36B4) was used for normalization. Each data point is the average of at least three independent experiments ± SEM.
To explain the differences in AhR expression between rat H4IIE cells and human HepG2 cells and T47D cells, we analyzed the promoter region of this gene. Two GRE-like sequences were identified that meet the criterion of being highly conserved between mouse and rat, but not human (Fig. 9A). The GRE starting at position −2317 has a consensus 3’ half site, while the more upstream high affinity site starting at position −7223 deviates at positions 4 and 6 from the canonical sequence [a/t]GT[c/t]CT (Nelson et al., 1999). Both sites were completely conserved between rat and mouse, except for a single C-T difference in the 5’ half site of the more downstream GRE. Furthermore, two putative XREs meeting the criterion of the consensus XRE [g/t][c/t]GCGTG[c/a/t] (Swanson et al., 1995) at positions −339 and −303 within the rat Ahr promoter were identified, which are also conserved between rat and mouse, but absent in the human Ahr gene (Fig. 9B). The presence of such XREs within the rat Ahr gene could not only explain the induction of AhR by TCDD itself (Fig. 7A and Table 2), but also the cooperative effect of ligated AhR and GR in the induction of rat AhR.

**DISCUSSION**

The results described in the present study clearly show that glucocorticoids enhance AhR signaling in rodent cells. These data are in accordance with previously reported studies showing the enhanced effects of glucocorticoids on TCDD signaling in rodents (Abott, 1995; Lai et al., 2004; Linder et al., 1999; Monostory et al., 2005). While the observed effects of glucocorticoids on dioxin signal transduction are mediated by GR in rodent cells (rat H4IIE cells and mouse Hepa1 1c1c7 cells), this is not the case in the human cells studied (HepG2 and T47D cells), although AhR and GR signaling pathways are both present and active in these human cell lines. Also in other human cell lines, no enhanced effects of glucocorticoids on AhR signaling have been observed (Monostory et al., 2005).

For all the studied endogenous AhR target genes (CYP1A1, CYP1A2, CYP1B1, GSTYa/GST-A1, HFH1, and NMOR), TCDD-induced mRNA expression is enhanced by dexamethasone in rat H4IIE cells, but not in human HepG2 cells and T47D cells. This enhancement of AhR target gene expression by glucocorticoids was reported earlier for CYP1A1 (Lai et al., 2004; Mathis et al., 1989; Sherratt et al., 1990; Pinaire et al., 2004; Xiao et al., 1995), GST (Xiao et al., 1995; Sherratt et al., 1990) and UGT (Xiao et al., 1995) in various types of rat cells, although the last study also reported negative regulation of glucocorticoids on the AhR target genes QOR and ALDH3C in rat hepatocytes (Xiao et al., 1995). It was proposed earlier that a functional GRE present in the first intron of CYP1A1 is responsible for the modulating expression of AhR target genes by glucocorticoids (Linder et al., 1999). In our study we do not find these effects in human cell lines. Furthermore, complex (pGudLuc) and minimal promoter (pDREtataLuc) reporter constructs show that the enhancing effects of glucocorticoids...
still can occur, even in the absence of the GRE in the CYP1A1 gene. In addition, as suggested earlier, it seems not likely that all these glucocorticoid regulated AhR target genes contain additional GREs in their promoters explaining these enhancing effects of dexamethasone, further strengthened by the fact that only the studied rat genes show this phenomenon. Therefore, the observed effects of glucocorticoids in dioxin signal transduction seem to be mediated by GR via direct induction of AhR in rat H4IIe cells, but not in human HepG2 and T47D cells. This is reflected by a 19 times induction of AhR mRNA when H4IIe cells are exposed to TCDD and dexamethasone, while TCDD or dexamethasone exposure alone resulted in three times and four times induction, respectively. These effects were not observed in the human cells studied. Furthermore, the observed dexamethasone-mediated enhancement of dioxin target gene induction was clearly shown to depend on a posttranscriptional process, likely AhR protein synthesis. This was shown earlier for CYP1A1 in H4IIe cells (Lai et al., 2004), but their results were not clear due to the early time point of exposure (no enhanced dexamethasone effect after 5 h), or super induction effects due to cycloheximide after 24 h of exposure. In our study, enhanced effects of dexamethasone on TCDD-induced CYP1A1 and luciferase expression were observed after 6 h which could blocked by Ru486 indicating the involvement of GR. The protein synthesis inhibitor cycloheximide could reduce the enhanced expression levels of CYP1A1 and luciferase to the levels of TCDD treatment alone, suggesting that the enhancing effect of dexamethasone via GR on TCDD target genes requires the synthesis of an additional protein such as AhR, which contains two GREs in its promoter region. Although both rat H4IIe cells and mouse Hepa1 1c1c7 cells show enhanced AhR signaling by glucocorticoids, this effect might be cell type specific. Although the experimental conditions were different, Brake et al. (1998) showed that AhR and CYP1B1 protein levels were reduced with a factor two after combined TCDD and dexamethasone exposure in rat mammary fibroblasts showing differences between fibroblasts and liver cell lines.

Higher AhR levels seem responsible for the enhanced induction of TCDD target genes in H4IIe cells. AhR levels can be limiting in cells and it has been demonstrated that over-expression of AhR in human neuro-2A cells and human H295R cells increased activities of AhR target genes Sim1 and StAR on reporter constructs, respectively (Sugawara et al., 2001; Yang et al., 2004). The GR-mediated upregulation of AhR is probably due to the presence of GREs in the rat AhR promoter but not in the human AhR promoter (this study). Murine AhR promoter analysis revealed a GC-rich, TATA-less promoter containing five putative Sp1-like binding sites (Garrison and Denison, 2000), well conserved in the human AhR promoter (Eguchi et al., 1994). In addition, a putative XRE was reported for the murine AhR promoter (Garrison and Denison, 2000). In our study, two GRE-like sequences were found that were specific for the rodent AhR genes. Recently, it

FIG. 8. Effect of cycloheximide on dioxin-induced CYP1A1, AhR, and luciferase expression in rat H4IIe-pGudLuc cells. H4IIe-pGudLuc cells were treated with increasing concentrations of TCDD (open bars) and TCDD in combination with dexamethasone (10 nM) (black bars) (left panel) or the same treatments in combination with 10 μg cycloheximide (CHX; right panel). Total RNA was isolated after 6 h and cDNA was prepared by reversed transcription. Induction of rCYP1A1 (A), luciferase (B), and rAhR (C) mRNA was determined by real-time quantitative PCR. Expression of the acidic ribosomal phosphoprotein P0 (36B4) was used for normalization. Each data point is the average of at least three independent experiments ± SEM.
was reported that functional GREs tend to be very highly conserved between species (Almon et al., 2005) and the present pair of sequences for rat and mouse qualifies as such, while the corresponding human sequence does not. Furthermore, two XRE-like sequences within the Ahr gene were found to be fully conserved between rat and mouse, but not in the human Ahr promoter at these positions, although here a putative XRE was found at position -7393 (data not shown). These XREs within the rat Ahr gene seem necessary for the induction of AhR by TCDD, and may also be involved in the cooperative effect of TCDD and glucocorticoids in the induction of rat AhR. Factual proof of functionality of both identified GREs and XREs should come from testing of Ahr induction in a relevant target cell or organ, characterization of the sequences in reporter gene assays, and/or chromatin immunoprecipitation.

From a practical point of view, the interactions of signal transduction pathways by dioxins and glucocorticoids could have consequences for quantitative dioxin analysis using the DR CALUX bioassay. Complex samples such as human serum samples, may contain a combination of both dioxin receptor agonists (such as TCDD, PAHs, and PCBs) and glucocorticoids (cortisol and prescription glucocorticoids such as dexamethasone) which would then overestimate the concentration of dioxins. An example of this possibility is the direct serum exposure or simple serum extract exposure to DR CALUX cells resulting in very high TCDD toxic equivalents (TEQs) compared to high-resolution gas chromatography–mass spectrometry TEQs (Ziccardi et al., 2000 and data not shown). In our laboratory, glucocorticoids were shown to be efficiently removed from matrices via clean-up procedures in the dioxin sample work-up in standard dioxin analysis with the DR CALUX bioassay (as examined using the GR CALUX bioassay (Sonneveld et al., 2005) measuring glucocorticoidal activity; data not shown).

In our study, insight has been gained into the interactions of signal transduction pathways by dioxins and glucocorticoids which provide explanations for known interactions between stress-related pathways and dioxin effects. The immunotoxic effects of TCDD, notably atrophy of the thymus and suppression of thymus-dependent immunity, are mediated by the AhR, expressed in the epithelial cells of the thymus (reviewed by Vos et al., 1998). The sensitivity of humans to the immunotoxic effects of TCDD, both in vitro and in vivo, is still controversial as contradictory data have been reported while for rodents clear TCDD-induced thymic atrophy (in vitro as well as in vivo) is described (Poland and Glover, 1980). Extrapolation of data from animal studies to man poses difficulties as the interspecies difference in sensitivity to TCDD is high. In other words, evaluation of the risks posed by dioxins to humans is hampered by the exceptionally large inter- and intraspecies differences occurring in laboratory animals for some of their effects. These differences culminate in acute lethality: for the most toxic dioxin TCDD, the hamster is about 1000-fold more resistant than the guinea pig and a difference of the same magnitude exists between a TCDD-sensitive rat strain, Long–Evans and a TCDD-resistant strain, Han/Wistar. The same dramatic differences in TCDD sensitivity within species exist for mice (Poland et al., 1994) and birds (Karchner...
et al., 2006). Besides these clear toxicity differences between rodents, also species differences between TCDD affinity for the AhR exist. In our study we found a decreased affinity of TCDD for the human AhR compared to rat and mouse AhR as reviewed earlier by Harper et al. (2006). Reporter gene assays showed that a dioxin reporter construct already could be induced at low TCDD levels in rat H4IIe cells and mouse Hepa1 1c1c7 cells but not in human HepG2 and T47D cells. In addition, EROD experiments showed picomolar range EC50 values for TCDD in H4IIe cells and Hepa1 1c1c7 cells, while human cells were 50–100 times less sensitive to TCDD. These differences in sensitivity to TCDD are believed to be caused by differences in the ligand-binding affinity of polymorphic AhR variants within and across species. In contrast, susceptibilities to the adverse effects of TCDD are not always necessarily attributable to polymorphism of the AhR gene: two rat strains with identical AhR structure showed a large difference in susceptibility (Kawakami et al., 2006), suggesting that the sequence of the AhR itself seems not always responsible for observed differences in terms of TCDD toxicity and therefore other mechanisms are likely involved. GR-mediated effects might be one of these mechanisms involved in differences in TCDD toxicity albeit both GR and AhR should be present in the target tissues/organisms (e.g., liver, thymus) and more importantly, the TCDD signal should be amplified by GR (e.g., upregulation of AhR). For this last proposed prerequisite we provided evidence (on a cellular level) that this might be specific for rodents but not for humans. Factual proof should come from rodent animal studies combining both TCDD and dexamethasone exposures, taking both toxicity and AhR target gene induction in appropriate organs (liver and thymus) as endpoints. Further evidence for AhR upregulation and consequent elevation of TCDD target genes comes from a study using human cells, although by another nuclear hormone receptor. Peroxisome proliferator activator receptor alpha (PPARalpha) activation potentiates AhR-induced CYP1A1 expression in human Caco-2 cells via induced AhR expression, although with very high concentration PPARalpha ligand (Fallone et al., 2005). The possible GR-mediated effect amplification of dioxin-like compounds (TCDDs, PCBs, PAHs) by induced levels of AhR in rodents suggests a higher sensitivity for rodents than for humans, especially when increased stress conditions (resulting in elevated cortisol levels) are present. Furthermore, these data consequently suggest the possibility that dioxin responses in rodent-based models for dioxin toxicity may overestimate the hazard in comparison to humans and may possibly have implications for risk assessment.

FUNDING

Dutch Ecogenomics Consortium and the Commission of the European Communities, integrated project “Newborns and Genotoxic exposure risks” (FOOD-CT-2005-016320).

ACKNOWLEDGMENTS

The authors wish to thank Sue Man for excellent technical assistance.

REFERENCES


