Disruption of contact inhibition in rat liver epithelial cells by various types of AhR ligands

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Introduction

The maintenance of a balance between cell gain and cell loss is essential for proper liver function. The exact role of aryl hydrocarbon receptor (AhR) in regulating cell proliferation and apoptosis of liver cells remains unclear, since ligand-dependent activation of AhR has been shown to induce cell cycle arrest, proliferation, differentiation or apoptosis, depending on the cellular model used[1]. AhR can directly interact with retinoblastoma protein in hepatic cells, forming protein complexes that can efficiently block cell cycle progression by inducing G_1 arrest, or to induce the expression of inhibitors of cyclin-dependent kinases, such as p27[1]. On the other hand, it has been suggested that AhR could play a stimulatory role in cell proliferation, either directly or by mediating a release from contact inhibition[2,3].

It is now generally accepted that progenitor cells exist in the liver, are activated in various liver diseases and can form a potential target cell population for both tumor initiating and tumor promoting chemicals[4]. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been found to release rat liver epithelial cells from contact inhibition by upregulating cyclin A expression and cyclin A/cdk2 activity[3]. Our previous studies have shown that a number of AhR ligands[5,6] can stimulate proliferation of confluent of rat liver epithelial "stem-like" WB-F344 cells. Such mechanism could play a role in liver tumor promotion. In the present study, we used flavonoid compounds that have been reported to act either as pure agonists, such as beta-naphthoflavone (BNF), or as partial/complete antagonists of AhR - alpha-naphthoflavone (ANF) and 3'-methoxy-4'-nitroflavone (3'M4'NF)[7,8], in order to investigate effects of AhR agonists/antagonists on confluent rat liver epithelial cells. The present study aimed to investigate the effects of model

flavonoids on the release of rat liver epithelial cells from contact inhibition, and on inducibility of cytochrome P450 1A1 (CYP1A1) mRNA expression, a model AhR-dependent target gene. The results are compared with data on the effects of both persistent and non-persistent AhR ligands on WB-F344 cells.

Methods and Materials

Determination of cell proliferation and cell cycle. WB-F344 rat liver epithelial cells were grown in modified Eagle's Minimum Essential Medium (Sigma Aldrich, Prague, Czech Republic)and only the cells at passage levels 15-22 were used throughout the study. The proliferative effects of flavonoids on confluent WB-F344 cells were determined as described previously[5]. Briefly, following the 72-h exposure of cells to test compounds, the medium was removed, cells were harvested with trypsin and counted with a Coulter Counter (Model ZM, Coulter Electronics, Luton, UK). Cells were then washed with PBS, and fixed in 70 % ethanol. Fixed cells, stained with Vindelov solution, were then analyzed on FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) for cell cycle distribution. Data were analyzed using ModFit LT version 2.0 software (Verity Software House, Topsham, ME).

Detection of CYP1A1 mRNA induction. Total RNA was isolated from cells exposed to TCDD, flavonoids or their combination, using the RNeasy mini kit (Qiagen, Valencia, CA) including treatment with DNase I (Qiagen). The amplifications of the samples were carried out in a final volume of 20 μL in a reaction mixture containing 10 μL of QuantiTect Probe RT–PCR Master Mix, 0.2 μL of QuantiTect RT Mix (Qiagen), 2 μL of solution of primers and probe, 5.8 μL of water and 2 μL of sample. The final concentration of the solution of each primer was 1.0 μM and probe was 0.2 μM. All probes were labeled with a 5´ FAM reporter and a 3´ BHQ 1 quencher. The amplifications were run on the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using the following program: reverse transcription at 50 °C for 20 min and initial activation step at 95 °C for 15 min, followed by 45-50 cycles at 95 °C for 0 s and 60 °C for 60 s. The primers and probe for rat CYP1A1 were: forward 5'-TGAGT TTGGGGAGGTTACTGGTT-3', reverse 5'-TGAAGGCATCCAGGGAAGAGT-3', probe 5'-ATACCCAGCTGACTTCATTCCTATCCTC CGTT-3' [9]. The primers and probe for the reference gene porphobilinogen deaminase (EC 4.3.1.8, GenBank accession number X06827) were: forward 5'-CCCAACCTGGAATTCAAGAGT ATTCG-3, reverse 5'-TTCCTCTGGGTGCAAAATCTGGCC-3', probe 5'-CCTCAACACCGCC TTCGGAAGCT-3'. Gene expression for each sample was

expressed in terms of the threshold cycle (C_t) and the final comparison of transcript ratios between samples was given as $2^{-\Delta\Delta C_t}$ [10].

Results and Discussion

The rate of proliferation of most non-transformed adherent cells decreases with increased cell density as cells become arrested in G_1 phase of cell cycle, which is a phenomenon known as contact inhibition. The loss of contact inhibition can lead to deregulated growth and is often associated with malignant transformation[11]. The liver progenitor cells could form a potential target cell population for tumor promoters. TCDD, a powerful liver tumor promoter, can induce a release from contact inhibition in liver "stem-like" cells[3]. The non-persistent AhR ligands, such as polycyclic aromatic hydrocarbons (PAHs) or heterocyclic PAHs, can also release rat liver epithelial cells from contact inhibition and this effect corresponds with their capacity to induce the AhR-mediated activity[5,6]. Moreover, we have found that dioxin-like PCBs, such as PCB 126, can stimulate rat liver epithelial cell proliferation (manuscript under preparation). The G_1/S-phase transition and S-phase progression are controlled through regulation of D-type cyclins, cyclin A and E through formation of specific cyclin/cdk complexes[12]. The release from contact inhibition in rat liver epithelial cells is associated with upregulation of cyclin A and increased cyclin A/cdk2 complex activity, which has been observed both after treatment with TCDD (Dietrich) and with PCB 126 (manuscript in preparation).

In the present study, we found that flavonoid compounds that function either as agonists or antagonists/partial agonists of AhR (ANF, BNF and 3'M4'NF) can all stimulate an increased cell proliferation in confluent WB-F344 cells in a concentration-dependent manner. As shown in Figure 1, all test chemicals induced a similar effect as TCDD, when used at concentration 1 μM. They induced both a significant increase of cells in S-phase (Fig. 1A) and a significant increase of cell numbers (Fig. 1B). In order to confirm that ANF and 3'M4'NF acted as antagonists in this cellular system, effects of flavonoids on induction of CYP1A1 mRNA expression were investigated. We found that BNF, a well known AhR agonist, induced CYP1A1 mRNA and it did not suppress induction of CYP1A1 mRNA by TCDD in WB-F344 cells (Figure 2). Contrary to that, both ANF and 3'M4'NF suppressed the levels of TCDD-induced CYP1A1 mRNA. Both compounds themselves also stimulated CYP1A1 mRNA expression, however 3'M4'NF induced much lower CYP1A1 mRNA levels than ANF.

As summarized in Table 1, the compounds that are not AhR ligands, such as PCB 153 or fluorene have no effect on proliferation of growth-inhibited liver epithelial cells. Contrary to that, potent AhR agonists such as TCDD or PCB 126 can induce a release from contact inhibition, which is associated with upregulation of cyclin A and increased cyclin A/cdk2 complex activity. Such mechanism could play a role in tumor promoting effects of AhR ligands in liver. Importantly, the present study showed that compounds that behave as partial AhR agonists/antagonists can induce a release of WB-F344 cells from contact inhibition. A number of dietary flavonoids are also known to be AhR agonists/antagonists[13], suggesting that these compounds, as well as environmental pollutants might alter cell cycle and they could potentially have impact on proliferation of the liver oval cells. Taken together, both the present data and several recent studies seem to suggest that a release from contact inhibition, observed in model rat liver progenitor cells in vitro, is a mode of action that is common to various types of AhR ligands. Future studies should investigate its significance in vivo, potential cell type or species specificity, as well as the mechanisms underlying this effect.

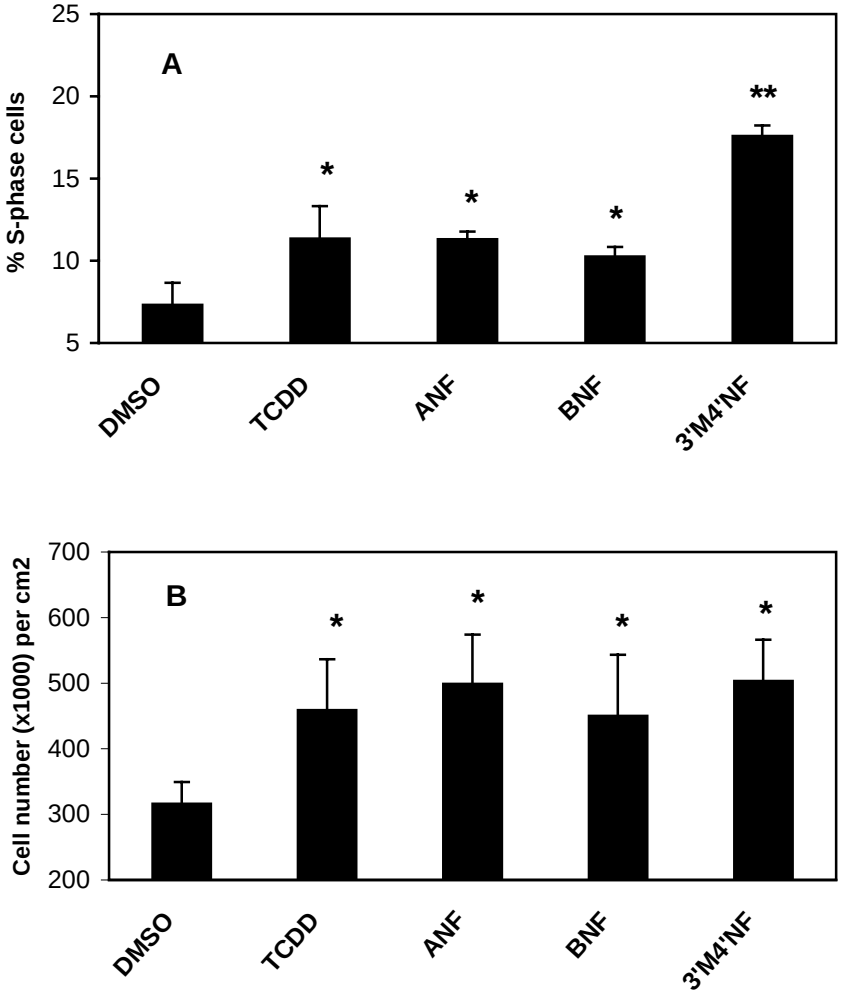

Figure 1: Effects of flavonoids on percentage of S-phase cells (A) and numbers (B) of contact-inhibited, confluent WB-F344 cells. Cells were incubated with TCDD (1 nM) or with flavonoids (1 μM) for 72 hours. The results represent means +/- SD from three independent experiments. * significantly different from control (DMSO) value (P<0.05). ** significantly different from control value (P<0.01).

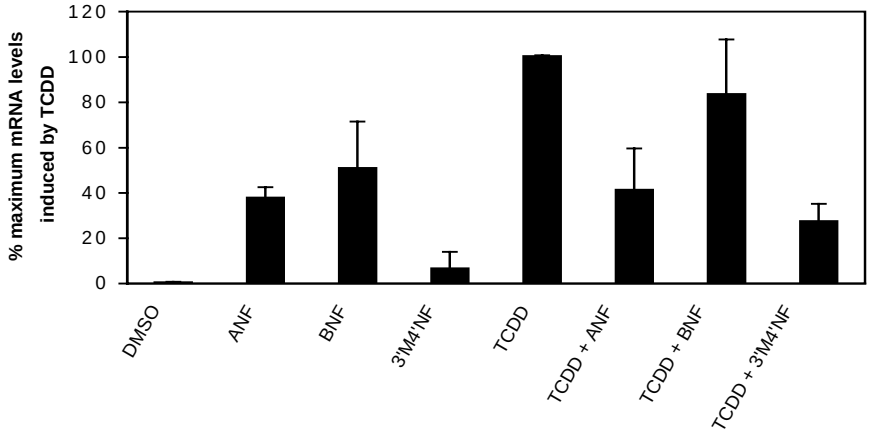

Figure 2: Effects of flavonoids on CYP1A1 mRNA levels in WB-F344 cells as detected by real-time quantitative RT-PCR. Cells were incubated with flavonoids (1 μM) and/or TCDD (100 pM) for 6 hours. The bars represent means +/- SD of two independent experiments.

Table 1: Summary of effects of selected AhR ligands on contact inhibition in rat liver WB-F344 cells

Compound	Increase of cell numbers	Increase of S-phase percentage	Increase of cyclin A levels and cyclin A/cdk2 activity
TCDD [3]	+	+	+
PCB 126 [a]	+	+	+
PCB 153 [a]	-	-	-
Benzo[b]fluoranthene [5]	+	+	n.a.
Fluorene [5]	-	-	n.a.
ANF	+	+	n.a.
BNF	+	+	n.a.
3'M4'NF	+	+	n.a.

[a] manuscript in preparation; n.a. – not analyzed.

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