RT-PCR AND SUPERINDUCERS, ARE THEY A TOOL TO IMPROVE THE EROD BIOASSAY?

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Introduction

Halogenated aromatic hydrocarbons such as 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) that bind specifically to the Aryl hydrocarbon receptor (AhR) promote the induction of specific enzymes, in particular, the cytochrome P4501A1. In the EROD bioassay the ethoxyresorufin-*O*-deethylase activity of the above mentioned cytochrome is determined.
It is well known that several families of compounds such as the artificial halogenated aromatic hydrocarbons and natural carotenoids are able to bind to the AhR with different levels of affinity. Additionally, some compounds, not able to bind to the AhR, increase their response magnifying the expression of cytochrome P4501A1[1,2]. These compounds are called superinducers because the AhR can be induced to higher activity. Compounds such as CHX (a protein synthesis inhibitor) or MG132 (reversible proteasome inhibitor) by decreasing the down-regulation mechanism of the AhR effected by TCDD[1,2] belong to this type of compounds. However, to our knowledge, there are no reports on studies with direct applications of these superinducers to bioassays based on the AhR induction. Therefore, this work aims at improving the sensitivity of the EROD bioassay based on the power of real time PCR (RT-PCR) as an alternative methodology to quantify the induction of cytochrome P450 1A1 as a first approach and additionally by using two selected superinducers: CHX and MG132. The micro-EROD bioassay allows the determination of dioxin-like compounds in environmental mixtures by induction of the cytochrome CYP1A1 activated through the AhR. According to previous studies, the AhR can be induced to higher activity with CHX by inhibition of protein synthesis[1,2]. Considering this, the hepatic cell line H4IIE used in the EROD bioassay was incubated with different CHX concentrations to study its potential to increase sensitivity in the bioassay. Previous reports show that the highest induction of the AhR response was observed with 10 µg/mL CHX. Thus, CHX was used here for incubation and pre-incubation at final concentrations of 5 µg/mL and 10 µg/mL[2,3]. MG132, acting by a different mechanism was also explored to increase the performance of the EROD bioassay. MG132 is known to reduce the degradation of ubiquitin-conjugated proteins in mammalian cells[3] and was used to pre-incubate HII4E cells before performing the classical bioassay.

Materials and Methods

Superinducers CHX was supplied by Sigma-Aldrich (Taufkirchen, Germany), and MG132 was acquired from Biozol Diagnostica (Eching, Germany).
Micro-EROD bioassay EROD assay using rat liver cell line (H4IIE) expressing cytochrome P4501A1 upon exposure to TCDD was performed according to Donato et al.[4] with adaptations[5]. As a modification, cells were incubated with CHX (10 µg/mL and 5 µg/mL) for 1, 2 ½, 5, and 24 hours together with the different TCDD standard concentrations (0.015-0.4 pg/well). Experiments with several pre-incubation conditions including the superinducers (CHX , MG132) and TCDD concentrations, were designed aiming at a maximal response after 24 and 72 hours incubation. After the pre-incubation, solutions were removed and the standard EROD procedure was followed with TCDD standards and two different types of blanks. Blanks consisting in cells incubated with no

additional chemicals except of medium with solvent (DMSO/Isopropanol 4:1) 0.005 % were used as a reference. Superinducer blanks identified as substrate blanks, consisted in CHX (10 µg/mL and 5 µg/mL) or MG132 (1 and 5 µM) in the medium of incubation.

Real Time PCR The primer design for *CYP1A1* was based on the GenBank sequence data (http://www.ncbi.nlm.nih.gov/). The template RNA was extracted (RNeasy Mini, QIAGEN, Hilden, Germany) and cDNA was synthesised according to manufacture´s instructions (QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). For quantification, the real-time 7900HT Instrument (Applied Biosystems, Darmstadt, Germany) was used with SYBR Green as a fluorescent reporter (QuantiFast SYBR Green PCR Kit, QIAGEN, Hilden, Germany). The data were normalized using the expression level of Actin mRNA.

Results and Discussion

RT-PCR for the detection of *CYP1A1* induction

The activation of the AhR-mediated response by TCDD at the *CYP1A1* gene level was tested in the range 0.0035-0.15 pg/well using RT-PCR. The method was successfully developed and the results showed good reproducibility (results not shown). However, in spite of a minor increase in sensitivity with respect to the classical EROD assay, the time and costs implicit in these techniques do not justify the scarce frame of improvement obtained. This leads us to study the use of potential superinducers in order to improve sensitivity in the RT-PCR detection and eventually in the classical EROD bioassay.

EROD and CHX superinduction

When the EROD bioassay was performed in the presence of CHX (5µg/mL and 10 µg/mL) no cytochrome P4501A1 activity was detected in any of the TCDD standard concentrations used, after 24 hours of incubation. Moreover, under these conditions, no activation of the AhR response was found at the mRNA level as analyzed by RT-PCR. Therefore, the micro-EROD bioassay in the presence of CHX was studied with lower incubation times (one to five hours) using detection by RT-PCR.

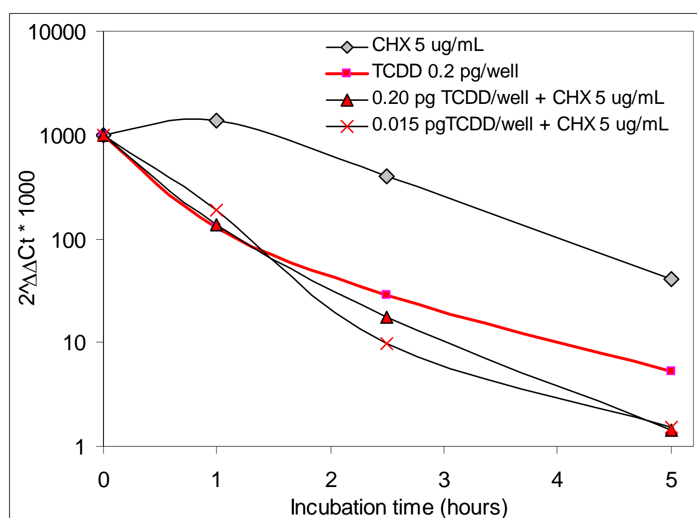

Figure 1: Time course of *CYP1A1* expression in H4IIE cells at different concentrations of TCDD (pg/well) with and without CHX (5 µg/mL).

Figure 1 shows the time course of the *CYP1A1* expression of H4IIE cells treated with CHX and TCDD after 5 hours incubation. It is noteworthy that some cytochrome P4501A1 induction was observed by CHX, when this

compound was the only inducer. Surprisingly, even at short incubation times, the agonist effect of TCDD is hindered by CHX addition and no TCDD dose-response curve could be outlined. The CHX damages the cells significantly within the first five hours of incubation, so these results discard the use of CHX as a superinducer under conditions where CHX is added simultaneously with TCDD during the incubation of H4IIE cells.

Pre-incubation with CHX

H4IIE cells were pre-incubated with cell media containing CHX (5 and 10 µg/mL) and the solution was removed before the classical EROD incubation with TCDD standards for 24 hours. However, this procedure gave no superinduction but a strong antagonist effect of the AhR-mediated response (Table 1).

Table 1: Effect of pre-incubation of H4IIE cells with CHX: ethoxyresorufin-*O*-deethylase activation (%) in relation to the TCDD reference standards without CHX, after 24 hours incubation.

Pre-incubation condition		***Incubation conditions***	***Relative activation***
Time (hours)	**CHX (µg/ml)**	**TCDD (pg/mL)**	**(%)**
1	10	30	ND
		40	ND
		120	-53
		200	-25
		300	-28
		800	-43
2	10	30	ND
		40	ND
		120	-67
		200	-36
		300	-29
		800	-51
1	5	30	ND
		40	ND
		120	-27
		200	-13
		300	-7
		800	-25
2	5	30	ND
		40	ND
		120	-32
		200	-14
		300	-13
		800	-39

ND: Not detected

Pre-incubation with MG132

This compound was less harmful than CHX to the cells but also exhibited an antagonistic effect when used simultaneously during the EROD assay, after 24 hours incubation time (data not shown). Therefore, we studied the effect of a pre-incubation step using both MG132 (1 and 5 µM) with and without TCDD. The pre-incubation times used were one, two, and four hours but the latter was no further investigated because of the considerable decrease in the cytochrome P4501A1 activity after two hours of pre-incubation. Both incubation times studied, (24 and 72 hours) showed similar results. Table 2 presents the EROD activity obtained after 72 hours incubation. Response values were calculated using the 4 parameter curve, and due to this, higher error in the calculations is observed at the lowest and highest standards of the curve where the graphic tends to be asymthotic. The results indicate that MG 132 exhibits a decreased response of the EROD activity also upon pre-incubation of TCDD

together with MG132.

Table 2: Effect of pre-incubation of H4IIE cells with MG132 and TCDD. Ethoxyresorufin-*O*-deethylase activation (%) is given in relation to the TCDD reference standards. EROD 72 hours.

Pre-incubation conditions			*Incubation conditions*	*Relative Activation**
Time (hours)	**TCDD (pg/well)**	**MG132 (µM)**	**TCDD (pg/mL)**	**(%)**
1	0.2	1	30	ND
			40	-75
			120	-17
			200	10
			300	-16
			800	1
2	0.2	1	30	-28
			40	-22
			120	-30
			200	-27
			300	-26
			800	-22

*Results are given after substracting the basal response of MG132 (MG132 blank) from the total response given by the TCDD standard with MG132.

Conclusions

The results obtained with these superinducers, well-known in the science field, were unexpected and somehow indicate the need of new knowledge regarding their interaction with the AhR-system and the expression of *CYP1A1*. Both CHX and MG132, able to generate AhR activation by different mechanisms are not able to improve the EROD performance under the current working conditions. This means that the way to search for other potential superinducers is opened again, and that this search has to be based not only on the study of compounds able to increase the concentration of AhR, but focused on other mechanisms of possible *CYP1A1* induction.

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