Development of an Exonuclease Protection Mediated PCR Bioassay for Sensitive Detection of Ah Receptor Agonists

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The aromatic hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biological and toxicological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related chemicals. Here we developed a novel method to detect the presence of AhR ligands using Exonuclease Protection Mediated PCR bioassay (EPM-PCR). This assay measures the ability of a chemical to activate AhR DNA binding in vitro. In the presence of AhR ligand, an expected length PCR product was observed on electrophoresis, but no signal was detected in the absence of ligand. Real-time quantitative PCR was performed to quantify DNA bound to ligand:AhR complex. We obtained a standard curve with TCDD concentration to bound DNA copies in the range of 0.01 pM–10 nM of TCDD. Minimal detection limit of the assay was below 0.01 pM TCDD, and the whole detection time was less than 5 h. In comparison to the chemical-activated luciferase gene expression (CALUX) bioassay, EPM-PCR bioassay is more sensitive and easier to perform. These results suggest that this assay is useful for detection and quantification of TCDD and related AhR ligands in a cell-free system without the use of radioactivity.

Key Words: Ah receptor; 2,3,7,8- TCDD; exonuclease III; S1 nuclease; real-time PCR.

Aromatic hydrocarbon receptor (AhR) is a ligand-dependent transcription factor involved in the regulation of several genes, including those for xenobiotic-metabolizing enzymes such as cytochrome P450 1A and 1B forms (Denison et al., 1998a; Whitlock, 1999). Exposure to 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD), the prototypical and most potent HAH and AhR ligand, results in a variety of toxicological and biological effects, such as alteration in cell proliferation, apoptosis, adipose differentiation, tumor promotion, immune function, vitamin A status, development, and reproductive functions (reviewed in Devito and Birnbaum, 1994). AhR ligands include a wide variety of ubiquitous and hydrophobic environmental contaminants. The well-known halogenated aromatic hydrocarbons (HAHs) and the nonhalogenated polycyclic aromatic hydrocarbons (PAHs) have the highest affinity to AhR and are able to induce gene expression in an AhR-dependent manner (Denison et al., 1998b; Safe, 1990). Recently, more evidence has shown that, unlike most ligand-dependent receptors, AhR can be bound and activated by structurally diverse ligands. Naturally occurring ligands must exist for AhR, such as endogenous ligands and dietary AhR ligands, although the majority of these chemicals appear to be relatively weak (reviewed in Denison et al., 2002). Identification and detection of AhR ligand is important for an in-depth description of AhR signal transduction and effects mediated by AhR.

Numerous analytical techniques have been developed to detect and quantify the AhR agonists in environmental, biological, and food samples. High-resolution gas chromatography/mass spectrometry (HR GC/MS) is the ‘gold standard’ for HAH analysis with accurate and specific measurement (Clement, 1991; Stephens et al., 1992). But the procedures are extremely costly and time consuming and require sophisticated equipment, which will not adapt for large numbers of samples to be screened. Additionally, this approach is unable to assess chemicals interactions and will not allow for the identification of other novel AhR agonists except HAHs (Safe, 1990; Van den Berg et al., 1998).

Currently, numerous AhR-based bioassays for identification and detection of AhR ligands have been developed in vitro (El-fouly et al., 1994; Garrison et al., 1996; Murk et al., 1996; Postlind et al., 1993; Wheelock et al., 1996). Whole cell systems, such as the chemical-activated luciferase gene expression (CALUX) (Garrison et al., 1996; Zhang et al., 2002) and ethoxyresolufin-O-deethylase (EROD) activity (Giesy et al., 1994; Kennedy et al., 1993; Safe, 1990) are sometimes represented as the next best system when compared with whole-body or in vivo systems. However, cell systems can be affected by the toxic chemical itself during the assay, thus causing confusing problems in the assay. Incorporation of metabolism in cell systems with uncertain consequences prolongs assay complexity and time. Thus, these drawbacks limit the utility of cell systems for screening purposes. Most cell-free bioassays require radioactivity, such as the gel retardation of AhR binding (GRAB) assay (Denison and Yao, 1991; Heath-Pagliuso et al., 1998;),...
or antibody of AhR or ligand (Santostefano et al., 1992; Wheelock, 1996), which are unsuitable for some laboratories.

Here a cell-free bioanalysis method, Exonuclease Protection Mediated PCR (EPM-PCR) bioassay, was established for detection of AhR ligands, based on the binding of the ligand:AhR complex to the specific DNA. EPM-PCR can provide indirect detection of ligands by quantification of the specific AhR-binding DNA, with no need for any DNA labeling and sophisticated equipments. This new bioassay not only has the higher sensitivity and specificity, but it is rapid and easy to perform.

MATERIALS AND METHODS

**Chemicals.** TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (PCB) were purchased from AccuStand (New Haven, CT). Benzo(a)anthracene (BA) and dimethylsulphoxide (DMSO) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Many of these chemicals are extremely toxic and/or carcinogenic, and they were prepared at 4°C until use. Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as the standard and adjusted to 10–15 mg/ml diluted in HEDGK buffer.

**Preparation of cytosol.** Male Sprague-Dawley rats (200–250 g) were exposed to 12 h of light and 12 h of dark daily and were allowed food and water. Treatment of animals was a standard operating procedure referring to the protocol described by Zhang (2002). In brief, HepG2 cells were engineered to contain a stably integrated DRE-driven luciferase reporter gene plasmid based on the vector pGL3. The recombinant HepG2 cells were incubated with DMSO (0.5%) or TCDD (0.1 μM–10 μM) for 24h at 37°C. The cells were lysed by luciferase lysis reagent (Promega), and luciferase activity was measured using a Lumate LB 9570 luminometer with luciferase assay reagent (Promega). The concentration of protein was detected by Bio-Rad method.

**Preparation of TCDD:AhR:DRE complex.** SD rat hepatocytic cytosol was incubated with DMSO (20 μl/ml), different concentrations of TCDD (0.01 pM–10 nM) or other AhR ligands (each of 10 μM), for 2 h at 20°C, followed by the addition of ~0.5 μg DREs probe and further incubation for 15 min. Every indicated chemical was diluted in DMSO solution.

**EMP-PCR bioassy.** The 10 μl aliquots of binding reaction mixture described above were mixed with 40 μl of 1.25× ExoSIII buffer, and preincubated for 5 min at 30°C, followed by addition of 100 U ExoIII and further incubation for 10 min. Next, 5 μl of ExoIII-treated aliquots was added to 15 μl S1 mix (200 μl mix containing 40 mM KAc (potassium acetate), pH 4.6, 340 mM NaCl, 1.35 mM ZnSO4, 0.8% glycerol, and 60 U S1 nuclease) to incubate for 30 min at room temperature. After the termination of digestion by addition of 2 μl S1 stop buffer (0.3 M tris-base, 0.05 M EDTA) and incubation for 10 min at 70°C to inactivate the enzyme, the sample of 10 nM TCDD was subjected to PCR with primers (F1, R1) or (F2, R2). Primer F2 (5'-CATTTAGCTAGTGCTC-3') and R2 (5'-CGTCTTATCGTCCATAC-3') were designed to amplify the residual DNA with less primer dimers and nonspecific products. The conditions of PCR were an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, 51°C for 45 s, and 72°C for 45 s, at last extended for 8 min. The target DNA products were identified by comparison to 100-bp DNA ladder on 2% agarose electrophoresis. For primers (F1, R1), the annealing temperature of PCR was 60°C.

**Real-time quantitative PCR.** Real-time PCR amplification was carried out in a 20-μl volume with 2-μl Lightcycler DNA Mastermix (Roche Molecular Biochemicals, Mannheim, Germany) containing Taq-polymerase, dNTPs (with dUTP instead of dTTP), PCR buffer, and the Sybr green I. MgCl₂ and primer concentrations were optimized for highest signal intensity and lower background. Here the concentration of MgCl₂ was 3 mM, and primer concentration was 0.75 μM. Different concentrations of 2-μl TCDD-treated samples were used as the templates. PCR were run in the LightCycler amplification and detection system (Roche Diagnostics). Protocol was done by 0 s denaturation at 95°C, 5 s annealing at 51°C, and 10 s elongation at 72°C for 40 cycles. Fluorescence was detected at the end of every 72°C extensive phase. Melting curve analysis was applied to all end PCR products after cycling protocol. The melting step consisted of denaturation at 95°C, cooling to 65°C for 10 s, then ramping to 90°C at 0.2°C/s, monitoring fluorescence continuously.

To determine the absolute copy numbers of the bound DNA in samples, ten-fold dilution series of dioxin probe (1 × 10² to 1 × 10⁵ copies ) were used in the real-time PCR. Threshold cycle (Ct) values are defined as the cycle at which Sybr green I fluorescence increases significantly above the background as a result of amplification formation.

**Results**

**Scheme of EPM-PCR.** Figure 1 shows the strategy of detection of the AhR ligands using EPM-PCR. The Ah receptor is a soluble intracellular protein that enhances the transcription of a number of genes. After activated by TCDD or other ligands, transformed AhR recognizes a specific DNA motif (DRE) flanked by several variable nucleotides upstream of the CYPIA1 gene (Denison 1988) and induces the gene expression. TCDD:AhR complex interacts with DRE in an one-to-one ratio (Denison and Yao, 1991), so the dioxin probe with DREs on both ends can bind two TCDD:AhR complexes per probe at saturation. Incubated with the activated AhR complex, both 3' ends of the dioxin probe were protected by ligand:AhR:DRE complex and resisted ExoIII cleavage (Wu, 1985). This meant that not only binding sites but also the sequences between two sites were protected against ExoIII in the presence of AhR ligands. Free DNAs were digested by ExoIII and S1 nuclease (Henikoff 1984). Only receptor protein-binding DNAs remained and were amplified by PCR.
Detection of AhR Ligands by EPM-PCR Bioassay

Figure 2 demonstrated that EPM-PCR assay successfully detects the indicated chemicals. In the presence of TCDD, the expected-length product was detected using the primer pairs (F1, R1) or (F2, R2) respectively (Fig. 2A, lane 3 and 4). In absence of TCDD, with DMSO used as a negative control, AhR was not activated, so all input probes were free and degraded by ExoIII and S1 nuclease. No band was observed on the agarose gel electrophoresis (Fig. 2A, lane 2). No-chemical input was tested on EPM-PCR as a blank control. The result was the same as the negative control (Fig. 2A, lane 5). Whatever primers were used, (F1, R1) or (F2, R2), the results of the control were the same. It showed that free probe was digested thoroughly, and it also meant that all PCR templates of the positive samples derived from the TCDD:AhR-binding DNAs. In addition to TCDD, several other ligands such as TCDF, PCB, and BA were successfully tested by EPM-PCR (Fig. 2B). It proved this system was amenable for detection of AhR ligands.

Quantification of TCDD by EPM-PCR Bioassay

For more information, Sybr green fluorescence real-time PCR was applied to quantify the TCDD:AhR-binding dioxin probe relative to different concentrations of TCDD. Calibration curves of three runs were obtained using different copies ranging from $10^2$ to $10^8$ copies of the dioxin probe as the templates in real-time PCR (Fig. A was shown in Supplementary Data). Primers (F2, R2) were chosen here. They all showed a linear relation ($r = -0.99$) between the log of template copy number ([log copy number]) and $C_T$ over the range of DNA copies examined. The PCR efficiencies ranged from 1.7 to 1.8. The samples of ExoIII/S1 digestion were quantified by...
The results demonstrated CT values decreased with the real-time PCR (Figs. B and C were shown in the Supplementary Methods section. Values represent the mean ± SD of three determinations. The induction response of TCDD was represented with the absolute copy numbers of AhR-binding DNA for EPM-PCR and with the luciferase activity for CALUX bioassay.

Comparison of EPM-PCR Assay and CALUX Assay

We have previously demonstrated that incubation of HepG2 cells with TCDD for 24 h results in a significant induction of luciferase activity (Zhang, 2002). A comparison of dose-response relationship and relative sensitivity of the CALUX and EPM-PCR for detecting TCDD is shown in Figure 3. EPM-PCR is approximately seven times more sensitive than the CALUX (EC\textsubscript{50} of ~3 × 10\textsuperscript{-12} M compared to ~2 × 10\textsuperscript{-11} M, respectively). The minimal detection limit is ten times lower (~1 × 10\textsuperscript{-14} M as compared to 1 × 10\textsuperscript{-13} M for the CALUX assay). And it has higher reproducibility (CV of 6–9% compared to 15–30%, respectively). Moreover, the EPM-PCR bioassay is as rapid as the CALUX, with detection time ~5 h, and is easy to analyze.

DISCUSSION

The objective of this research was to develop a sensitive bioassay for quantification of the toxic dioxin-like chemicals that could be used easily by a wide variety of researchers. The method described here quantifies TCDD indirectly by detecting the transformed AhR-binding DNA in vitro. It is similar to other AhR-DNA binding bioassays for TCDD, in that it utilizes DNA-binding transformation of the AhR as the discriminatory event.

The standard protocol of the exonuclease protection assay was described by Wu (1985). It detected protected DNA (containing the nonspecific nucleic acid) on the agarose electrophoresis directly after ExoIII digestion. This assay is unfeasible for detecting the trace protein-binding DNA, due to low sensitivity, and is incapable of distinguishing the specific and nonspecific DNA. PCR is a good technique for amplifying the trace DNA, but it is not suitable for classic exonuclease protection assay. The high frequency of false-positive results is achieved because the end-blunt DNA, digested to 70–90% of completion by ExoIII, remains a short double strand even after extensive degradation (Richardson et al., 1964). The ‘single-stranded’ oligonucleotides produced by ExoIII can be amplified by PCR. With ExoIII digestion alone, the results had no significant difference between the negative control and indicated chemicals when PCR was performed. Even if the digestion time was extended to 60 min, the results were similar (data not shown). In this case, the addition of S1 nuclease resolved the problem of high background value. The free DNA treated with ExoIII was deleted quickly by S1 nuclease and was not be amplified by PCR. The utilization of PCR improved the sensitivity of EPM-PCR and made it feasible to detect the trace dioxins.

To clarify the specificity of EPM-PCR bioassay, the mutated DNA probe (without DRE) replaced the indicated dioxin probe in EPM-PCR, and no signal was observed. Poly(dI/dC) was preincubated with the samples to eliminate nonspecific protein-binding DNA in the subsequent reaction steps. In fact, no differences on the result of PCR with and without the addition of poly(dI/dC) in the reaction mixture were detected. It may be because, even if the nonspecific protected DNA exists, it cannot be detected in the present system by the primers used. In fact, EPM-PCR is applicable to a variety of cell lines or species. The crude protein extract also contains other proteins that may preferentially bind to the DNA probe input. If there was such binding, specific protected DNA was confirmed by melting curve analysis of PCR product. Taqman and molecular beacon quantitative PCR with higher specificity are good alternatives to measure the AhR-binding dioxin probe. EPM-PCR assay avoids the loss of TCDD:AhR-binding DNA and interference of the free or nonspecific DNA by eliminating, not separating, the free DNA from bound DNA, which improved the specificity.

Besides TCDD, EPM-PCR assay was also used to examine the ability of other three ligands to directly stimulate AhR transformation and DNA binding in vitro (data not shown).
Dose-response curves demonstrated the rank order in potency was TCDD > TCDF > PCB > BA, which was consistent with their toxic equivalency factors (TEFs).

By applying of ExoIII and S1 nuclease digestion in the AhR-binding DNA bioassay, EPM-PCR provides sensitive and specific detection and quantification of AhR ligands. Compared with other existing methods for dioxin detection, EPM-PCR bioassay has advantages, such as nonradioactivity, sensitivity and simplicity. It is useful to screen the positive from large-scale of samples or measure novel AhR agonists. However, it doesn’t provide structural information of the agonists like GC/MS analysis. So addition of the structure-based approach is necessary for identification of an unknown AhR agonist.

SUPPLEMENTARY DATA

Figures A, B, and C are available online at www.toxsci.oupjournals.org.

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REFERENCES


