An *in vitro* system for the detection of compounds that can interfere with the expression of steroidogenic cytochrome P450 (CYP) enzymes

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Introduction

A strong interest exists in the development of rapid and sensitive screening tools to detect the ability of chemicals (either environmental contaminants or commercial products) to interfere with hormonal function, which may lead to reproductive problems and other toxicities related to sexual differentiation, growth and development. Current research has focused mainly on the effects of environmental contaminants on the function of the estrogen and androgen receptors. However, many other mechanisms of potential interference with endocrine functions exist, including effects on enzyme systems involved in steroid production and breakdown.

Our working hypothesis is that certain chemicals can cause hormonal disruption and reproductive toxicity by interfering with the function of key enzymes involved in steroid synthesis and breakdown. The main objective is to develop sensitive methods for the rapid screening of chemicals that interfere with these enzyme systems. We are evaluating the human NCI-H295 adrenocortical carcinoma cell line as an *in vitro* tool for this purpose. The H295 cell line has been characterized in detail and shown to express most of the key enzymes necessary for steroidogenesis [1-4]. These include the cytochrome P450 enzymes (CYP) CYP11A (cholesterol side-chain cleavage), CYP11B1 (steroid 11β-hydroxylase), CYP11B2 (aldosterone synthetase), CYP17 (steroid 17β-hydroxylase), CYP19 (aromatase) and CYP21B2 (steroid 21-hydroxylase).

A number of approaches can be considered to measure potential effects on steroidogenic enzymes. These include the measurement of catalytic activities using selective substrates, the determination of levels of messenger RNA using northern blotting and, for greater sensitivity, reverse transcriptase-polymerase chain reaction (RT-PCR), and the measurement of levels of enzyme protein using immunoblotting techniques. This abstract introduces the H295*R* (a subpopulation of H295 that grows in a monolayer [3,4]) adrenocortical cell line as an *in vitro* screening tool to measure steroidogenic interferences and describes some initial findings of potential effects of two environmental contaminants on the expression of three steroidogenic enzymes, CYP11A, CYP17 and CYP19, using RT-PCR.

Materials and Methods

H295R cells were obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown in 75 cm^2 flasks under culture conditions published previously [3,4]. Cells were grown until almost confluent and then trypsinized and seeded into 6-well plates at a concentration of 1.0 cm^2/ml (assuming that an almost confluent flask contained 75 cm^2 of cells). This method led to reproducible cell densities among experiments. Each well contained 4 ml of medium. Cells were exposed to various concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) administered as 1000-fold stock solutions in dimethyl sulfoxide (DMSO). Control cells were exposed to 4 µl of DMSO. After 24 h, cells were additionally exposed to 300 µM of 8-bromo-cyclic AMP (8-Br-cAMP) for another 24 h. Then, RNA was isolated using the RNA Insta-Pure System (Eurogentec, Belgium) and stored at -70°C. RT-PCRs were performed using the Access RT-PCR System (Promega, USA). The purity of the RNA preparations was verified by denaturing agarose gel electrophoresis; the RNA preparations were found acceptable for RT-PCR when their A260 (nm)/A280 (nm) ratios were greater than 1.8. Total RNA concentrations of the preparations were determined from their absorption at 260 nm. Suitable primer pairs for CYP11A1, CYP17 and CYP19 were obtained by entering their cDNA sequences from the European Molecular Biology Laboratories database into the software program Geneworks (version 2.4; IntelliGenetics, USA). PCR conditions, such as annealing temperature and Mg^{2+} concentration were optimized empirically for each primer pair. Primer pairs for each CYP produced a single amplification product in the form of a sharp band of the theoretically expected fragment length in H295R cells. Serial dilutions of total RNA concentrations were amplified using the three primer pairs to determine the 'linear' range of the PCR reaction, in order to make semi-quantitative inferences. As further control, RT-PCR was performed using primers for β-actin mRNA. Amplification products were detected using agarose gel electrophoresis and ethidium bromide staining. Intensity of the ethidium bromide stains were quantified using a FluorImager (Molecular Dymanics, USA).

Results and Discussion

In initial experiments in JEG-3 cells, RT-PCR demonstrated the ability of 8-Br-cAMP to induce mRNA levels for CYP11A and CYP19 dose- and time-dependently; 300 µM 8-Br-cAMP increased the amplification response by 2.5- and 5.5-fold, respectively after 24 h. 4-hydroxy-androstenedione (4-HA), an inhibitor of the catalytic activity [5] and to a lesser extent of the expression of CYP19 [6] reduced the amplification response for CYP19 by 50% without an effect on CYP11A. The treatments did not affect the level of β-actin mRNA.

In H295R cells, 8-Br-cAMP induced CYP11A, CYP17 and CYP19 by about 2.5-, 2- and 10-fold, respectively, after 24 h, without any effect on β-actin expression; 4-HA had no effect. The levels of induction of the CYP mRNAs determined by RT-PCR were similar to those observed by other investigators using northern blotting [1], indicating the ability of the RT-PCR method to provide semi-quantitative information under our conditions. An amplification product was readily detected for CYP11A and CYP17 using an initial RNA concentration of 0.5 ng per PCR for untreated H295R cells; for CYP19 a 100-fold greater concentration of RNA was required. These findings are consistent with the much greater expression of CYP11A and CYP17 than of CYP19 observed in H295 cells by others [1,3,4].

Table 1. Effect of TCDD on 8-Br-cAMP-mediated induction of the expression of steroidogenic CYPs.

TCDD (nM)	8-Br-cAMP	8-Br-cAMP + TCDD		
	----------(relative amplification response %)----------			
		CYP11A	*CYP17*	*CYP19*
1	100	99	not determined	111
10	100	70*	118	59
100	100	61**	96	57*

* - *lower than in 8-Br-cAMP-treated cells ($p < 0.1$).*

** - *lower than in 8-Br-cAMP-treated cells ($p < 0.05$).*

Exposure of H295R cells to various concentration of TCDD led to a reduction in the ability of 8-Br-cAMP to induce CYP11A ($p < 0.05$) and possible CYP19 ($p < 0.1$), but not CYP17. This effect was dose-dependent (Table 1) and strongest at the greatest tested concentration of 100 nM TCDD (Fig. 1). Benzo*a*pyrene (B*a*P) at 1 μM had a similar effect on CYP11A ($p < 0.02$), but not on CYP19 (Fig. 2).

In summary, these results indicate that the H295R adrenocortical carcinoma cell line, in combination with the semi-quantitative RT-PCR method, is suitable for the measurement of potential effects of xenobiotics on the expression of steroidogenic cytochrome P450 enzymes.

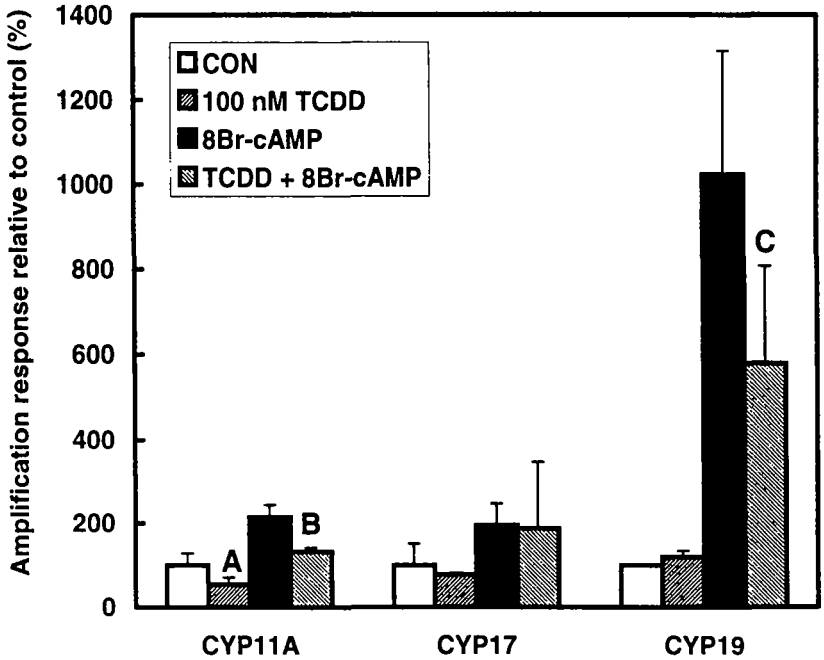

Figure 1. *Effect of 100 nM TCDD on the level of mRNA for several steroidogenic enzymes in untreated and 8-Br-cAMP-treated H295R human adrenocortical carcinoma cells. **A** - lower than in control cells ($p < 0.1$). **B** - lower than in 8-Br-cAMP-treated cells ($p < 0.05$). **C** - lower than in 8-Br-cAMP-treated cells ($p < 0.1$), using two-tailed Student t-test. The treatments did not affect the level of β-actin mRNA.*

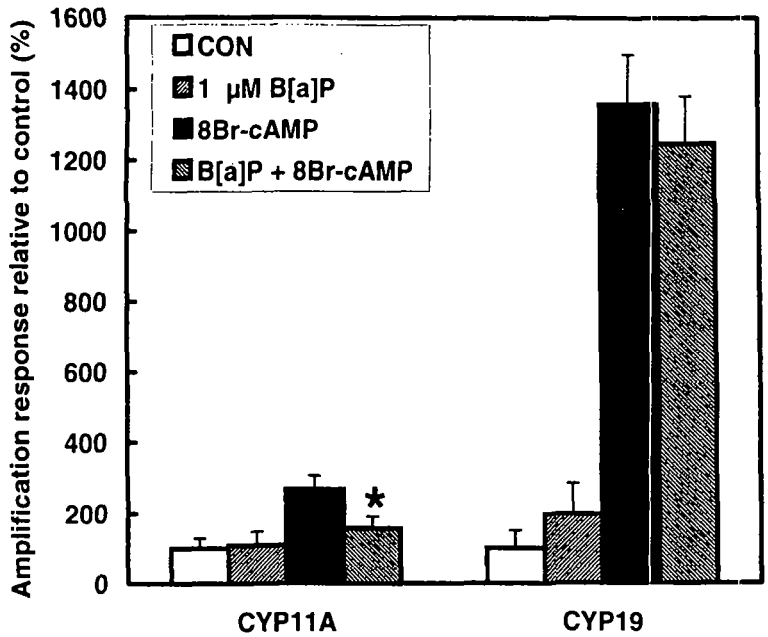

*Figure 2. Effect of 1 μM BaP on the level of mRNA for the steroidogenic enzymes CYP11A and CYP19 in untreated and 8-Br-cAMP-treated H295R human adrenocortical carcinoma cells. * - lower than in 8-Br-cAMP-treated cells ($p < 0.02$), using two-tailed Student t-test. The treatments did not affect the level of β-actin mRNA.*

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