ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR BY 1-BENZYLIMIDAZOLE: A NON-COPLANAR COMPOUND

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Introduction

Numerous foreign chemicals (xenobiotics) induce the expression of a broad spectrum of cytochrome P450-dependent enzymes involved in xenobiotic metabolism[1]. In fish, one of these P450 isoenzymes is the xenobiotic-metabolizing cytochrome P4501A (CYP1A)[2]. Transcriptional activation of *CYP1A* is regulated by the aryl hydrocarbon receptor (AhR). Ligands bind to the AhR in the cytoplasm of the target cell, thereafter the ligand-activated receptor undergoes a transformation process whereby it forms a heterodimer with the AhR nuclear translocator protein (ARNT) and is translocated into the nucleus. The nuclear receptor complex interacts with specific xenobiotic-responsive elements (XREs) located upstream of the *CYP1A* gene, leading to stimulated transcription of the gene, elevated *CYP1A* mRNA, and increased levels of CYP1A1 protein and its catalytic activity[3,4]. AhR ligands are polycyclic, aromatic compounds that take a planar conformation into a rectangle of approximately 3 Å x 10 Å [5]. The prototype inducer of CYP1A ideally fulfilling the structural requirements of an AhR ligand is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Further inducers include coplanar polychlorinated biphenyls (PCBs), for instance 3,3',4,4',5-pentachlorobiphenyl (PCB 126), planar aromatic hydrocarbons, for instance benzo[a]pyrene, or planar flavonoid compounds such as ß-naphthoflavone (ßNF).

Imidazoles are a large group of compounds used normally as antifungal agents, both clinically as well as in agriculture, due to their capacity to inhibit cytochrome P450-mediated ergosterol synthesis[6]. Also imidazoles have been reported to be inducers of CYP1A, and as a consequence, it has been assumed these compounds are able to take a coplanar conformation[7]. However, from the point of view of structural organic chemistry this assertion is questionable. The main objectives of this work are, (1) to compare the capacity of an imidazole compound (1-benzylimidazole, BIM) to take a coplanar conformation with that of the prototype AhR ligands ßNF and TCDD, and (2) to study the CYP1A induction caused by ßNF and BIM in rainbow trout primary hepatocytes.

Methods and Materials

All the computational work was performed in a Silicon Graphics O_2 R5000 computer, with Irix 6.5 operating system, and in a double-processor PC computer, running Linux operating system. The computational conformational study of TCDD, ßNF, and BIM was carried out in the vacuum (1.0 as effective dielectric constant), using a hybrid Hartree-Fock/density functional (HF/DT) methodology that employs the B3LYP functional[8] and 6-31G(d) basis set as implemented in GAUSSIAN 98 suite of programs[9]. The calculations were carried out up to a high convergence, using the *tight* option in the GAUSSIAN 98 program. The starting geometries for the quantum chemical calculations were obtained through conformational searches using the Sybyl program.

Hepatocytes were isolated from sexually immature male and female rainbow trout (250-350 g weight) from a local trout farm. Trouts were maintained in 200 l steel tanks in the facilities of the Umweltforschungszentrum, (Centre for Environmental Research), Leipzig, Germany. (Water temperature: 14-16ºC). Hepatocyte isolation was performed following a two-step perfusion technique as described by Braunbeck and Segner[10], and were cultured in M199 medium (Sigma, USA) following exactly the same method as described previously[11], using Falcon 24-well plates (Beckton Dickinson, Oxnard, CA). ßNF, aNF, and BIM (Sigma, USA) were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) and added to the culture media of hepatocytes to achieve the final desired concentration. Final solvent concentration in the assay was 0.1%.

CYP1A transcription levels were assessed by means of RT-PCR. Total RNA was isolated from hepatocytes using TRIZOL Reagent (Life Technologies, Gibco BRL, USA). For the reverse transcriptase (RT) reaction, the first strand cDNA Synthesis Kit (Boehringer Mannheim, Germany) was used, and the PCR was performed with the Taq DNA polymerase kit from SIGMA (USA) as previously described[11]. A 270 bp fragment of the CYP1A gene, and a 540 bp fragment of b-actin, used as housekeeping gene for the RT-PCR, were amplified using adequate primers[11].

Co-treatments of ßNF and aNF were carried out in six different experiments, and co-treatments of BIM and áNF were performed in two different experiments. For each independent isolation, every treatment was done using either duplicates or triplicates.

Results and Discussion

A computational conformational study on TCDD, ßNF, and BIM was conducted to evaluate the likelihood of these molecules to take a planar conformation. The computational study on TCDD yielded two nearly planar structures as the energy minima (Figure 1a). The energies of these two conformers are identical: one of the conformers is indicated in Figure 1a, and the other one is its mirror image. These structures are slightly bent, being the dihedral angles between each oxygen atom and the vicinal peri-carbons about 175º. Given the small energy barrier (less than 3 KJ.mol^{-1}), for the interconversion (through a planar structure) between the two bent-conformers of TCDD, both structures must be in equilibrium, and the average conformation of TCDD is the planar one.

As expected, the fused tricyclic ring system of ßNF has a coplanar conformation. Additionally, rotation around the single bond connecting the phenyl ring and the fused tricycle is possible. We have analyzed the energy difference between the conformation with the four rings in the same plane (Figure 1b) and the conformation with the phenyl ring in a perpendicular arrangement (dihedral angle = 90º), finding that the energy difference is lower than 5 KJ.mol^{-1}; thus, it is very likely that bNF binds the AhR in a totally coplanar conformation (as in Figure 1b).

BIM has a very different conformational behavior than the other molecules. The most stable conformer for BIM has the imidazole ring and the phenyl ring in different planes (Fig 1c): the dihedral angles between the central carbon and the imidazole ring are 71º and –113º, and between the central carbon and the phenyl ring are 66º and –115º. Any conformation having the imidazole and the phenyl ring in the same plane is more than 46 KJ.mol^{-1} less stable than the conformer depicted in Figure 1c. Consequently, a coplanar structure for BIM is unlikely.

The transcriptional activation of CYP1A by xenobiotics is typically mediated through ligand binding to the AhR[12]. Ligands with a high affinity for the AhR, for instance TCDD or ßNF are hydrophobic aromatic compounds that are planar, and can be accommodated within a rectangular binding site of approximately 3 Å x 10 Å [5]. Although the computational studies performed here showed that BIM was not able to take a coplanar conformation, it has been reported that BIM similarly to other imidazole compounds, is able to induce CYP1A[7].

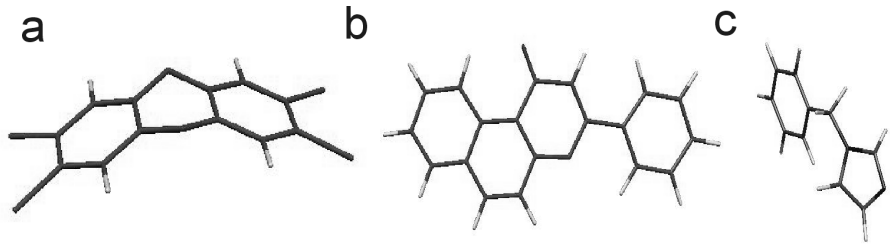

Figure 1. Molecular conformations of (a) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD, (b) ß-naphthoflavone, ßNF, and (c) 1-benzylimidazole, BIM. (Calculated as described in Methods).

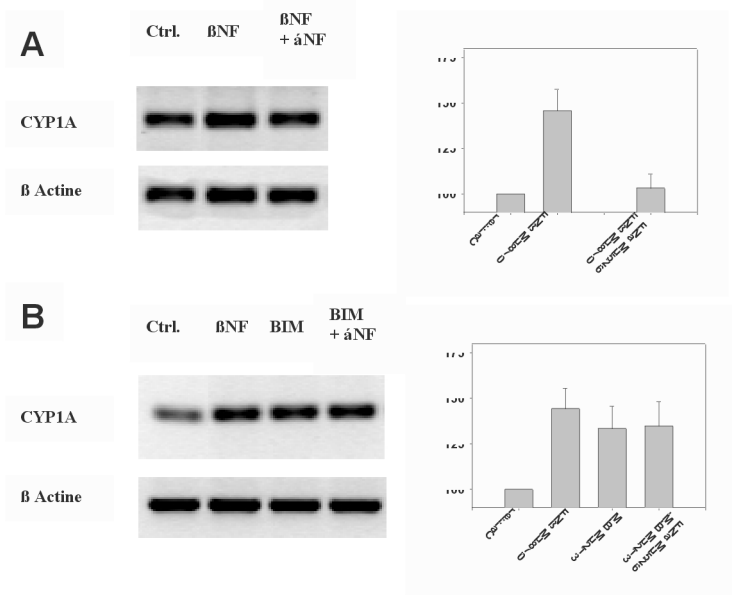

Figure 2. Influence of á-naphthoflavone (áNF) on cytochrome P4501A transcription levels in rainbow trout hepatocytes treated (A) with ß-naphthoflavone (ßNF), or (B) with 1-benzylimidazole (BIM).

In this work, the capacity of ßNF and BIM to induce CYP1A was studied using rainbow trout hepatocytes, and the transcription level of the CYP1A gene was estimated by means of RT-PCR. Cells treated with 0.78 µM ßNF or with 3.12 µM BIM exhibited a clearly enhanced expression of *CYP1A* gene compared to the control cells (Fig. 2a, and 2b, respectively). Therefore, BIM is an inducer of CYP1A although the result of the computational analysis indicates that BIM does not fulfill the structural requirements of a typical AhR ligand. In order to establish whether CYP1A induction occurs via ligand binding to the AhR, co-incubation experiments with the AhR partial agonist aNF were performed. If CYP1A induction takes place via ligand binding to the AhR, the presence of aNF should (partially) inhibit the elevation of CYP1A mRNA. When the cells were co-treated with 0.78 µM ßNF

and 6.25 µM aNF, CYP1A mRNA levels were not increased (Figure 2a). On the contrary, co-exposure of 3.12 µM BIM with 6.25 µM áNF did not lead to reduced levels of CYP1A mRNA (Figure 2b).

The results presented indicate that a non-coplanar compound, such as BIM, is able to activate the AhR. At the same time, the lack of effect of aNF on the CYP1A induction caused by BIM suggests that AhR activation provoked by BIM does not occur through direct binding to the ligand binding domain of the AhR, comparable to the induction mechanism of typical AhR ligands, such as ßNF and TCDD. Other chemicals such as carbaryl[13] and benzimidazoles[14] are able to activate AhR, but in competition assays no ligand binding was observed. It has been proposed that these compounds could activate the AhR following a model similar to that of the ligand-independent activation of steroid-receptors[15]. Steroids can bind to membrane receptor(s), stimulating intracellular phosphorylation pathways and modifying ATP levels in cells. In our system, a similar mechanism could be responsible of the CYP1A induction caused by BIM. This suggestion agrees with results of experiments carried out in rat liver showing that BIM failed to bind with high affinity to the cytosolic AhR[16].

Acknowledgements

Financially supported by Spanish MCYT (Project BQU2001-2270) and CAM (Project 08.5/0056/2000-2); and a “Ramón y Cajal” contract from the Spanish MCYT to JM Navas.

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