RESVERATROL IS A COMPETITIVE ANTAGONIST ON THE ARYL HYDROCARBON RECEPTOR.

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Halogenated and/or polycyclic aryl hydrocarbons (dioxin, benzo(a)pyrene (BaP), are industrial chemicals which bind the aryl hydrocarbon receptor (AhR) and which we will refer to as **AhR ligands**. Such compounds, which are also present in cigarette smoke, are environmental toxicants which have been shown to cause immunosuppression (1), cancer and ischemic heart diseases (2). In addition, they have been linked to DNA damage (3), endothelial cell damage and to the development of atherosclerosis (4). While screening for dioxin antagonists potentially usable in human medicine, we noticed that wine contains a variety of phenolic compounds with a chemical structure ressembling that of flavonoid ligands of the arylhydrocarbon receptor (AhR) with antagonistic abilities. We hypothesized that a component of red wine might have antagonistic activity on the AhR. We show here that the trihydroxystilbene resveratrol, found in red wine, is a pure AhR competitive antagonist and has the requisite properties of potency and non-toxicity to be a clinically useful prophylactic agent against arylhydrocarbon induced pathology.

Methods.

Chemicals : TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) was a gift from Dr S. Safe (Texas AM University, Texas). 2,3,7,8-tetrachloro (1,6-^{3}H) dibenzo-p-dioxin, 28 Ci/mmole was purchased from Terrachem (Lenexa, KS). All other chemicals were purchased from Sigma (France).

Transfection and reporter assay. T-47D cells were grown and transiently transfected as described (5). Stable transfection with a construct bearing a dioxin responsive element linked to the TK-CAT reporter gene and CAT assays were as described (5).

Immunoblotting experiments were performed as described (5).The rabbit polyclonal antibody against cytochrome P450 1A1 was from Daiichi Pure Chemicals Co (Japan).

Whole cell binding assay was performed as described (6).

In vivo antagonism. Groups of three female Sprague-Dawley rats (3-6 months old) were treated by subcutaneous injection of : 1 mg/kg BaP/DMBA ; 1 mg/kg BaP/DMBA plus 1 mg/kg resveratrol ; 1 mg/kg BaP/DMBA plus 5 mg/kg resveratrol ; vehicle alone (olive oil) or no treatment for the controls. Injections were performed on days 1 and 7. Rats were killed on day 11. Lungs and kidneys were removed and snap frozen in liquid nitrogen. Tissue samples were homogeneized and submitted to immunoblotting.

Results.

We used the T47D cell line stably transfected with a dioxin responsive reporter gene to challenge dioxin-mediated transactivation with various wine compounds. Among tested molecules, only the phytoalexin resveratrol inhibited dioxin-driven induction of transcription in this model (Fig. 1).

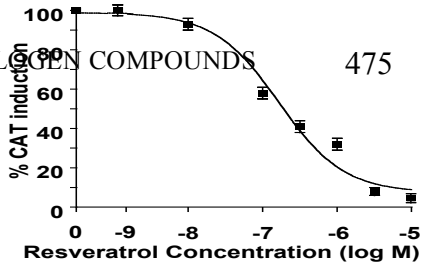

Figure 1: Resveratrol inhibits dioxin-mediated transactivation in a dose-dependent manner. T-47D were treated with 10^{-9} M dioxin alone or in the presence of resveratrol at concentrations ranging from 10^{-9} to 10^{-5} M. 100 % dioxin transactivation was obtained by comparison of cells treated with 10^{-9} M dioxin versus control cells receiving 0.1 % ethanol.

Competition binding in T47D and Hep G2 cells (Fig. 2) showed that resveratrol displaced labeled dioxin from the AhR in both cell lines, with a 50 % Effective Concentration (EC 50) of 10^{-7} M. Total TCDD binding was 360 fm/mg protein in T47D cells and 150 fm/mg proteins in HepG2 cells. These *in-vitro* effect of resveratrol were confirmed *in vivo* by measuring cypA1 production in T-47D cells by immunoblottting (Fig. 3). Dioxin strongly induced cyp1A1 and resveratrol blocked the induction.

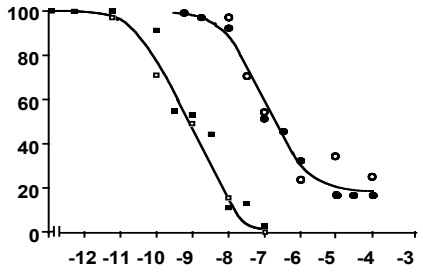

Figure 2: Binding competition assays for the arylhydrocarbon receptor (AhR). Whole cell binding competition assay performed with labeled dioxin (2 nM) versus unlabeled dioxin (squares) or resveratrol (circles) on T47D cells (closed symbols) or HepG2 (open symbols). Percentage of total binding is shown plotted against the log of competitor concentration.

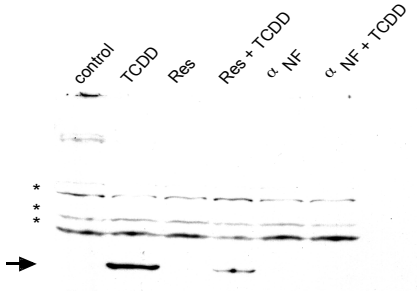

Figure 3: Resveratrol inhibits induction of cytochrome P4501A1 by dioxin in T-47D cells. Cells were treated by ethanol, 10^{-10} M dioxin (TCDD), 5.10^{-6} M resveratrol (Res), a combination of dioxin and resveratrol (TCDD + Res), 10^{-6} M α-naphthoflavone (α-NF) or a combination of dioxin and α-naphthoflavone (TCDD + α-NF). Cell extracts were submitted to Western blotting analysis as described. Arrow points to the specific CYP 1A1 band, stars to non-specific bands.

This effect of resveratrol was not an isolated phenomenon : we repeated our experiment on two known dioxin-sensitive genes: Interleukin 1β and the promoter in the HIV1 long terminal repeat (LTR). Resveratrol strongly repressed the stimulatory effects of dioxin on pre-interleukin 1b expression detected by immunoblot in RL-95-2 cells (not shown). The HIV1-LTR promoter CAT construct transfected in T-47D cells was induced 3-fold by dioxin, and this was abolished by resveratrol (Fig. 4). Gel retardation experiments showed that resveratrol induced a similar degree of DNA binding of AhR compared to dioxin (not shown). Binding was specific as shown by its suppression upon addition of an anti-AhR antibody during preincubation. Neither resveratrol nor

α-NF inhibited dioxin-mediated AhR DNA binding. The inhibitory activity of resveratrol therefore takes place during the interaction between AhR and the transcriptional complex.

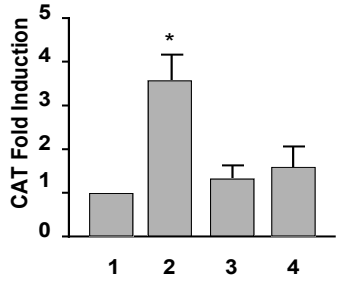

Figure 4: Resveratrol inhibits the transactivation of HIV-CAT by dioxin. T47-D cells were transiently transfected with a HIV (-453/+82)-CAT construct bearing the promoter from the HIV long terminal repeat Cells were treated with 10^{-9} M dioxin (lane 2), 5.10^{-6} M resveratrol alone (lane 3) or in combination (lane 4), Lane 1 : controls. Transfections were performed in triplicate. *: p=0.01 by one way ANOVA on ranks followed by Dunnett's test for multiple comparisons using Sigmastat (SPSS, Chicago, IL).

Finally, we extended the results obtained *ex-vivo* on the effects of AhR ligands on CYP 1A1 protein production by assessing these effects as well as the protective effects of resveratrol *in vivo* in various tissues of the rat. Female Sprague-Dawley rats were treated as described in Methods with a combination of BaP and DMBA (dimethylbenzanthracene), two AhR ligands found in cigarette smoke, plus or minus resveratrol. As can be seen in Figure 5, BaP/DMBA elicited CYP 1A1 expression in lung and kidney and this induction was totally suppressed by resveratrol. Moreover, the competition was complete in conditions of equal concentrations of BaP/DMBA and resveratrol, showing a greater efficiency of resveratrol *in vivo* when compared to *ex vivo* experiments. This observation suggests that resveratrol may be metabolized into a more active compound in the intact animal, thus acting as a pro-drug.

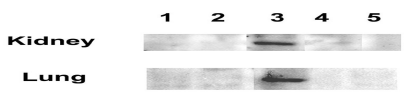

Figure 5: Effect of resveratrol on the induction of CYP1A1 in rat lung and kidney by AhR ligand treatment *in vivo*. Lane 1 : control animals. Lane 2 : vehicule only (olive oil). Lane 3 : 1 mg/kg BaP/DMBA. Lane 4 : 1 mg/kg BaP/DMBA plus 1 mg/kg resveratrol. Lane 5 : 1 mg/kg BaP/DMBA plus 5 mg/kg resveratrol. Lane 4 : 5 mg/kg resveratrol. Each lane contains 30 µg (lung) or 60 µg (kidney) of whole tissue extract proteins. Western blot was performed as in Figure 3.

Discussion.

Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexin, in a variety of spermatophyte plants including eucalyptus, peanuts and grapes (7). Resveratrol is present in red wine in concentrations between 1 and 8 mg/L (8). It is not water-soluble. Resveratrol has previously been proposed as being responsible for the cardioprotective effects of red wine leading to the so-called « French Paradox » (9). Resveratrol was later dismissed as having no additive effect on platelet aggregation and lipid metabolism when compared to alcohol alone (10).

Arylhydrocarbons have been implicated in a variety of diseases including cancer and immunosuppression (1,2). Epidemiological studies of carcinogenic, inflammatory and/or cardiovascular effects of dioxins have proven hard to show unequivocal results. It appears now that insufficient duration of follow-up in these studies may explain these difficulties. Indeed, recent studies on the Seveso population now detect an increase in cardiovascular mortality (11) that was rarey reported previously (2). AhR ligands such as dioxins, benzo(a)pyrene or DMBA (present in cigarette smoke) interact with AhR present in endothelial cells and other cell lineages of the cardiovascular system where they have been shown to cause endothelial cell damage and dysfunction through ROS generation secondary to CYP1A1 activation (4). AhR ligands, through ROS generation , are also able to cause peroxidation of low density lipoproteins (LDL) leading to the formation of cytotoxic oxysterols (12). Accordingly, AhR ligands (and especially benzo(a)pyrene and DMBA from tobacco smoke) have historically been suspected to be involved in atherogenesis (13).

We demonstrate here that resveratrol is able to compete with dioxin for AhR binding, and efficiently counteract the effects of AhR ligands on gene expression without any agonist activity. Our data suggest that resveratrol may prevent the toxic effects of AhR ligands on the cardiovascular system and other organs through a novel mechanism of antagonizing ligand binding to the AhR and preventing inflammatory endothelial cell damage subsequent to upregulation of genes such as CYP 1A1. In support of our hypothesis of a link between AhR activation and atherogenesis, it has been reported that leukocyte activation during the inflammatory processes of atherogenesis result in the activation of AhR and in its subsequent ability to transactivate CYP 1A1 expression in the absence of a clearly identified ligand (14). Clinical and epidemiological studies correlating resveratrol blood levels with cardiovascular protection will be necessary to confirm our hypothesis. Although less efficient against dioxin-mediated transactivation than α-naphthoflavone, resveratrol has the advantage over the latter compound of being devoid of any known toxicity (15). We propose : a) that resveratrol antagonism of AhR activation, linked to wine consumption, may be the cause for the so-called « French Paradox »; b) that since AhR ligands exert deleterious effects on the cardiovascular system as an important feature of their long-term toxicity, resveratrol could be used for the large-scale prevention of a variety of adverse effects of these environmental toxicants on the population.

References.

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