Natural Ah receptor agonists in the human diet : beneficial food components or unperceived risk factors?

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**Natural Ah receptor agonists in the human diet:
beneficial food components or unperceived risk factors?**

Pim de Waard

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Natural Ah receptor agonists in the human diet: beneficial food components or unperceived risk factors?

Proefschrift

ter verkrijging van de graad van doctor
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**Ich weiss es wohl, bejahrt und noch Student,
Bemooster Herr! Auch ein gelehrter Mann
Studiert so fort, weil er nicht anders kann.
So baut man sich ein mässig Kartenhaus,
Der grösste Geist baut's doch nicht völlig aus.**

Mephistopheles in Goethe's Faust

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LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon nuclear translocator
BaP	Benzo[<i>a</i>]pyrene
CPE	Citrus pulp extract
CYP	Cytochrome p450
DMSO	Dimethyl sulfoxide
DR CALUX	Dioxin Receptor Chemically-Activated LUCiferase eXpression
DRE-CAFLUX	Dioxin Responsive Element-driven Chemical Activated FLUorescent protein eXpression
EGFP	Enhanced green-fluorescent protein
EROD	Ethoxyresorufin-O-deethylase
GJE	Grapefruit juice extract
GST	Glutathione-S-transferase
I3C	Indole-3-carbinol
ICZ	Indolo[3,2- <i>b</i>]carbazole
NAhRA	Natural AhR agonist
NER	Nucleotide excision repair
NQO	Quinone oxidoreductase
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
UDPGT	Uridine diphosphate glucuronosyl transferase

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General introduction

FOOD, HEALTH AND TOXICOLOGY

The human diet is mainly based on food items containing carbohydrates, proteins and fat of animal or plant origin for acquiring energy and growth. Besides these macronutrients, food contains a large amount of micronutrients and other compounds of different nature, which notwithstanding its relative small part in weight can have a great impact on health. Animal products can contain e.g. minerals, hormones and vitamins, and vegetables and fruits contain besides the micronutrients many secondary metabolites like tannins, alkaloids, glucosinolates, polyphenols, coumarins and terpenes. This diverse group of phytochemicals causes the coloration, flavoring and odorization of the plant parts, and can achieve attractive or repellent effects on organisms. For humans, many phytochemicals can show both healthy and toxic effects, and many are used as medicines and poisons (Renwick *et al.*, 2004; Rietjens *et al.*, 2005). Since the complex nature of foods and the varying influences on human health, there is a growing interest in optimizing the human diet for health purposes in the form of food supplements, functional foods and nutraceuticals (Bagchi, 2006). However, not always is the mechanism of action of these bioactivating food ingredients elucidated, and many of them can show both toxic and healthy effects depending on the circumstances. Thus understanding toxicology plays an important role in determining when an enrichment of a food component in the human diet will be safe (Verschoyle *et al.*, 2007). In this thesis, a group of food chemicals of natural origin has been investigated which is considered as beneficial for human health, but at the same time shows a bio-activity that can be related to the toxic mechanism of well-known environmental pollutants like dioxins and polychlorinated biphenyls (PCBs), namely activation of the aryl hydrocarbon receptor (AhR).

THE ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AhR) was originally identified and characterized due to its central role in the cellular response to many planar aromatic hydrocarbons. This receptor is a cytosolic receptor protein of the bHLH-PAS (basic-helix-loop-helix/Period-Aryl hydrocarbon receptor nuclear translocator-Single minded) family of heterodimeric transcriptional regulators. Its well conserved gene is expressed in most cell types of mammals, birds, fish, and invertebrates. The AhR is a transcriptional regulator and it is suggested that it plays physiological roles in cell proliferation and differentiation, in liver and immune system homeostasis and in tumor development, however, the endogenous ligand(s) and the functional role of the receptor activation are still largely unknown (Hankinson, 1995; Hahn, 2002; Barouki *et al.*, 2007). The AhR received much attention for its role in the toxicity of the dioxins, a well-known group of environmental pollutants of which TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is the strongest agonist and activator in the picomolar concentration range. Many different species of laboratory animals showed a wide range of toxicological responses upon exposure to TCDD, e.g. tumor promotion, carcinogenesis, endocrine disruption, immunotoxicity and teratogenicity, which are related to activation of the AhR (Fernandez-Salguero *et al.*, 1996; Mimura and Fujii-Kuriyama, 2003; Steenland *et al.*, 2004). However, a large difference in susceptibility between species is observed (Safe, 2001; Bock and Kohle, 2006). As for humans, it

is known that after accidental exposure to relatively high concentrations of TCDD, like during the disaster in Seveso, Italy 1977, chloracne is evidently proven, and tumor promotion of certain cancer types is highly probable, because of an increased cancer incidence and mortality at all cancer sites after dioxine exposures rather than at a specific site (Steenland *et al.*, 2004; Landi *et al.*, 2005).

Figure 1 shows a simplified diagram of the activation mechanism of the AhR. In the cytosol, the AhR is bound to two heat shock proteins (hsp90), and the proteins XAP2 (X-associated protein 2) and p23 (23-kDA co-chaperone protein). When a ligand, e.g. TCDD, enters the cell, it binds to the AhR and the protein complex moves to the nucleus of the cell. After entering the nucleus, the chaperon proteins detach and the AhR-ligand complex binds to the aryl hydrocarbon nuclear translocator protein (ARNT). Together, they bind to the DNA at dioxin responding elements (DRE) located in the promoter region of many AhR responding genes, e.g. the phase I biotransformation

Fig. 1. Schematic presentation of the AhR activation. See the text for an explanation.

L	Ligand
AhR	Aryl hydrocarbon receptor
hsp90	Heat shock protein 90
p23	23-kDA co-chaperone protein
XAP2	X-associated protein 2
ARNT	Aryl hydrocarbon nuclear translocator
DRE	Dioxin responsive element
CYP	Cytochrome p450
UDPGT	Uridine diphosphate glucuronosyl transferase
GST	Glutathione-S-transferase
NQO	Quinone oxidoreductase

P450 cytochromes CYP1A1, CYP1A2 and CYP1B1, and the phase II biotransformation enzymes glutathione-S-transferase P1 (GSTP1), uridine diphosphate glucuronosyl transferase 1A6 (UGT1A6) and NAD(P)H quinone oxidoreductase 1 (NQO1) (Hankinson, 1995; Denison *et al.*, 2002; Barouki *et al.*, 2007). These induced enzymes may play an important role in the detoxification of xenobiotic compounds like polycyclic aromatic hydrocarbons (PAHs), which are also agonists of the AhR, and may therefore stimulate their own metabolic conversion (Baird *et al.*, 2005). TCDD is metabolized very slow in the human body and accumulates in fatty tissues, which is thought to be the main reason for its toxic potency (Bock and Kohle, 2006). The estimated half-life of TCDD in rats is 21 days (Pohjanvirta *et al.*, 1990), and in humans even 6-7 years (Maruyama and Aoki, 2006). In some animal models, TCDD has particularly toxic potency in the developing embryo, causing structural malformations such as hydronephrosis and cleft palate (Couture *et al.*, 1990). The more sustained or prolonged AhR activation by TCDD probably caused the observed cleft palate in mice, while the suspected endogenous potent AhR ligand ITE (2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, isolated from porcine lung tissue) did not show this effect (Henry *et al.*, 2006).

Low amounts of dioxin-like compounds in food or environmental samples can be measured by gas chromatography techniques with mass spectrometry detection (GC-MS), but for high throughput screening of AhR agonist activity in samples, the DR-CALUX[®] (Dioxin Receptor Chemically-Activated LUCiferase eXpression) reporter gene bioassay has been developed. Rat hepatoma H4IIE cells have been stably transfected with the reporter vector pGudLuc1.1 containing a dioxin-responsive element-driven firefly luciferase gene. The induction of luciferase by AhR-agonists can be detected by addition of luciferin and measurement of the resulting bioluminescence (Aarts *et al.*, 1995; Garrison *et al.*, 1996). The amount of persistent dioxin-like AhR agonists is expressed as TEQs (toxic equivalents), which is the concentration of a dioxin-like congener multiplied by its toxic equivalency factor (TEF) to relate its toxicity to that of TCDD (Van den Berg *et al.*, 1998). The tolerable daily intake of dioxin-like compounds has been established by the WHO at 2 pg TEQ/kg/day or 140 pg TEQ/day for a person of 70 kg (JECFA, 2002). Similarly the EC Scientific Committee for Food set the tolerable weekly intake to 14 pg TEQ/kg/week, taking into consideration the accumulating effect (SCF, 2001).

NATURAL AHR AGONISTS

In 1971 Wattenberg discovered that laboratory rats showed an increase in AhR activity (by then called induction of benzo[a]pyrene hydroxylase or arylhydrocarbon hydroxylase) after feeding with cruciferous vegetables (Wattenberg, 1971). Analysis of the food ingredients revealed that the compounds which were responsible for the AhR activation were indoles, especially indole-3-carbinol (I3C) (Loub *et al.*, 1975). These are metabolites from one of the omnipresent cruciferous glucosinolates, namely glucobrassicin. This secondary plant metabolite is rapidly transformed into I3C, glucose, isothiocyanate and sulphate by the enzyme myrosinase. This enzyme is stored in separate compartments of the plant cells and is released after crushing of the plant tissue, implying that I3C

is also formed after consumption of glucosinolate containing vegetables (Virtanen, 1967; Broadbent and Broadbent, 1998a). I3C undergoes self-condensation reactions in the acid environment of the stomach and forms a number of dimers, trimers and other types of heterocyclic aromates (Fig. 2). Whereas I3C is not a strong AhR activator by itself, one of the acid reaction products, indolo[3,2-*b*]carbazole (ICZ) is a potent AhR activating compound. However, it is only formed in very low amounts (ca 8 ng/mg I3C). It is still unclear which compounds of the acid reaction mixture are the most important in the AhR activation (Bjeldanes *et al.*, 1991; De Kruif *et al.*, 1991; Rogan, 2006). Nowadays, many different natural AhR agonists (NAhRAs) and sources of NAhRAs have been found, e.g. the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole (Wei *et al.*, 1998),

Fig. 2. The metabolism of glucobrassicin into bioactive acid reaction products. Of the many acid reaction products formed in the stomach, only the four most important ones are shown. ICZ, indolo[3,2-*b*]carbazole; LT, linear trimer; CT, cyclic trimer; DIM, diindolyl methane.

Table 1. Some AhR-activating compounds identified in the human diet (Denison and Nagy, 2003; van Ede *et al.*, 2008).

Compound	Origin
7,8-Dihydrorutacarpine	Citrus fruits
Carotinoids (canthaxanthin, astaxanthin, β -apo-8'carotenal)	Many plants, mushrooms, algae
Curcumin	Curry spice
Dibenzoylmethanes	Licorice
Flavonoids (quercetin, diosmin, tangeretin, tamarixetin)	Many vegetables and fruits
Furocoumarins (bergamottin, bergapten)	Citrus fruits
Indole-3-carbinol	Cruciferous vegetables

indirubin from natural dye compounds (Adachi *et al.*, 2001), furocoumarins, like bergamottin, and polymethoxyflavones, like tangeretin, from citrus fruits (Canivenc-Lavier *et al.*, 1996; Baumgart *et al.*, 2005) and extracts of many fruits, vegetables and herbals from the human diet, suggesting a high exposure of humans (Amakura *et al.*, 2003a; Jeuken *et al.*, 2003; van Ede *et al.*, 2008). A number of AhR-activating compounds in these food items have been identified (Table 1). Still, ICZ is one of the strongest AhR binding and activating NAHRAs (Denison and Nagy, 2003).

TOXIC VERSUS BENEFICIAL EFFECTS OF AHR AGONISTS

During the early days of NAhRA discovery, the induction of CYP1A1 by the activation of the AhR, was thought to be the main cause of the healthy, anti-cancer properties of cruciferous vegetables (Wattenberg, 1971). The induction of biotransformation enzymes, also called detoxification enzymes, was thought to be a first step in eliminating toxic compounds quickly from the cell. However, the CYP1A1 induction is also related to the cancer formation process caused by PAHs, of which benzo[*a*]pyrene (BaP) is a well-known example. BaP itself is not a potent toxic compound, but biotransformation enzymes like CYP1A1 convert it into reactive electrophilic metabolites as e.g. benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE), which can easily bind to the DNA to form DNA adducts that may cause mutations and ultimately may lead to cancer (Baird *et al.*, 2005). Against these toxic effects several cellular defense mechanisms exist. Phase II enzymes like glutathione-S-transferase (GST) and uridine diphosphate glucuronosyl transferase (UDPGT) can transform the electrophilic compounds into products which can be eliminated rapidly from the cell. In addition, DNA repair enzymes, like the nucleotide excision repair (NER) system, remove the bulky BaP-DNA adducts from the DNA (Hanawalt *et al.*, 2003; Baird *et al.*, 2005). This dynamic balance of metabolic pathways (Fig. 3) may be influenced by AhR agonists, and may thus influence the carcinogenic risk, because these agonists induce both phase I and phase II biotransformation enzymes and may direct the balance into a beneficial way or into an opposite way.

TCDD is considered to be a carcinogen in view of its tumor promoting properties that have been established in many animal experiments (Steenland *et al.*, 2004). Also I3C has been investigated for its modulation of cancer inducing processes, and many experiments in rat, mouse and trout showed a tumor suppressive effect in breast, liver or colon. However, a number of experiments

Fig. 3. The dynamic balance of possible metabolic routes of benzo[a]pyrene. CYP, cytochrome P450; GST, glutathione-S-transferase; UDPGT, uridine diphosphate glucuronosyl transferase; NER, nucleotide excision repair.

showed a tumor promotive or enhancing effect in liver or colon in these animal models, especially when I3C was given after the tumor initiating chemicals (Verhoeven *et al.*, 1997; Broadbent and Broadbent, 1998b; Dashwood, 1998; Aggarwal and Ichikawa, 2005). This indicates that under certain circumstances the NAhRAs can induce similar tumor promotive mechanisms as xenobiotic AhR-agonists like TCDD. In view of the dualistic character of the role of the AhR, it is unfortunate that only a few studies compared the effects of I3C directly with TCDD. A number of *in vitro* and *in vivo* experiments show comparable effects of exposures to I3C or ICZ and TCDD, both toxic and beneficial. Both types of agonists show beneficial anti-estrogenic properties (Liu *et al.*, 1994; Safe, 1995), inhibition of gap-junctional intercellular communication (GJIC), which is considered to play a role in tumor promotion (Herrmann *et al.*, 2002), increased COX-2 expression leading to enhanced prostaglandin E2 production and colon cancer progression (Sherratt *et al.*, 2003), increased oxidative DNA damage (Bjeldanes *et al.*, 1991; Chen *et al.*, 1995; Park *et al.*, 1996; Pohjanvirta *et al.*, 2002), and toxic reproductive effects in rats (Wilker *et al.*, 1996). These observations emphasize the importance for more knowledge of the mechanisms that lies behind the effects of NAhRAs and that can be used for proper risk-benefit analyses.

A complicating factor in the risk-benefit analysis of NAhRA consumption is that humans are usually exposed to these compounds in complex mixtures like foods (Denison and Nagy, 2003; Jeuken *et al.*, 2003). Apart from NAhRAs, food products can also contain considerable amounts of AhR antagonists. Furthermore, some NAhRAs can show antagonistic effects on AhR activation by xenobiotic agonists, or inhibition of AhR induced enzymes (Amakura *et al.*, 2003b). This raises the question to what extent these antagonists may influence the toxic effects of dioxin-like compounds.

EPIDEMIOLOGY OF AhR AGONIST INDUCED HEALTH EFFECTS

In contrast to the predominantly higher tumor incidence caused by dioxins found in epidemiological studies (Steenland *et al.*, 2004), high intake of NAhRA containing foods show tumor suppressive effects in most epidemiological studies (Steinmetz and Potter, 1996; Verhoeven *et al.*, 1996; Heber, 2004). However, a number of epidemiological studies on fruit and vegetable consumption does not show such a clear tumor suppression, and sometimes even tumor promotive effects are shown (Verhoeven *et al.*, 1996; van Gils *et al.*, 2005; Lynn *et al.*, 2006).

It may be very difficult to identify the main cause of a tumor suppressive effect. For instance, according to the WCRF-report “Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective”, cruciferous vegetables show a negative correlation with endometrial, oesophageal and oral cancer, but it is not clear whether this is caused by indoles or isothiocyanates, the other glucosinolate metabolites (WCRF/AICR, 2007). Isothiocyanates show a strong induction of phase II enzymes like GST and corresponding anti-oxidative effects by activating the electrophile-responsive element (Seow *et al.*, 2002). Possibly, the combination of these food constituents is causing the protective effect. Besides, also harmful effects of consumption of cruciferous vegetables have been found. Figure 4 shows a number of cohort studies of consumption of cruciferous vegetables with both negative and positive effects on colon cancer (Lynn *et al.*, 2006).

The modern trend to supply possible chemoprotective ingredients from fruit or vegetables in purified form and in sometimes extremely high concentrations as compared to normal dietary intake, makes it even more urgent to evaluate the health effects of NAhRA intake in epidemiological studies (Noonan and Noonan, 2006). For instance, the intake of 500 mg I3C several times a day, instead of a few mg in a meal of broccoli or Brussels sprouts, and thus without the assumed beneficial effects of the isothiocyanates, theoretically could push the balance towards undesirable (toxic) side effects. A number of clinical trials have been carried out to examine the chemoprotective effects of I3C in women on breast and cervical cancer (Aggarwal and Ichikawa, 2005). One of the main findings was an increase of the estrogen metabolite ratio in urine of 2-hydroxyestrone to 16 α -hydroxyestrone, which is a surrogate endpoint biomarker for reduced risk of breast cancer and caused by AhR-dependent CYP1A1/1A2 activation (Bradlow *et al.*, 1996; Aggarwal and Ichikawa, 2005). Together with an increase of the 2-/16 α -hydroxyestrone ratio, reduced precancerous lesions of the cervix were found in women (Bell *et al.*, 2000). No toxic or other side effects were found, but these studies are directed to breast and cervical cancer, and no conclusions can be drawn on the possible effects on the onset or outgrowth of other types of cancer (Aggarwal and Ichikawa, 2005).

Fig. 4. Cohort studies of colon cancer and cruciferous vegetable consumption. Values are relative risks and 95% CI represented by horizontal bars. F, females; M, males (Lynn *et al.*, 2006).

TOXICOGENOMICS AND BIOMARKERS

Despite more than 30 years of research on dioxin toxicity, the mechanistic pathways of TCDD-toxicity have not yet been revealed. The introduction of genomics tools in the field of toxicology offers new opportunities to study the possible toxic effects of NAhRAs. A relatively new tool for elucidating biological pathways involved in toxicological processes is the use of microarray technology (Aardema and MacGregor, 2002; Orphanides, 2003). With this technology, the expression of thousands of genes, called the transcriptome, can be determined simultaneously. After exposure of cells, tissues or laboratory animals, the mRNA is extracted from the cells of interest and transferred into fluorescent labeled cRNA or cDNA. Microarrays, which are chips or glass slides with thousands of different probes consisting of single strand cDNA molecules or oligonucleotides representing the genes, are exposed to the cRNA or cDNA samples. Each cRNA or cDNA hybridizes to its complementary sequence on the microarray and the fluorescent signal is measured. Difference in mRNA induction can be measured between exposed and control samples, and the resulting differential gene expression profiles can reveal certain affected biological pathways (Schena *et al.*, 1995; Lockhart *et al.*, 1996). RNA amplification techniques allow the use of very small amounts of RNA, in the order of nanomolars, to be extracted and purified. Figure 5 shows an example of such a procedure for a 22,000 spot oligonucleotide microarray of Agilent Technology, which we have used for gene expression profiling in this thesis. Shortly, the mRNA of extracted and purified RNA from e.g. exposed cells is converted into cDNA by use of reversed transferase (MMLV) and oligo dT-promotor primer. Subsequently, by addition of T7 RNA polymerase, NTPs and the dye Cy5-CTP to the cDNA, the cRNA is amplified and labeled. After purification, the cRNA can be hybridized to the microarray and the fluorescent dye signal can be measured with a fluorescence scanner.

Usually, the expression of a small number of genes is also measured using the real-time reverse transcription polymerase chain reaction technique (RT-PCR) for confirmation of microarray studies. In this assay, the expressed mRNA from one or a few genes is reverse transcribed into cDNA, which is subsequently labeled and amplified in a cyclic way. The amount of fluorescent product is measured after each cycle and accurately quantified (Bustin, 2002).

Fig. 5. Principle of the Agilent microarray fluorescent cRNA synthesis procedure (Agilent Technologies). Sample A is the exposed specimen, sample B is the control or a reference. See text for further explanation.

One of the main paradigms in toxicogenomics research is that certain profiles of gene expression can reveal mechanisms of toxicity and can be used as biomarkers for the involvement of specific toxicological pathways (Decristofaro and Daniels, 2008). In this way, the gene expression in cells exposed to NAhRAs can be compared to the expression profiles after exposure of the same type of cells to TCDD or other known AhR-agonists with a high toxic potential, like certain PAHs. This approach may elucidate whether or not the NAhRAs have similar effects at the transcriptome level as the toxic AhR-agonists or show significant different expression profiles, possibly indicating their beneficial potency. Shortly before the experiments started for this present thesis, a few microarray studies had been performed on human liver and airway epithelial cells after exposure to TCDD. Multiple genes were affected, especially concerning xenobiotic metabolism, cell cycle and cell proliferation. However, a clear understanding of the complicated molecular mechanisms governing the toxic effects of TCDD was not reached and a common biomarker for AhR-activation was not yet revealed (Puga *et al.*, 2000; Frueh *et al.*, 2001; Martinez *et al.*, 2002).

AIM AND OUTLINE OF THE THESIS

Based on the information presented above, it can be concluded that there is quite some uncertainty about potential hazardous and beneficial effects of NAhRAs in food and food supplements:

- There is convincing evidence that the toxic effects of dioxins and dioxin-like compounds are mediated by activation of the AhR pathway.
- Humans are frequently exposed to considerable amounts of NAhRAs in the diet.
- Some *in vitro* and *in vivo* experiments show dioxin-like toxicity of NAhRAs.
- Epidemiological studies of NAhRA-containing foods are neither conclusive about beneficial nor about harmful aspects of these types of foods.

The aim of this thesis is to investigate the similarities of NAhRAs with xenobiotic AhR agonists like TCDD and BaP at the level of gene expression and genotoxicity, and to determine if these effects of NAhRAs can be shown in humans. These insights may be helpful to understand the risks versus benefits that are related to intake of NAhRAs via the human diet. The emphasis will be put on those food categories which are thought to show beneficial health effects, like cruciferous vegetables and citrus fruits.

The outline of the study is as follows:

First, the effects on gene expression modulation in the human colon cancer cell line Caco-2 upon exposure to NAhRAs, BaP and TCDD is examined, in order to find similarities between these types of compounds at the transcriptomic level. As NAhRAs are chosen an extract of grapefruit juice, in the absence of a positive identification of the bioactive component, and ICZ, a representative pure component of the acid reaction mixture of I3C, originating from cruciferous vegetables. Typical

AhR responsive sets of genes found *in vitro* will be used as biomarkers for AhR-agonist exposure *in vivo* (**Chapter 2**).

Secondly, an investigation of the presence and amounts of natural AhR agonists in common consumed food items in The Netherlands is performed. For this purpose, the extracts of a number of frequently consumed food items is examined for AhR-agonistic activity using the DR CALUX[®] bioassay. The results are used to make an estimation of the daily exposure to NAhRAs in the human diet (**Chapter 3**).

Then, freshly isolated human lymphocytes are *ex vivo* exposed to xenobiotic AhR-agonists and NAhRAs to affirm the AhR-related gene expression profiles, found in the human Caco-2 cell line, in an easily obtainable human tissue. A human intervention study is followed, in which volunteers are offered diets enriched in NAhRA-containing foods. Blood and urine samples are collected before and after the intervention to determine AhR-related effects on gene expression in blood cells and on activity of biotransformation enzymes (**Chapter 4**). Because AhR-related effects in blood are very small, the AhR-activity in plasma was analysed with two reporter gene bioassays: DR CALUX[®], a commonly used screening assay for dioxin-like compounds in foods and environmental samples, versus DRE CAFLUX (Dioxin Responsive Element-driven Chemical Activated FLUorescent protein eXpression), a relatively new comparable gene reporter assay, with a promising technique to potentiate very low AhR-activity signals (**Chapter 5**).

The (anti-)genotoxic effects of NAhRAs and TCDD are investigated by determining their influence on BaP-DNA adduct formation in Caco-2 cells. Both NAhRAs and TCDD induce phase I and II biotransformation enzymes like CYP1A1, CYP1B1 and NQO1, which can convert BaP into reactive DNA-binding metabolites, a mechanism to cause tumor formation, but can also remove potential toxic compounds quickly from the cell. Effects at the transcription level of a number of phase I and II enzymes, and some nucleotide excision repair genes are compared with effects on the toxic endpoint of BaP-DNA adduct formation (**Chapter 6**).

Finally, **Chapter 7** contains a summary of the results and a general discussion.

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2

Gene expression profiling in Caco-2 human colon cells exposed to TCDD, benzo[a]pyrene, and natural Ah receptor agonists from cruciferous vegetables and citrus fruits

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ABSTRACT

Cruciferous vegetables and citrus fruits are reported to possess health-beneficial properties, but also have been shown to contain natural aryl hydrocarbon receptor (AhR) agonists (NAhRAs). Binding to the AhR is widely assumed to activate the main pathway by which dioxins, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exert their toxicity. To establish whether or not activation of the AhR pathway by NAhRAs and dioxin-like substances results in similar cellular responses, gene expression profiles induced in Caco-2 cells were studied using microarray analysis. Cells were exposed to indolo[3,2-*b*]carbazole (ICZ), an acid reaction product from cruciferous vegetables, and to extracts of citrus pulp and grapefruit juice. Gene expression profiles induced by these NAhRAs were compared to those of the xenobiotic AhR agonists TCDD and benzo[*a*]pyrene (B[a]P). Over 20 genes were found more than 1.5 times up- or downregulated by TCDD, and the expression of most of these genes was modulated in the same direction and to a similar extent by B[a]P and the NAhRAs. Results were confirmed by RT-PCR, and many of these genes may be involved in dioxin-related toxic effects. In conclusion, this *in vitro* study showed similar effects induced by NAhRAs, TCDD and B[a]P at the transcriptome level in a human intestinal cell line.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a transcription factor which is activated by ligand binding, and is expressed in most human tissue types. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototype AhR ligand and the most potent xenobiotic AhR agonist known. Upon ligand binding, the AhR-ligand complex is translocated into the nucleus of the cell and functions as a transcription factor binding to certain enhancer elements, called dioxin-responsive elements (DREs). This results in altered expression levels of AhR-responsive genes, among them the genes encoding for the biotransformation enzymes CYP1A1, CYP1A2, CYP1B1, UDPGT1A6, and GSTP1 (Hankinson, 1995; Denison and Nagy, 2003). Most if not all toxic effects of TCDD and other dioxin-like compounds are thought to be mediated by activation of AhR-regulated gene expression, e.g. chloracne, tumor promotion, immunosuppression and teratogenesis (Fernandez-Salguero *et al.*, 1996; Mimura and Fujii-Kuriyama, 2003; Steenland *et al.*, 2004).

Besides environmental contaminants like certain dioxins, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), also AhR agonists from plant and other biotic origins are known (Denison and Nagy, 2003). Cruciferous vegetables like Brussels sprouts, cabbage and broccoli contain e.g. AhR agonist precursors in the form of glucosinolates, which are converted via indole-3-carbinol (I3C) into I3C condensation products during food preparation, consumption and subsequent incubation in the acid environment of the stomach (Bjeldanes *et al.*, 1991; Broadbent and Broadbent, 1998). Examples of these compounds are diindolylmethane (DIM) and indolo[3,2-*b*]carbazole (ICZ), of which the latter is a very potent AhR agonist (Kleman *et al.*, 1994; Bergman *et al.*, 2001). Also many other food items appear to contain high levels of AhR-activity, as judged by the DR CALUX[®] bioassay (Jeuken *et al.*, 2003). Recently it was reported that hexane extracts of citrus products show strong AhR agonist activity (Hoogenboom, 2002). It is known that citrus fruits contain furocoumarins, like bergamottin, and polymethoxyflavones, like tangeretin, which were reported to be AhR agonists (Canivenc-Lavier *et al.*, 1996; Baumgart *et al.*, 2005).

Considering that both NAhRAs and persistent xenobiotic AhR agonists trigger the same gene regulatory pathway, it may be expected that the gene expression patterns induced by both groups of AhR agonists will be similar to a certain extent. Several studies describe AhR-mediated adverse effects of NAhRAs, especially for the I3C metabolite ICZ, such as inhibition of gap-junctional intercellular communication (GJIC), which is considered to play a role in tumor promotion (Herrmann *et al.*, 2002), increased COX-2 expression leading to enhanced prostaglandin E2 production and colon cancer progression (Sherratt *et al.*, 2003), and increased oxidative DNA damage (Bjeldanes *et al.*, 1991; Chen *et al.*, 1995; Park *et al.*, 1996; Pohjanvirta *et al.*, 2002). However, because NAhRAs were found to induce beneficial gene expression effects, such as phase II biotransformation and antioxidant enzyme induction (Chen and Yang, 2002), inhibition of phase I-mediated procarcinogen activation (Wen *et al.*, 2002; Sarkar and Li, 2004), and antiestrogenic effects in human breast cancer cells (Liu *et al.*, 1994), they are actually considered to be natural cancer-chemopreventive agents. Furthermore, in an *in vivo* study in TCDD-sensitive rats relatively high doses of ICZ did not produce any of the

acute toxicity effects of TCDD, although moderate CYP1A1 induction in the liver was observed (Pohjanvirta *et al.*, 2002).

It is generally assumed that the apparent discrepancy between the toxicity of TCDD and compounds like ICZ is caused by their great difference in persistence in biological systems (Chen *et al.*, 1995; Pohjanvirta *et al.*, 2002). Pohjanvirta *et al.* estimated that the elimination half-life of ICZ in rats is less than a day, possibly in the order of a few hours, whereas the half-life of TCDD in rats is estimated at 21 days (Pohjanvirta *et al.*, 1990), and in humans even 6-7 years (Maruyama and Aoki, 2006). It cannot be excluded that the large difference in biostability between NAhRAs and persistent xenobiotic AhR agonists may result in differences between the gene sets modulated by both groups, and that selective AhR modulators as described by Safe *et al.* (Safe *et al.*, 1999) do exist within the large group of NAhRAs. None of these possible differences have been comprehensively studied thus far. This issue is even more relevant regarding the increasing popularity of dietary supplements with large amounts of pure I3C or DIM, especially if consumed on a regular basis (Zeligs, 2002). As a first step on examining this issue, the objective of the present study was to determine the gene expression profiles in the human intestinal epithelial cell-line Caco-2 after exposure to ICZ or extracts from citrus fruit products, and compare these with the profiles obtained after exposure to well known xenobiotic AhR agonists. These included the persistent dioxin TCDD as well as the readily biodegradable compound benzo[*a*]pyrene (B[a]P). The Caco-2 cell line was chosen since it is generally used as a model system to study the effect and absorption of compounds in the small intestine. By using microarrays, the expression of thousands of genes can be examined simultaneously. The more the transcriptome profiles look alike, the more likely it is that the compounds under investigation may exert similar biologic, pharmacologic or toxic effects (Aardema and MacGregor, 2002). This may also lead to the identification of a set of gene expression biomarkers for TCDD- or NAhRA-like effects. This study will focus on effects on the transcriptome level, but follow-up studies with e.g. human volunteers will need to take into account other possibly important factors, such as proteomics, absorption and metabolism.

MATERIALS AND METHODS

Preparation of extracts of citrus products

Citrus pulp, used for animal feed, was originally imported from the USA, grapefruit juice (1 litre of juice is obtained from ca 2.5 kg of grapefruit) was bought at a local supermarket. Five gram of pulp or 10 ml of juice were mixed with 20 ml of methanol/water 85/15, and after 30 min extracted twice with 20 ml of n-hexane/diethyl ether 97/3 following mixing for 1 h. Hexane-layers were collected, evaporated and the residue dissolved in 40 µl of DMSO (Merck, Darmstadt, Germany). Ten ml of water (Ultra Pure) was used for a blank extraction. Because the extracts contain a mixture of compounds, concentrations are expressed as gram extracted sample (pulp, juice or water) per litre medium used to expose the cells.

HPLC-analysis of extracts of citrus products

Twenty μ l of extract in DMSO was dissolved in 1 ml of methanol, and 100 μ l was analyzed on an HPLC-system (Waters, Milford, USA) using an in-house developed method. In short, samples were eluted over a Supelco LC-18-DB column, 250 mm x 4.6 mm/5 μ m, gradual elution of 0.8 ml/min. in methanol/water 10/90 -90/10 in 40 min. UV-detection of analytes was performed by diode array (Waters) at 325 nm. NAhRA components in the extracts were identified on the basis of co-elution with purified standards of the polymethoxy flavones sinensetin, tetramethylscutellarein, nobiletin, heptamethoxyflavone and tangeretin, and the furocoumarin bergamottin (all purchased from APIN Chemicals, Abingdon, UK), and in addition, by comparison of the UV-spectra. For concentration determination calibration curves of the standards were made.

Cell cultures and exposures

DR CALUX[®] cells (rat H4IIE hepatoma cells stably transfected with pGudluc1.1, an AhR-regulated luciferase reporter plasmid) (Aarts *et al.*, 1995) were grown in α -MEM (Sigma, St-Louis, USA), containing 10% fetal bovine serum (GIBCO, Breda, The Netherlands) and 1% penicillin/streptomycin (Sigma), at 37°C and 5% CO₂.

The Caco-2 human colorectal adenocarcinoma cell-line (ATCC, Manassas, USA) was grown in DMEM (Sigma), containing 10% fetal bovine serum and 1% penicillin/streptomycin, at 37°C and 5% CO₂.

Cells were seeded 24 h before exposure in 6-well plates (microarray assay, 5.10⁵ cells/well) or in 48 well-plates (DR CALUX[®] and EROD assays, 5.10⁴ cells/well) and exposed for 24 h to sample solutions in DMSO added to the culture medium. Concentration ranges were made of 1-5000 pM TCDD (purity 98%, Schmidt BV, Amsterdam, The Netherlands), 0.03-300 nM ICZ (purity >95%, synthesized as described by Bergman (Bergman, 1970) and checked for quality by HPLC, NMR, IR and MS, and with a reference sample (A kind gift of prof. J. Bergman, Department of Chemistry, Royal Institute of Technology, and Department of Biosciences at Novum, Huddinge, Sweden), 0.01-10000 nM benzo[a]pyrene (purity >98%, Aldrich, Steinheim, Germany), 3-625 g/l citrus pulp extract, and 5-1250 g/l grapefruit juice extract for the DR CALUX[®] and EROD assays. For the microarray analysis, cells were exposed to 0.5% DMSO, 500 pM TCDD, 1 μ M B[a]P, 100 nM ICZ, 208 g/l citrus pulp extract, 417 g/l grapefruit juice extract or 417 g/l blank extract. These concentrations are based on a near maximum AhR activation to obtain a high as possible signal in the microarray experiments. The final concentration of DMSO in all exposures was 0.5% (V/V).

DR CALUX[®] assay

The Dioxin Receptor - Chemical-Activated LUCiferase gene eXpression assay was performed as described previously (Bovee *et al.*, 1998). After exposure of the cells in 48-well microtiter plates the medium was removed and cells were washed with PBS. Cells were lysed using Cell Culture Lysis Reagent (Promega), centrifuged and 20 μ l of lysed solution of each well was transferred to a 96 wells plate. Luminescence was measured with a LuminoskanAscent (Thermo Labsystems, Finland) upon

addition of 0.1 ml of assay mixture (20 mM Tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM EDTA, 33.3 mM DTT, 261 μM Coenzyme A, 470 μM Luciferin, 530 μM ATP, pH 7.8). By use of the program SlideWrite V6 and the formula $a_0/(1+(x/a_1)^{a_2})$, which describes the AhR activation process suitably, EC_{50} and maximal response values were calculated.

EROD assay

The ethoxyresorufin-O-deethylase assay was performed according to Burke and Mayer (Burke and Mayer, 1974) with modifications, as described below. After exposure of the Caco-2 cells in 48-well microtiter plates the medium was removed and cells were washed twice with EMEM (Sigma, St-Louis, USA). A total of 150 μl of 5 μM ethoxyresorufin (Sigma) was added and the cells were incubated for 30 min. Subsequently, 50 μl of the solution was transferred into a 96 wells plate, 50 μl of 2 mg/ml sulfatase (Sulfatase from *Helix pomatia* type H-1, sulfatase > 10,000 U/g, β -glucuronidase > 300,000 U/g, Sigma) in 0.2M sodium acetate (pH 5) was added and incubated for one hour at 37°C and 5% CO_2 . After incubation, 175 μl of 0.15 M Tris-HCl pH 7.8 was added and fluorescence was measured by a spectrophotometer (LS 50B, Perkin Elmer, excitation 510 nm, emission 586 nm).

RNA isolation

After exposure total RNA was isolated from the cells using Trizol (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. Purity and integrity of the RNA were checked by gel electrophoresis and UV-absorption.

cDNA microarray

Microarray analysis was performed using an indirect labeling method as described previously (Pellis *et al.*, 2003). Twenty μg of total RNA per sample was incubated with SuperScript II Rnase H reverse-transcriptase (Invitrogen) and aminoallyl-dUTP (Sigma) was incorporated and coupled to Cy5 monofunctional dye (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Universal Human Reference RNA (Stratagene, La Jolla, USA) was used as a reference and after reverse transcription as described above, coupled to Cy3 monofunctional dye (Amersham). Before hybridization, the Cy3- and Cy5-labeled samples were mixed 1:1. Samples were hybridized to silylated slides (CEL Associates, Houston, TX), printed by a PixSys 7500 arrayer (Cartesian Technologies, Durham, NC) with 2304 spots of cDNA derived from a subtracted cDNA library, enriched for genes which are expressed in differentiated and undifferentiated Caco-2 cells (Nur *et al.*, 2002). Because a number of cDNA spots was corresponding to the same genes, approximately 1000 different genes were represented on the slides. After hybridization, the spots were scanned with the ScanArray Express HT scanner (Packard Biosciences, Boston, USA). The fluorescence signals were quantified using ArrayVision software (Imaging Research, Ontario, Canada), exported to Microsoft Excel, and normalized first by correcting the Cy5 signals with the Cy3 internal standard signals. Subsequently, the median of the adjusted Cy5 values was used to correct for possible differences between experiments with respect to the efficiency of labeling and the amount of label. The corrected values

were used for principle component analysis (PCA, GeneMath, Applied Maths, St-Martens-Latem, Belgium), a dimensioning technique that produces two- or three-dimensional plots in which the entries studied are spread according to their relatedness. The discriminant analyses calculates the best discriminating components for groups that are defined by the user, in our case the duplicate samples. For characterisation of the TCDD-induced gene expression profile, all genes of which the values of the duplicate exposures differed more than twice were excluded from analysis, and values of different spots representing the same genes were averaged. Because of the duplicate exposures of the cells to the samples, no statistical significance analysis was performed. Function of genes of interest were obtained from websites on <http://us.expasy.org/sprot> and <http://www.ncbi.nlm.nih.gov>.

Real-time RT-PCR

Two µg of total RNA was reverse transcribed into cDNA using 0.5 µl random hexamer primers (0.5 µg/µl) and 1 µl of 200 U/µl M-MLV reverse transcriptase (Invitrogen Life Technologies, Breda, The Netherlands) in a final volume of 20 µl. For amplification of β -actin, CYP1A1, CYP1B1, ODC1, MMP1, XAG, AR, DJ850, GLDC, and FGG (see for abbreviations) the following primers were used:

β -Actin: forward 5'-GCTGGCCGGGACCTGACTGACTAC-3';

reverse 5'-CGCTCGGCCGTGGTGGTGAA-3';

CYP1A1: forward 5'-CCATGTCGGCCACGGAGTTTC-3';

reverse 5'-CATGGCCCTGGTGGATTCTTCA-3';

CYP1B1: forward 5'-TTCCAGTGAGGGCAGCAGGTGTCCA-3';

reverse 5'-GCGGGTTTAGCGGCAAGGGTCGTT-3';

ODC1: forward 5'-ATGTGGGTGATTGGATGCTCTTTGA-3';

reverse 5'-TGCTGGCATCCTGTTCTCTACTTC-3';

MMP1: forward 5'-CAAACCCAAAAGCGTGTGA-3';

reverse 5'-ATCTCTGTGCGCAAATTCGTAAGC-3';

XAG: forward 5'-CAGAAATTGGCAGAGCAGTT-3';

reverse 5'-GCAGGTTGTAAGCATAGAGAC-3';

AR: forward 5'-GTCTCCACTCGCTCTTCCAACACC-3';

reverse 5'-GAGCCGAGTATCAAGAGCGACAGC-3';

DJ850E9: forward 5'-GGCCTAACCCCATACCAGCAGT-3';

reverse 5'-GTATTTCCCTCTTCCCCCTTTCTCT-3';

GLDC: forward 5'-GCGGCAGATCAATTTTCGGCTTTTTT-3';

reverse 5'-GAACGGGCTGGTCCTCTTGAACACA-3';

FGG: forward 5'-CCTGGGACAATGACAATGATAAGT-3';

reverse 5'-TTCCAAGTGGCCCAAATAATG-3'.

β -Actin was used as the internal control for differences in efficiency during the reverse transcription. The real-time PCR was carried out using the iCycler PCR machine (Biorad, Veenendaal, The

Netherlands) and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). Of each sample 6 dilutions were made based on the original amount of RNA: 0.781, 1.563, 3.125, 6.25, 12.5, and 25 ng cDNA in 5 μ l. Amplification was performed with 5 μ l diluted sample cDNA, 1 μ l of each primer (10 μ M) and 2x QuantiTect SYBR Green Master Mix in a final volume of 25 μ l. The PCR products were checked by melt curve peak analysis and by gel electrophoresis. Gene expression levels were calculated from the threshold cycle values and expressed as the ratio with the β -actin expression level.

RESULTS

Composition of the citrus extracts

The composition of the extracts from dried citrus pulp and grapefruit juice was analysed by HPLC-DAD to determine the content of reported and putative AhR agonists. The concentrations of these compounds, expressed in μ M in exposure medium of the microarray experiment, are shown in Table 1. The medium with extract of citrus pulp (CPE) contained 5 different polymethoxyflavones in the lower micromolar range as well as 0.4 μ M of the furocoumarin bergamottin, while the medium with extract of grapefruit juice (GJE) contained much less polymethoxyflavones but 6.9 μ M bergamottin. The chromatograms of both extracts revealed one other compound which has not yet been identified.

Determination of the relative AhR-activating potency

To assess their relative potencies to activate the transcription factor function of the AhR, the DR CALUX[®] assay was used to determine the response induced by citrus pulp and grapefruit juice extracts, ICZ, TCDD and B[a]P (Fig. 1). This reporter gene system utilises rat H4IIE hepatoma cells stably transfected with an AhR-controlled luciferase reporter gene, and enables quantification of the level of AhR-mediated gene expression. After 24 hours of exposure the EC₅₀ of TCDD, being the concentration inducing a half-maximal response, was 15 pM. For ICZ a maximal response as high as that for TCDD was found with an EC₅₀ of 8 nM, and B[a]P showed a maximum of 40% of that of TCDD and an EC₅₀ of 89 nM. CPE and GJE showed maximum luciferase expression activation of ca. 60% and 50%, respectively, compared to TCDD, although in the case of CPE no plateau level was reached. The extraction blank (ExBl) did not show any activation (Fig. 1).

Since the DR CALUX[®] is based on H4IIE rat hepatoma cells, whereas the gene expression studies were carried out in human Caco-2 cells, the validity for Caco-2 cells of the optimal exposure levels established on the basis of the DR CALUX[®] assay could be seriously compromised by substantial differences in ligand binding affinity and transactivation properties between the rat and human AhR. Therefore, the 7-ethoxyresorufin-O-deethylase (EROD) induction assay was used to measure the activity of the AhR-responsive enzyme Cytochrome P4501A1 in Caco-2 cells (Fig. 2). The EC₅₀ of TCDD for EROD induction in Caco-2 cells was 150 pM, which implies that this human cell-line is tenfold less sensitive to AhR activation as compared to the H4IIE rat cells, in which similar EC₅₀ values were found for EROD and luciferase induction (Hoogenboom *et al.*, 1999). Within the

Fig. 1 A **Fig. 1 B**

Fig. 1. AhR agonist activity of TCDD, B[a]P and ICZ (A), and Citrus pulp and Grapefruit juice extracts (B) in rat H4IIE DR CALUX* cells. Exposure time was 24 h, and results are expressed as percentage of the maximum response induced by TCDD. Error bars represent the standard deviation of triplicates. CPE, citrus pulp extract; GJE, grapefruit juice extract; ExBl, extraction blank.

ICZ concentration range used, the EROD induction did not reach a maximum, but extrapolation of the fitted curve indicated that only ca 74% of the maximum response level reached by TCDD could be attained at higher ICZ concentrations with an EC_{50} of ca. 42 nM. B[a]P had a maximum EROD induction level of 28%. The activation decreased above 300 nM, most likely due to the EROD activity-inhibiting properties of B[a]P (Bosveld *et al.*, 2002) (Fig. 2).

Table 1. Composition of the citrus pulp and grapefruit juice extracts as determined by HPLC

	Conc. (μ M)	
	CPE	GJE
Sinensetin	0.9	<0.1
Tetramethylscutellarein /nobiletin	1.8	<0.1
Heptamethoxyflavone	4.3	<0.1
Tangeretin	1.7	0.1
Bergamottin	0.4	6.9

Note. Concentrations are calculated as μ M in the exposure medium of the Caco-2 cells used for the microarray analysis. The medium contained the equivalent of 208 gram dried citruspulp or 417 gram grapefruit juice per liter. Tetramethylscutellarein and nobiletin could not be separated and therefore the sum of their concentrations is given.

CPE, citrus pulp extract; GJE, grapefruit juice extract.

Fig. 2 A **Fig. 2 B**

Fig. 2. EROD activity induced by TCDD, B[a]P and ICZ (A), and Citrus pulp and Grapefruit juice extracts (B) in human Caco-2 cells. Exposure time was 24 h, and results are expressed as percentage of the maximum response induced by TCDD. Error bars represent the standard deviation of triplicates. CPE, citrus pulp extract; GJE, grapefruit juice extract; ExBl, extraction blank.

CPE showed an EROD activation of 25% of the TCDD-maximum, but did not reach a plateau level within the tested concentration range. GJE did not induce a clearly increased activity (Fig. 2). It was hypothesized that this could be due to the presence of EROD-inhibiting compounds, like bergamottin (Baumgart *et al.*, 2005). In order to confirm this, the EROD-induction by mixtures of TCDD and GJE was measured. Indeed, Fig. 3 shows a concentration-dependent inhibition of the effect of TCDD by the grapefruit juice extract.

Modulation of gene expression by NAhRAs and xenobiotic dioxin-like compounds

To investigate the overlap in the gene expression profiles induced by NAhRAs and by the persistent reference compound TCDD or the readily biodegradable Ah-receptor agonist B[a]P, Caco-2 cells were exposed during 24 hours to 500 pM TCDD, 1 μ M B[a]P, 100 nM ICZ and citrus-extracts (208 g/l citrus pulp extract, 417 g/l grapefruit juice extract or 417 g/l blank extract) at concentration levels based on a significant response level in the DR CALUX[®] and EROD analysis with Caco-2 cells. The logarithm of the microarray fluorescence signals was imported into the GeneMaths program, and principal component analysis with discriminant was performed and plotted in a 3-D diagram, in which a total gene expression profile is represented by a dot (Fig. 4). The profiles for TCDD, B[a]P and the NAhRAs are clearly separated from those of the solvent DMSO and the blank extraction which cluster together. In particular the gene expression profiles induced by ICZ, GJE, and one of the B[a]P duplicates appear to plot very close to TCDD.

Fig. 3. Concentration-dependent inhibition of EROD-activity in Caco-2 cells by grapefruit juice extract (GJE). A TCDD-spiked blank extract did not show EROD inhibition (ExBl + 500 pM TCDD, $P > 0.05$, Student's t-test), while the inhibiting effects of both GJE concentrations + 500 pM TCDD were highly significant ($P < 0.001$). Error bars represent the standard deviation of triplicate exposures. The maximum effect of TCDD is the same as shown in Fig. 2.

In order to look for the genes affected by TCDD treatment versus treatment with the solvent only, all genes which were up- or down-regulated more than 1.5 times by TCDD were selected. In this way 22 TCDD-responsive genes were identified (Table 2). Table 2 includes the induction factors observed with the other compounds and extracts tested, and the functions and involvement in metabolic pathways of the responsive genes. CYP1B1 was added as no 23, despite an induction factor of only 1.4, because it is known from literature to be strongly up-regulated by TCDD (Hankinson, 1995; Spencer *et al.*, 1999; Martinez *et al.*, 2002) and showed a larger induction factor by using RT-PCR (see next paragraph). Most of the responsive genes that were found to be up-regulated by TCDD, are known to be involved in biotransformation, growth, cell proliferation, stress or the acute phase response.

In Fig. 5, gene expression ratios versus DMSO or ExBl are plotted from strongest up-regulation to strongest down-regulation by TCDD. The gene expression ratios found for TCDD (Fig. 5A) lie well above the profile for the DMSO control, and B[a]P and ICZ induced very similar gene expression profiles compared with TCDD. The citrus extracts were plotted against the blank extract (Fig. 5B). As shown in Fig 5A, the gene expression induced by the extract of the blank sample plotted against DMSO generated a profile which presented an insignificant experimental noise around the DMSO-line, indicating that the extraction procedure as such had not introduced any effect on gene

Fig. 4. Three-dimensional plot of principal component analysis (PCA) with discriminant from all 2304 spots of the Caco-2 microarray after 24h exposure of Caco-2 cells to TCDD, benzo[a]pyrene, ICZ and citrus extracts. The total gene expression pattern is visualized as a numbered dot; equal numbers represent duplicate exposures to the tested compound. X, Y and Z-axes represent the 1st, 2nd and 3rd principal component resp.

expression. As shown in Fig. 5B, the CPE and GJE up- or down-regulated most of the 23 dioxin-responsive genes in the same direction as TCDD. For 5 of these genes (AR, SLC20A1, FOS, PDZK1 and GLDC), exposure to CPE showed no or little effect, while GJE appeared not to affect 7 of these genes (MMP1, AR, DJ850E9, CYP1B1, GLDC, DIPP and SCAMP1). GLDC was also not affected by B[a]P and ICZ, and thus appeared to be specific for TCDD exposure. Genes that were more than 1.5 times up- or down-regulated by ICZ or the citrus extracts, but less than 1.5 times by TCDD, are not presented here, because they are unlikely to be dioxin toxicity-related genes and although interesting, beyond the scope of this study. However, preliminary analysis within this gene category showed that again mainly small differences between the responses induced by the NAHRAs and by TCDD were found (results not shown).

Real-time RT-PCR confirmation

Quantitative real-time Reverse Transcription PCR was used in order to validate the results of the microarray experiment. For this purpose 10 genes were selected: 9 of the 23 dioxin responsive genes and β -actin as a housekeeping gene for normalization. Nearly all results of the microarray analysis that were checked by real-time RT-PCR were clearly confirmed with respect to the up- or down-regulation of the gene expression (Fig. 6). The only qualitative difference was observed for

Table 2. Genes up- or downregulated upon exposure of Caco-2 cells to TCDD, benzo[*a*]pyrene and NAhRAs, their induction factors as determined by microarray analysis, and their biological functions

Gene name	Gene symbol	Acc. no. GenBank	Gene induction factors				Function of protein	
			TCDD	B[a]P	ICZ	ExBl	CPE	GJE
Growth/proliferation								
Ornithine decarboxylase	ODC1	NM002539	2.1	1.5	1.8	0.8	1.4	2.1
Matrix metalloproteinase 1	MMP1	NM002421	1.8	1.7	1.6	0.9	1.4	1.1
Anterior gradient 2 Xenopus laevis homolog	XAG	AF088867	1.7	1.3	1.6	0.8	1.4	1.8
Amphiregulin	AR	NM001657	1.7	1.5	1.4	1.3	1.0	0.9
Non-specific cross-reacting antigen	NCA	M18728	1.7	2.0	1.5	1.0	1.7	1.2
RNA helicase 2	DDX21	AF261917	1.6	1.4	1.5	0.9	1.3	1.7
Proto-oncogene protein c-fos	FOS	NM005252	1.6	1.2	1.4	0.9	0.9	1.3
Novel C2H2 type zinc finger protein	DJ850E9	AL121758	1.6	1.5	1.3	1.1	1.1	1.1
PDZ domain containing 1	PDZK1	NM002614	0.6	0.7	0.6	0.9	1.0	0.8
Secretory carrier membrane 1	SCAMP1	NM004866	0.5	0.7	0.7	0.8	0.6	1.0
Stress/Acute phase respons								
Heat shock protein 86	HSP86	X07270	1.8	1.5	1.8	1.2	1.4	1.5
Heat shock protein 89	HSP89	AF028832	1.7	1.2	1.6	0.8	1.7	1.5
Fibrinogen alpha	FGA	M64982	0.5	0.7	0.7	1.3	0.4	0.6
Diphosphoinositol polyphosphate phosphohydrolase	DIPP	AK026295	0.5	0.4	0.6	0.7	0.7	0.9
Fibrinogen gamma	FGG	AF118092	0.3	0.3	0.3	1.3	0.2	0.4
Xenobiotic metabolism								
Cytochrome P450 1A1	CYP1A1	NM000499	9.6	9.5	4.7	1.2	24.1	5.5
UDP-glucuronyltransferase 1A6	UDPGT	NM001072	1.5	1.8	1.1	0.9	2.1	1.4
Cytochrome P450 1B1	CYP1B1	NM000104	1.4	1.2	1.4	1.1	1.3	0.9
Glutathione S-transferase alpha-2	GSTA2	NM000846	0.6	0.8	0.7	1.1	0.7	0.8
Other								
Solute carrier family 20A1	SLC20A1	NM005415	1.6	1.5	1.4	1.1	0.8	1.3
Transferrin receptor	TFRC	AF187320	1.5	1.2	2.0	0.9	1.3	1.5
Ornithine aminotransferase	OAT	NM000274	0.6	0.6	0.7	0.9	0.6	0.9
Glycine decarboxylase	GLDC	M63635	0.6	0.9	0.9	1.0	0.8	1.2

Note. Those genes are shown that were up- or downregulated 1.5 times or more after 24 hours of exposure to TCDD (500 pM), except for CYP1B1, which is included since its expression is known to be increased by TCDD. For TCDD, B[a]P and ICZ, the induction factors are calculated as the ratio of the normalized hybridization signal versus the solvent DMSO, and for CPE (citrus pulp extract) and GJE (grapefruit juice extract) as the ratio versus the extraction blank ExBl. All results are averages of duplicate exposures.

Fig. 5 A

Fig. 5 B

Fig. 5. Gene expression profiles from microarray results. Expression characteristics are shown for CYP1B1 and 22 dioxin-responsive genes that were found by microarray analysis to be up- or down-regulated 1.5 times or more upon exposure of Caco-2 cells to 500 pM TCDD. A: Gene induction factors calculated versus the solvent DMSO upon exposure to TCDD, B[a]P, ICZ and ExBl. B: Gene induction factors calculated versus the extraction blank (ExBl) upon exposure to extracts of citrus pulp (CPE) and grapefruit juice (GJE). All dots represent averages of duplicate exposures.

Fig. 6 A

Fig. 6 B

Fig. 6. Gene expression characteristics of nine genes as determined by real-time RT-PCR. A: Gene induction factors calculated versus the solvent DMSO upon 24h of exposure of Caco-2 cells to TCDD, B[a]P, ICZ and ExBl. B: Gene induction factors versus the extraction blank (ExBl) upon exposure of Caco-2 cells to extracts of citrus pulp (CPE) and grapefruit juice (GJE). Error bars represent standard deviations of 2 to 5 measurements.

Table 3. Induction factors of nine genes responsive in Caco-2 cells upon exposure to TCDD, B[a]P or NAhRAs as determined by real-time RT-PCR, or microarray analysis without or with background subtraction (MA and MA-BS, respectively).

	TCDD						B[a]P						ICZ					
	mean	sd		n	MA	MA-BS	mean	sd		n	MA	MA-BS	mean	sd		n	MA	MA-BS
CYP1A1	218.1	47.9	***	4	9.6	51.8	205.2	81.5	***	4	9.5	53.0	63.3	5.4	***	4	4.7	22.4
ODC1	1.50	0.31	**	4	2.1	2.4	1.38	0.28	**	4	1.5	1.9	1.28	0.28	*	4	1.8	1.8
MMP1	3.55	0.88	***	4	1.8	3.7	3.47	0.61	*	2	1.7	2.9	2.68	0.12	**	2	1.6	2.6
XAG	1.13	0.20		4	1.7	1.5	1.57	0.44	**	4	1.3	1.3	1.32	0.19	**	4	1.6	1.2
AR	2.02	0.31	***	5	1.7	2.2	1.48	0.59	*	5	1.5	2.1	1.31	0.22	**	5	1.4	1.8
DJ850E9	1.47	0.02	***	2	1.6	1.4	2.38	0.09	**	2	1.5	1.9	1.71	0.24	*	2	1.3	1.3
CYP1B1	12.25	2.43	***	5	1.4	6.5	14.38	7.14	**	4	1.2	4.9	4.99	2.72	**	4	1.4	6.0
GLDC	0.64	0.15	*	2	0.6	0.9	0.66	0.03	**	2	0.9	1.1	0.60	0.00	***	2	0.9	0.8
FGG	0.12	0.01	***	2	0.3	0.3	0.10	0.02	***	2	0.3	0.3	0.11	0.02	***	2	0.3	0.3

	ExBI						CPE						GJE					
	mean	sd		n	MA	MA-BS	mean	sd		n	MA	MA-BS	mean	sd		n	MA	MA-BS
CYP1A1	1.54	0.42	*	3	1.2	0.9	326.0	156.3	**	4	24.1	165.6	49.2	7.0	***	4	5.5	34.0
ODC1	0.48	0.13	***	4	0.8	0.9	3.15	0.93	***	4	1.4	1.4	1.89	0.59	**	4	2.1	1.9
MMP1	0.40	0.13	***	5	0.9	1.0	6.33	1.87	*	2	1.4	2.3	2.20	0.89		2	1.1	1.4
XAG	0.84	0.18	*	4	0.8	0.7	1.20	0.31		4	1.4	1.3	1.19	0.40		4	1.8	1.5
AR	0.46	0.08	***	5	1.3	1.8	2.48	0.63	***	5	1.0	0.8	1.96	0.49	***	5	0.9	0.9
DJ850E9	1.12	0.28		2	1.1	0.9	1.81	0.46		2	1.1	1.4	1.37	0.15	*	2	1.1	1.5
CYP1B1	0.81	0.41		3	1.1	4.5	21.58	6.06	*	2	1.3	1.5	6.58	2.13	***	4	0.9	1.0
GLDC	0.87	0.25		2	1.0	1.1	0.79	0.30		2	0.8	0.7	0.75	0.24		2	1.2	1.0
FGG	0.60	0.10	*	2	1.3	1.2	0.24	0.13	**	2	0.2	0.2	0.32	0.01	***	2	0.4	0.4

Note. * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$ (Student's t-test; when $n=2$, it can only be considered as an indication); n, number of RT-PCR replicates; CPE, extract of citrus pulp; GJE, extract of grapefruit juice.

XAG, for which the RT-PCR, in contrast to the microarray, did not show up-regulation for most of the samples. To rule out malfunction of the used primer pair, another primer pair for XAG was developed with a different annealing site in the mRNA, however with the same results. Possibly, the up-regulation detected on the microarray was a false positive.

In addition, a few quantitative discrepancies were observed. In contrast to the non-conclusive microarray results, the genes for MMP1, AR, and DJ850E9 were found to be clearly up-regulated by GJE when measured by RT-PCR. AR was also found up-regulated by CPE (Table 3). Another remarkable result was that the ratio of 12.3 measured with RT-PCR for CYP1B1 induction by TCDD is much higher than the microarray result of 1.4, and also for the other compounds high ratios were found (5.0 - 21.6). The same holds for CYP1A1 (ratio for TCDD of 218 by RT-PCR versus 9.6 by microarray) and, to a lesser extent, for MMP1. A likely explanation for these discrepancies is that for the microarray analysis background subtraction had not been applied, because this resulted in a much higher variability than found with the overall signal values. Since CYP1A1, CYP1B1 and MMP1 had very low baseline expression levels, which produced only a small signal increase, subtraction of the background from the microarray signal has a relatively large influence on the exposed to control signal ratio. When including background subtraction in the analysis of the microarray data, the ratios found for these 3 genes increased considerably (Table 3). The lack of CYP1B1 induction, a known AhR-regulated gene, by TCDD, B[a]P and NAhRAs, as indicated by some microarray results, was contradicted by the RT-PCR control (Table 3).

In contrast to the microarray analysis, the RT-PCR also revealed that the 1.6 times down-regulation of GLDC by TCDD was not specific for TCDD, because all the other compounds were shown to down-regulate this gene too by 1.3-1.7 times (Table 3).

DISCUSSION

Many food items have been shown to contain high levels of Ah-receptor agonist activity, as judged by the DR CALUX[®] bioassay (Jeuken *et al.*, 2003). Furthermore several natural occurring compounds, or their acid reaction products, have been confirmed to be AhR-agonists (Bjeldanes *et al.*, 1991; Baumgart *et al.*, 2005). Currently, it is still unclear what causes the apparent profound difference in biological effects between NAhRAs and xenobiotic AhR agonists, and whether this is just a matter of the persistence of the compound in living cells and organisms, or whether additional differences in gene expression effects or of other nature are more crucial. As a result, it is hard to predict on the basis of current knowledge whether a substantial increase in the consumption of NAhRAs because of their presumed beneficial health effects would imply a risk for unexpected adverse AhR-mediated side effects.

Gene expression profiling using microarray technology may provide valuable, transcriptome-wide information about potential similarities or differences in the gene expression effects of compounds (Aardema and MacGregor, 2002). As a first step in evaluating the hypothetical adverse effects of NAhRAs in humans, the purpose of this study was to compare gene expression patterns in a human cell-line exposed to NAhRAs in comparison to those elicited by the well-known xenobiotic AhR agonists TCDD and benzo[*a*]pyrene (B[*a*]P). The latter compound was included in these studies, because it is rapidly metabolised compared with TCDD, and is thus expected to be comparable to NAhRAs in this respect. Therefore, if NAhRAs would turn out to induce a gene expression pattern which is clearly more related to that of B[*a*]P than to that of TCDD, this would indicate that the biological stability is a possible determinant of the difference in biological effect observed between NAhRAs and persistent AhR agonists such as TCDD. However, under the present conditions and despite the relatively long incubation period of 24 h, both the NAhRAs and B[*a*]P were found to display a gene expression profile similar to that of TCDD (Table 2, Fig. 5 and Fig. 6). This precludes any conclusion concerning the role of compound stability in the difference in biological effects between NAhRAs and TCDD. This may be a reflection of the predominance on the gene expression level of the dioxin-like toxic effects described for B[*a*]P-like PAHs, such as immunotoxicity and teratogenicity (Burchiel and Luster, 2001), indicating that NAhRAs may have at least the potential to cause similar effects.

TCDD exposure leads to various effects in which up- and down-regulation of many different genes appear to play a role, although a clear mechanistic view of TCDD-related effects has not been obtained thus far. The most extensively investigated genes known to be affected by exposure to dioxins are those involved in phase I and II biotransformation, like cytochrome P450s 1A1, 1A2, 1B1, and UDP-glucuronyl transferase 1A6 (Hankinson, 1995; Nebert *et al.*, 2000). Biotransformation enzymes play an important role in the detoxification of xenobiotic compounds, but some of them may also cause

negative effects on health, e.g. due to the production of toxic metabolites like genotoxic electrophiles and oxygen radicals (Agundez, 2004). In our study with the microarrays CYP1A1, CYP1B1 and UDPGT 1A6 were upregulated by all tested AhR agonists. Also many other genes, such as those related to signaling pathways, transcription factors, differentiation, apoptosis, cell development, cell adhesion, cancer and metastasis have been shown to be differentially regulated by TCDD (Hankinson, 1995; Nebert *et al.*, 2000; Puga *et al.*, 2000; Frueh *et al.*, 2001). Ten out of the 23 dioxin responsive genes in this study appear to be involved somehow in cell proliferation and are modulated by TCDD in the direction that they may stimulate tumor formation. ODC1, up-regulated 1.3-3.2 times by all tested AhR agonists (Table 3), is found elevated in human colorectal tumors and polyps (Radford *et al.*, 1990), and the use of ODC1 inhibitors has been proposed as a strategy for colon cancer prevention (Martinez *et al.*, 2003). MMP1, up-regulated 2.2-6.3 times by all test samples (Table 3), is a collagenase and plays a key role in matrix deposition and tissue remodeling that occurs constantly in both normal and diseased conditions. The expression of MMP1 is an important factor related to tumor invasion and metastasis in colorectal carcinoma (Malhotra *et al.*, 2002). This gene is also up-regulated by TCDD in the human HPL1A cell-line and in normal human keratinocytes (Martinez *et al.*, 2002; Murphy *et al.*, 2004). In the study of Murphy *et al.*, activation of the AhR pathway by TCDD was shown to interfere with the retinoic acid signaling in the skin, which may play a role in the development of skin disorders like acne. Puga *et al.* relate AhR-activity with retinoic acid function and the teratogenic effect of cleft palate induction in offspring of mice after TCDD administration (Puga *et al.*, 2005). Amphiregulin (AR) was up-regulated 1.3-2.5 times by all compounds and extracts (Table 3). The gene product is a member of the epidermal growth factor family and an autocrine growth factor for human keratinocytes. The expression of AR is increased in psoriatic epidermis and considered central in the development of this skin disease (Shoyab *et al.*, 1989; Cook *et al.*, 1997). AR is also a specific ligand of ERBB (encoding a tyrosine kinase growth factor receptor), and ERBB pathway dysregulation is associated with breast cancer (Bieche *et al.*, 2003). A role of AhR-mediated AR dysregulation in breast cancer development would also be consistent with the notion that TCDD might have an inhibitory effect on mammary carcinogenesis (Safe *et al.*, 2000; Safe and Wormke, 2003). DJ850E9 is a novel C2H2-type zinc finger protein which represents a large class of DNA-binding proteins and transcription factors and may be involved in hematopoiesis, oncogenesis, and immune responses (Zhang *et al.*, 2001). Nonspecific cross-reacting antigen (NCA) functions as an intercellular adhesion molecule and overexpression can contribute to colon tumorigenesis (Ilantzis *et al.*, 2002). Increase of mRNA levels of the proto-oncogene *c-fos* by exposure to TCDD has been shown in mouse hepatoma cells (Hoffer *et al.*, 1996), and by B[a]P in human prostate carcinoma cells (Kizu *et al.*, 2003). Fibrinogen alpha (FGA) and fibrinogen gamma (FGG) were down-regulated by all AhR agonists and this down-regulation by TCDD was already reported in human HepG2 cells (Wang *et al.*, 1996; Frueh *et al.*, 2001). Fibrinogen acts as a cofactor in platelet aggregation, but is also an acute-phase protein (Koenig, 2003). Down-regulation of FGG may be related to the immunosuppressive activity of TCDD.

Most of the genes found to be affected in our study are theoretically involved in disease processes, and possibly also in some of the reported toxic effects, especially tumor promotion, exerted by I3C and ICZ *in vitro* and *in vivo* (Dashwood, 1998; Herrmann *et al.*, 2002; Yoshida *et al.*, 2004). As with most, if not all, *in vitro* studies, our results can not be extrapolated directly to human *in vivo* exposure, because of the lack of factors at the tissue and organ level present in the intact organism. At the same time, the concentrations of NAhRAs and their tissue distribution in the human body are unknown. However, a preliminary and rough estimate of the internal exposure can be performed by using the dose-response curves of TCDD and e.g. the grapefruit juice extract. According to the DR CALUX[®] results, the amount of AhR agonists in one glass of grapefruit juice would show the same DR CALUX[®] response as 2000 pg TCDD. This would be a substantial amount, for the tolerable daily intake was established by the WHO at 14 pg TCDD equivalents (TEQ)/kg bw/week, which corresponds to 140 pg TEQ/day for a person of 70 kg (JECFA, 2002). Human studies showed that consumption of cruciferous vegetables or pills with pure indole-3-carbinol appear to affect the AhR pathway (Kall *et al.*, 1996; Sepkovic *et al.*, 2001). So, despite that NAhRAs need much higher concentrations than TCDD for AhR activation, and probably have much shorter half-lives, humans can have a regular, and therefore prolonged, exposure to effective concentrations in the diet, especially when considering food supplements with a high NAhRA content.

Although most of the genes affected by NAhRAs in our study were also affected by TCDD (and vice versa), and possibly play a role in certain diseases, a microarray study, such as presented here, does not provide conclusive insight in the health impact of NAhRAs, because it is only directed to the transcriptome level. To enable conclusions on possible toxic effects, additional information on the impact of the observed transcriptional effects on the proteome level and toxic endpoints is necessary (Heijne *et al.*, 2003). The inhibition of the EROD-activity by GJE (Fig. 3) is an example where the up-regulation of the expression of a gene (*CYP1A1*) did not result in an increased expression of the encoded biological function (EROD enzymatic activity). It is not unlikely that NAhRAs also have post-translational modulating effects on the biological functions of proteins other than *CYP1A1*. Furthermore, it needs to be clarified to what extent the gene expression profiles obtained in the Caco-2 cell-line may be extrapolated to AhR-responsive cells and tissues *in vivo* in the context of the complex physiological interactions and kinetics within the complete human body. Since our microarray did only cover about 1000 different genes, it cannot be ruled out that the effect of NAhRAs on the expression of other genes causes differential biological effects as compared with TCDD. Notwithstanding that, our results show that coordinated expression of e.g. *CYP1B1*, *MMP1*, *AR* and *FGG* genes may serve as a biomarker for exposure to AhR agonists.

In conclusion, this study shows that NAhRAs, TCDD and B[a]P modulate the expression of a largely overlapping set of genes in Caco-2 cells, and that, for the set of genes represented on the microarrays used, a clear distinction between the gene expression profiles induced by these different AhR agonists can not be demonstrated. This implies that the thus far reported differences in biological activity between NAhRAs and xenobiotic AhR agonists are either the result of differences in gene expression effects beyond the scope of this study, or caused by effects beyond the transcriptome level, or due

to the kinetics of these compounds in the body. Further investigations should therefore be directed towards a more comprehensive transcriptome-wide comparison of the gene transcription effects of NAhRAs versus xenobiotic AhR agonists, complemented by the assessment of the levels and biological activities of the proteins encoded by the identified responsive genes, and studies on the fate and effects of these compounds in animals and humans. This strategy would provide insight into the balance between healthy and unhealthy pathways affected. Based on these new insights, a sound risk-benefit assessment for regular intake of food items with high NAhRA content can be made.

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Ah receptor agonist activity in frequently consumed food items

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ABSTRACT

The aryl hydrocarbon receptor (AhR) receives much attention for its role in the toxicity of dioxins and dioxin-like PCBs. However, also many other compounds have been reported to bind and activate the AhR, of which natural food components are of special interest from a human health perspective. Using the DR CALUX[®] bioassay, we screened extracts from many food items, frequently consumed in The Netherlands, to estimate the intake of natural AhR-agonists (NAhRAs). Using the prototypical AhR-agonist TCDD as standard, we estimated that the daily intake of NAhRAs may be considerably higher than the reported intake of dioxins and dioxin-like PCBs. Potatoes, cruciferous vegetables, bread, hamburgers and grapefruit juice contained most NAhRAs. Food preparation and acid treatment can show a significant effect on the AhR-activation. Interaction of natural and xenobiotic AhR-agonists should be taken into account when performing risk-benefit analysis of both types of compounds.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a cytosolic ligand-activated transcription factor, which is present in many cell types of mammals, including humans. At the moment, there is still no consensus about the biological function of this receptor. However, it received much attention for its role in the toxicity of dioxins, especially the most potent activator 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hankinson 1995; Safe 2001).

Not only contaminants like dioxins, PCBs and PAHs can bind to the AhR, but also a large number of compounds of natural origin can bind to and activate this receptor. Surprisingly, these compounds can have very different chemical structures, which indicates that this receptor has a rather promiscuous ligand binding site (Denison and Nagy 2003; Safe 1998). One of the most investigated natural AhR agonists (NAhRAs) is indole-3-carbinol (I3C), which is formed in cruciferous vegetables (Brussels sprouts, broccoli, cabbage) by an enzymatic reaction after crushing of the plant tissues. During consumption, I3C undergoes self-condensation into a number of oligomers and indolo[3,2-*b*]carbazole (ICZ) in the acid environment of the stomach. Although ICZ is formed in very low amounts (ca 0.0002%), its AhR activating potency is relatively high (Bjeldanes and others 1991). Also many other food items have been shown to contain relatively large amounts of NAhRAs, of which the chemical identity of the receptor-activating compounds is often unknown (Jeuken and others 2003).

In contrast with the prototypical AhR agonist TCDD and related toxic compounds, the NAhRAs do apparently not show clear toxic effects at normal dietary exposure levels; on the contrary, they even seem to have beneficial health effects. Many researchers attribute this to the induction of a number of phase I and II detoxifying biotransformation enzymes, like the cytochrome P450s CYP1A1, CYP1A2, GSTP, and NQO1 (Kim and Milner 2005; Rogan 2006). However, these enzymes are induced by the activation of the AhR, and are thus also induced by TCDD and other toxic dioxin-like contaminants. A distinctive property of the NAhRAs seems to be their rapid clearance from the body, resulting in an essentially transient effect on the expression of AhR-regulated genes as compared to the more persistent xenobiotic AhR agonists (Chen and others 1995; Pohjanvirta and others 2002). Another property that seems to distinguish many NAhRAs from e.g. dioxins is their ability to inhibit the enzymatic function of P450 enzymes, and even to inhibit AhR activation by TCDD (Amakura and others 2002; Pohl and others 2006). This raises the question whether humans are exposed to such an amount of NAhRAs that they could inhibit the possible toxic effects of compounds like dioxins and PAHs or that they may present a risk hazard because of their AhR agonist activity.

The purpose of our study was to examine the AhR-activating properties of a number of food items frequently consumed in The Netherlands, in order to identify sources of NAhRAs and relate their intake by humans to that of dioxin-like AhR-activating food contaminants. We prepared the food samples in the way they are consumed normally by Dutch consumers, and we included treatment of the samples with hydrochloric acid (HCl) to mimic the acid environment of the stomach. A low pH could theoretically hydrolyse unstable NAhRAs and deactivate them, but on the other hand, some

natural compounds, like I3C, can be transformed by acidification into relatively strong AhR agonists such as ICZ. Comparing the AhR-activation by the NAhRAs, as measured with the DR CALUX⁺ assay, with the activity of TCDD, and relating this to the Dutch Food Consumption Survey of 2003 (Hulshof and others 2004), provided us with an estimation of the theoretical load of NAhRAs in the human diet. Because of the difficulty to measure every food item, this is likely to result in an under-estimation of the actual intake.

MATERIALS AND METHODS

Chemicals

Methanol, n-hexane, diethyl ether, sulphuric acid and silica gel 60 (70-230 mesh) were purchased from Merck (Darmstadt, Germany), luciferin assay mixture from Promega (Madison, WI, USA), cyclohexane and ethylacetate from Biosolve (Valkenswaard, The Netherlands).

Preparation of food extracts

All food items were bought at local supermarkets. Solid foods were grinded by a food chopper T71 (Moulinex, Woerden, The Netherlands). Five gram of fresh solid food or 10 ml of liquid food was mixed with 20 ml of methanol/water 85/15, and after 30 min of soaking, extracted twice with 20 ml of n-hexane/diethyl ether 97/3 by mixing for 1 h in an overhead mixer (REAX 2, Heidolph, Germany), followed by centrifugation at 3000 rpm for 10 min. Hexane-layers were collected, evaporated in a Speedvac (Hetavac VR-1, Birkerød, Denmark) and the residue dissolved in 40 µl of DMSO (Merck, Darmstadt, Germany). In order to mimic the acid environment of the stomach, in addition grinded samples were 1:1 mixed with 0.1 M HCl, adjusted to pH=1 with 1 M HCl (Merck, Darmstadt, Germany), and mixed for an hour before addition of the methanol/water. When relevant, samples were boiled before grinding according to common Dutch kitchen practises, and afterwards, also the boiling water was collected for examination. Extracts from food samples containing fat, like bread and meat, were defatted in order to prevent incomplete mixing with the medium. Extract residues containing 40 µl DMSO were dissolved in 15 ml of eluent (cyclohexane/ethylacetate 1/1 v/v), of which 12.5 ml were inserted into a gel permeation chromatography system (Gilson). Fat was separated by a BioBeads SX3 column (i.d. 2.5 cm, length 60 cm, 200-400 mesh), and the eluted fraction from 44 to 107 min was collected and evaporated until 40 µl DMSO. To check for contamination with dioxin-like persistent AhR agonists, hexane-extracts of a number of samples were transferred on a column containing 10 g acid silica (33% H₂SO₄), on which NAhRAs are removed (Hoogenboom and others 2006b) and subsequently tested for residual AhR-agonist activity by DR CALUX⁺ analysis.

Cell culture and treatment

DR CALUX⁺ cells (rat H4IIE hepatoma cells stably transfected with pGudluc1.1, an AhR-regulated luciferase reporter plasmid) (Aarts and others 1995) were grown in α-MEM (Sigma, St-Louis, USA), containing 10% fetal bovine serum (GIBCO, Breda, The Netherlands) and 1% penicillin/streptomycin (Sigma), at 37°C and 5% CO₂. Cells were seeded 24 h before exposure in Costar 48

well-plates ($5 \cdot 10^4$ cells/well) and exposed for 24 h to sample solutions in DMSO added to the culture medium. During each experiment, a calibration curve was made of 0.5-500 pM TCDD (Schmidt BV, Amsterdam, The Netherlands). The undiluted sample concentration of the solid foods was equivalent to 1250 mg fresh product per ml exposure medium, except for the meat samples and fish, of which the concentration was 625 mg/ml. The undiluted concentration of the liquid samples was 2500 mg/ml. Samples were also diluted 3, 9, 27 or 81 times. The final concentration of DMSO in all exposures was 1.0 % (V/V). In addition, a number of samples were also exposed for 4, 8 and 16 h.

DR CALUX® assay

The Dioxin Receptor - Chemical-Activated Luciferase gene eXpression assay was performed as described previously (Bovee and others 1998). After exposure of the cells in 48-well microtiter plates the medium was removed and cells were washed with PBS. Cells were lysed using Cell Culture Lysis Reagent (Promega), centrifuged and 20 µl of lysed solution of each well was transferred to a 96 wells plate. Luminescence was measured with a LuminoskanAscent (Thermo Labsystems, Finland) upon addition of 0.1 ml of luciferin assay mixture (20 mM Tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM EDTA, 33.3 mM DTT, 261 µM Coenzyme A, 470 µM Luciferin, 530 µM ATP, pH 7.8). Using the program SlideWrite V6, the curve $y=a_0/(1+(x/a_1)^{a_2})$, which describes the AhR activation process suitably (Hoogenboom and others 2006a) was fitted for the data and the response maximum of TCDD and the TCDD luciferase induction equivalents (TCDD-IEQs) of the food extracts were calculated.

Statistics

Statistical analyses were performed by use of the 2-side Student's t-test. Effects of acidification or boiling of groups of foods were tested paired.

RESULTS

By use of the DR CALUX® bioassay, the Ah-receptor activity was measured in extracts from a number of food items, regularly consumed in The Netherlands. To rule out the effects of possible contamination with dioxins and other persistent AhR agonists, a number of samples with relatively high luciferase induction has been examined by use of the acid silica clean up procedure which destroys the more labile natural AhR agonists. None of the acid silica treated samples showed a significant induction of the luciferase reporter gene expression, except for the flatfish, which showed a CALUX activity of about one-third of the non acid silica treated sample (results not shown). To establish whether the food extracts showed a similarly persistent response as TCDD, the CALUX cells were also exposed for 4, 8 and 16 h to a number of samples. Most of the samples induced luciferase 20-70 times more during 4 h and 8 h, than during 24 h of exposure, both at the 3 (Figure 1) and 27 times dilutions (results not shown). The 16 h exposures showed less difference with 24 h (1-3 times). Apparently, the AhR agonists in all samples are less persistent in activity than TCDD, probably because of their faster metabolism or faster increase in response compared with TCDD.

Fig. 1. Luciferase induction in rat H4IIEpGudluc1.1 cells determined by the DR CALUX[®] assay, expressed as % of the maximum induction by TCDD after 4, 8, 16 and 24 h of exposure to 3 times diluted extracts of a number of frequently consumed food items. Bars: mean of triplicate exposures $\pm$ sd.

DR CALUX[®] response in food items

Figure 2 to 6 show the AhR activity in extracts of a number of food items frequently consumed in the Netherlands, as measured by the DR CALUX[®] assay after 24 h of exposure. All samples were tested in dilution ranges, but for clarity reasons not all dilutions are shown. The influence of acidification has been determined for most of the food samples, and where relevant according to the habitual Dutch food preparation, also the effect of boiling has been studied. The results are reviewed in the same order of product groups as described in the Dutch Food Consumption Survey of 2003 (Hulshof and others 2004). The limit of detection of the used DR CALUX[®] assay is 0.5 pM TCDD (Hoogenboom and others 2006a), which corresponds with a luciferase induction of about 1% of the maximum induction caused by TCDD. In this study, a luciferase induction of 2% is assumed to be a significant activation of the AhR, corresponding to a limit of quantitation (LOQ) of 0.1 pg TCDD-luciferase induction equivalents (TCDD-IEQ) per gram of undiluted sample. After boiling the vegetables, the boiling water was collected and also examined for AhR activation, but the luciferase induction by these water extracts was negligible.

Potatoes. French fries contained a considerable amount of inducing compounds (up to 28% induction, Figure 2). At three dilutions, the acidification reduced the luciferase activity significantly by 20-30%. Potatoes showed a significant luciferase activity of about 4-7% of the TCDD maximum at the highest concentration. Boiled potatoes showed significant less induction than acidified raw potatoes (Figure 3).

Fig. 2. DR CALUX^{*} response in a dilution range of fried (black) and fried plus acidified (grey) French fries extracts. The exposure time was 24h. Bars: mean of triplicate exposures $\pm$ sd, * $P < 0.05$, ** $P < 0.01$

Lettuce, carrot and pea. The DR CALUX^{*} response of these vegetables was near or below 1% of TCDD maximum at the highest concentration (results not shown).

Bell pepper. Three different color types of bell pepper were examined. Undiluted samples showed an activity of 4-6 % of TCDD maximum. Acidification of the boiled vegetables increased the activity significantly (Figure 3).

Cruciferous vegetables. Four types of cruciferous vegetables have been examined: Brussels sprouts, broccoli, Savoy cabbage and white cabbage. All of them showed a relatively strong luciferase induction. Although Brussels sprouts tended to be the most strongly activating vegetable, the differences were not significant and the mean of all types has been plotted in Figure 3. As expected in view of the known acid induced NAhRAs like I3C in cruciferous vegetables, acidification increased the response significantly by 2.8 (acidified vs raw) to 2 (boiled and acidified vs raw) times. Boiling did not have a large effect on luciferase induction.

Paste, rice and corn. Penne, macaroni, spaghetti and rice but not corn could induce the DR CALUX^{*} response by 2-7%. Acidification increased the response of penne, and decreased the response of rice significantly (Figure 4).

Bread. Bread showed a significant response of 15-20% at the highest concentration (Figure 4). Acidification decreased the response, but not significantly. Wheat flour was very low in AhR activity (results not shown).

Meat and fish. Very high levels of AhR activity, up to 80% of the TCDD maximum, were observed with hamburgers and the flatfish plaice, and to a lesser extent with fried minced beef (Figure 5). Raw minced beef showed no significant response and frying also increased the response of chicken breast and hamburger, but decreased the response of steak significantly. Acidification of the fried samples had no significant influence on the luciferase induction.

Fig. 3. DR CALUX* response in extracts of raw (black), raw plus acidified (grey), boiled (white) and boiled plus acidified (striped) potatoes, bell pepper and cruciferous vegetables. The exposure time was 24h. Bars: mean of four (potatoes, undiluted), three (bell pepper, undiluted, three different color types), or eleven (cruciferous vegetables, three times diluted, four different types) samples of vegetables $\pm$ sd, * $P < 0.05$

Fig. 4. DR CALUX* response in three times diluted extracts of boiled (black) and boiled plus acidified (grey) pastas, rice, corn and bread. The exposure time was 24h. Bars: mean of triplicate exposures $\pm$ sd, * $P < 0.05$

Fig. 5. DR CALUX* response of undiluted extracts of raw (black), fried (grey) and fried plus acidified (striped) samples from minced beef, steak, pork, chicken breast, hamburger and flatfish (plaice). The exposure time was 24h. Bars: mean of triplicate exposures $\pm$ sd, * $P < 0.05$, ** $P < 0.01$

Fruit juices. Undiluted orange juice showed a moderate activity of up to 30% of the TCDD maximum, while grapefruit juice highly induced the luciferase activity up to ca. 40% at low concentrations (Figure 6). Undiluted samples of grapefruit juice extracts were clearly cytotoxic, and also the 3-times dilutions were less active. Acidification of these fruit juices had no significant effect on the AhR induction, probably also because of the low pH of the juices themselves (pH=3.2).

Human exposure with NAhRAs

To estimate the amount of AhR-activating substances found in the human diet, we calculated their contribution to the DR CALUX* induction relative to the induction by TCDD, and expressed it as pg TCDD luciferase induction-equivalents (TCDD-IEQ, Table 1). In this way TCDD has been used as a normalization standard for AhR-activity as measured by DR CALUX*, enabling comparison with AhR-agonist levels in food items reported in literature. We emphasize that this does not imply a comparison to TCDD-toxicity. It was decided to use only the 24 h exposure data. Because the slope of the dose-response curves varies considerably between food extracts and TCDD, lower and upper limits of the DR CALUX* inductions were determined over the range of food extract dilutions tested, and the means of the representatives of the product groups are compared with the mean daily consumptions of the food items as reported in the Dutch Food Consumption Survey of 2003 (Hulshof and others 2004). Where appropriate, only acidified and boiled samples were taken into

Fig. 6. DR CALUX[®] response in a dilution range of orange juice (black), acidified orange juice (grey), grapefruit juice (white) and acidified grapefruit juice (dark grey) extracts. The exposure time was 24h. Bars: mean of seven (orange juice) of three (grapefruit juice) different types $\pm$ sd. At the 81 times dilution only grapefruit juice has been measured.

account. Table 1 shows that the total TCDD-IEQ amounts to 1545 pg/day at the lowest estimation, and 6235 pg/day at the highest. Of all tested food extracts, potatoes, cruciferous vegetables, bread, hamburgers and fruit juices are the most important sources of AhR agonist activity for the average consumer. The 81-times diluted grapefruit juice extract showed the highest relative DR CALUX[®] activity of 41 pg TCDD-IEQ per g of product (results not shown). Because of the relatively small sampling of food items in our study, the overall exposure of the Dutch people to these kind of bioactive compounds will probably be significantly higher.

DISCUSSION

Many compounds of natural origin appear to be AhR-agonists and extracts of many food items were found to have a significant influence on the AhR activity as measured by *in vitro* and *in vivo* assays (Amakura and others 2002; Amakura and others 2003; Jeuken and others 2003; Leibelt and others 2003; Nishiumi and others 2006; Pohl and others 2006). Some of these compounds and mixtures showed both activating and inhibiting effects on the AhR pathway. Very high estimates of AhR activating potential by natural food components have been reported (Jeuken and others 2003; Safe 1998). However, a systematic inventory of the exposure of humans to NAHRAs in food has not been made. By analyzing a variety of food items frequently taken by Dutch consumers, and including the effect of food preparation and acidification, we showed in this study that the intake of

Table 1. Estimated range of exposure of the Dutch population to NAhRAs from a number of frequently consumed food items, expressed as DR CALUX[†] pg TCDD-IEQ/day. The food items are prepared according Dutch standards, and acidified to mimic the gastric environment. Levels based on the 24h incubations of the DR CALUX[†] bioassay.

VCP-2003 Product group	Samples	VCP-2003 Consumption g/day	Range pg TCDD-IEQ/g		Daily pg TCDD- IEQ/day	
			Lower	Upper	Lower	Upper
Potatoes	Mean of potatoes [*]	96	1.4	2.4	130	226
Leaf vegetables	Lettuce	15	0.0	0.1	0	2
Fruit vegetables	Bell pepper [*]	28	0.2	0.9	6	25
Tuberous vegetables	Carrot [*]	7	0.0	0.0	0	0
Crucifers	Mean of crucifers [*]	23	1.8	4.5	40	103
Peas, beans	Pea [*]	3	0.0	0.0	0	0
Flour	Wheat flour	1	0.8	0.8	1	1
Paste, rice	Mean of paste, rice [*]	46	0.7	0.7	31	32
Bread	Bread	151	0.4	14.5	60	2190
Fresh meat	Mean of fresh meat	44	0.8	1.1	36	47
Meat products	Hamburger [‡]	60	10.5	24.9	630	1494
Fish	Plaice (flatfish) [‡]	4	4.2	6.1	17	24
Fruit juices	Mean of fruit juices	159	3.7	13.1	594	2088
Total, pg/day					1545	6232

^{*} Samples boiled

[‡] Samples fried

NAhRAs through the diet is probably not as high as previously thought (Jeuken and others 2003; Safe 1998), but still much higher than the intake of persistent xenobiotic AhR agonists (Baars and others 2004).

French fries, cruciferous vegetables, bread, hamburgers and citrus juices showed the most important AhR agonist activity of the food extracts we examined. Many natural compounds which can cause the AhR-activity in these food items may not have been identified yet. In cruciferous vegetables, the AhR agonists are attributed to the acid reaction products of indole-3-carbinol (I3C). In baked or fried foods, like fries, bread and meat products, the activity may be caused by compounds which originate from the formation of polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines or Maillard products during the food preparation process at high temperatures. Also blood rests in meat may contribute to the AhR activity; heme degradation products like bilirubin and biliverdin are reported as AhR agonists. Polyphenols and furocoumarins are probably responsible for the shown activity in citrus fruit products (Denison and Nagy 2003).

Expressing the levels of NAhRAs in food in TCDD-IEQs may suggest that the responsible components represent a certain level of AhR-mediated toxicity according to the TEQ (toxic or dioxin equivalent) approach as described by Safe (Safe 1998). However, we emphasize that we only used TCDD-IEQs to compare AhR activity, because the NAhRAs do probably not meet some of the important requirements for the TEQ-principle, namely additivity, and more important, persistence

(Safe 1998). However, TCDD is a very potent agonist, and shows very reproducible results in the DR-CALUX[®] assay, which makes it an ideal standard for the expression of relative AhR agonist activity. This implies that our results can not be interpreted as direct measurements of toxicity of food components, but only reveal the AhR activation effects, expressed as TCDD-IEQs or luciferase induction equivalents. Because the slopes of the dose-response curve of NAhRAs and PAHs differ from that of dioxins, the dilution of the samples has an important effect on the results of the calculation of pg TCDD-IEQs/g sample (Windal and others 2005). Because of the probably fragile nature of many natural compounds, it is important to include effects like food preparation and the effect of acidification in the gastric environment on the levels of AhR agonist activity. Frying and acidification can have a significant influence on AhR activity, boiling to a much lesser extent and the loss of AhR agonists in the boiling water was negligible.

The exposure time is another important factor in AhR activity measurement. Natural compounds, but also some PAHs like benzo[a]pyrene, can be metabolized very quickly in comparison with TCDD (Chen and others 1995; Machala and others 2001; Pohjanvirta and others 2002). In our study, the luciferase induction after 4 h exposure to TCDD has reached only about 30% of its maximum after 24 h, whereas the induction by PAHs and NAhRAs is higher at 4h than at 24h of exposure. As a result, a relatively short exposure time of 4 h will show a much higher luciferase induction by NAhRAs and PAHs compared with TCDD, than after 24 h of exposure. This may explain the large difference between some results of Jeuken *et al.* (2003) and ours. They found e.g. 640 pg TCDD-IEQ/g in potato, where we found 1.4-2.4 pg TCDD-IEQ/g (Table 1). However, based on our measurement with the 4h exposure time, we found in line with Jeuken *et al.* a much higher amount of 110-332 pg TCDD-IEQ/g in potato. The interpretation of DR-CALUX[®] measurements on extracts of food items is thus very complicated, and we do not pretend to give a conclusive opinion about their eventual toxic potential in the human diet. Important, however, is to obtain an estimation which shows whether the exposure to NAhRAs is considerably higher than to xenobiotic AhR agonists such as dioxins and other persistent compounds in the human diet, or much lower and negligible. In 1999, the estimated intake of the sum of dioxins and dioxin-like PCBs in The Netherlands was 1.2 pg WHO-TEQ per kg body weight (bw) per day, which would be for a person weighing 70 kg 84 pg WHO-TEQ. Of this population was 8% exposed to intake levels above the tolerable weekly intake for dioxins and dioxin-like PCBs of 14 pg WHO-TEQ per kg bw per week, or over 140 pg WHO-TEQ per person per day. The average dietary intake of these xenobiotic AhR agonists in 1978 was nearly 700 pg WHO-TEQ per person per day (Baars and others 2004). These levels are that far below the theoretical levels estimated by Jeuken *et al.* (e.g. 96000 pg TCDD-IEQ per serving of 150 g potato), or our study (1545-6235 pg TCDD-IEQ/day for part of the average daily diet), that the regular intake of NAhRAs should be seriously evaluated with regard to the biological meaning of the level of AhR activation that they may cause. On the one hand, the omnipresence of the competing natural AhR agonists, together with the presence of many AhR antagonists (Amakura and others 2002; Amakura and others 2003; Baumgart and others 2005) in food, may appear as a prophylaxis against xenobiotic intoxication via the AhR. On the other hand, several human studies showed

AhR responsive effects, like CYP1A2 induction, by several non-dioxin like AhR agonists such as NAhRAs from cruciferous vegetables (Kall and others 1996; Murray and others 2001), I3C (Reed and others 2005), the anti-ulcer drug omeprazole (Rost and others 1992), and heterocyclic aromatic amines (Sinha and others 1994). This seems to be in contrast with a regular exposure to numerous AhR agonists in the human diet, which would constantly elevate the AhR activity. Another major problem in this is, that the exact pathways by which the xenobiotic dioxins and dioxin-like PCBs exert their toxicity are not well understood (Guzelian and others 2006; Steenland and others 2004). This makes it very difficult to understand to what extent NAhRAs, in amounts effective to cause AhR related effects, e.g. CYP1A2 induction by concentrated food additives, also can initiate dioxin-like toxic pathways, especially when slow processes like tumor promotion are involved. Besides tumor suppressing capacity, high doses of NAhRAs given to laboratory animals have sometimes also shown tumor promoting capacity (Dashwood 1998), and the accumulation effect of persistent fat-soluble xenobiotic AhR agonists could be mimicked by constant or regular NAhRA exposure.

In conclusion, this study confirms to a certain extent that the human diet contains a large amount of AhR-activating substances of natural origin, which triggers questions about the potential effects in humans, also in relation to the intake and adverse effects of xenobiotic dioxins and dioxin-like PCBs. In order to elucidate the potential toxicity or health-beneficial properties of these relatively large amounts of bioactive dietary components, the toxic pathway(s) of dioxins in humans should be elucidated first. This would give indications at what critical steps of the toxic pathway(s) the NAhRAs would have a significant dioxin-like toxic effect or on the contrary, induce totally different or even beneficial health effects, and what ultimately the predominant effect or the balance of all these effects would be.

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4

A human intervention study with foods containing natural Ah-receptor agonists does not significantly show AhR-mediated effects as measured in blood cells and urine

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ABSTRACT

Binding and activation of the aryl hydrocarbon receptor (AhR) is thought to be an essential step in the toxicity of the environmental pollutants dioxins and dioxin-like PCBs. However, also a number of natural compounds, referred to as NAhRAs (natural Ah-receptor agonists), which are present in e.g. fruits and vegetables, can bind and activate this receptor. To study their potential effects in humans, we first investigated the effect of the prototypical AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the gene expression in *ex vivo* exposed freshly isolated human lymphocytes, and compared the resulting gene expression profile with those caused by the well-known NAhRA indolo[3,2-*b*]carbazole (ICZ), originating from cruciferous vegetables, and by the hexane extract of NAhRA-containing grapefruit juice (GJE). TCDD and ICZ induced a similar gene expression profile in the lymphocytes, and both significantly up-regulated CYP1B1 and TIPARP (TCDD-inducible poly (ADP-ribose) polymerase) mRNA.

Next, we performed a human intervention study with NAhRA-containing cruciferous vegetables and grapefruit juice. The expression of the prototypical AhR responsive genes CYP1A1, CYP1B1 and NQO1 in whole blood cells and in freshly isolated lymphocytes was not significantly affected. Also enzyme activities of CYP1A2, CYP2A6, NAT2 and XO, as judged by caffeine metabolites in urine, were unaffected, except for a small down regulation of NAT2 activity by grapefruit juice. Examination of blood plasma with DR CALUX[®] showed a 12% increased activity 3h and 24h after consumption of cruciferous vegetables, but did not show a significant effect of grapefruit juice consumption. We conclude that intake of NAhRAs from food may result in minor AhR-related effects measurable in human blood and urine.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a protein present in the cytoplasm of most human and other mammalian cells. Binding of the environmental contaminant and potent agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in the translocation of the AhR to the nucleus of the cell, heterodimer formation with the AhR nuclear translocator (ARNT) and binding to dioxin-responsive elements in DNA. Transcription activation results in induction of the biotransformation enzymes CYP1A1, CYP1A2, CYP1B1, GSTP1, NQO1 and UDPGT1A6 (Hankinson, 1995; Nebert *et al.*, 2000; Denison and Nagy, 2003). Activation of the AhR is thought to be the main trigger for the toxic effects of TCDD, such as tumor promotion, chloracne, immunosuppression, endometriosis, reduced sperm counts and teratogenesis (Fernandez-Salguero *et al.*, 1996; Van den Berg *et al.*, 1998; Mimura and Fujii-Kuriyama, 2003; Steenland *et al.*, 2004).

Besides environmental contaminants like dioxins, dioxin-like PCBs and certain PAHs, also a number of natural ingredients in vegetables and fruit can bind to and activate the AhR. Examples of these compounds or mixtures are indolo[3,2-*b*]carbazole (ICZ), a gastric-acid reaction product of indole-3-carbinol (I3C), originating from cruciferous vegetables (Bergman *et al.*, 2001), and the dye indigo and its by-product indirubin originating from plant fermentation (Sugihara *et al.*, 2004). Also hexane extracts of citrus products and many other food items (Hoogenboom, 2002; Jeuken *et al.*, 2003) show increased activity in the DR CALUX[®] bioassay, a luciferase reporter-assay designed to detect the presence of AhR-agonists. In contrast to the toxic effects of TCDD, these natural AhR agonists (NAhRAs) in foods are often thought to have health promoting effects, like radical scavenging or tumor suppression (Broadbent and Broadbent, 1998; Yu *et al.*, 2005). This is in agreement with many studies reporting beneficial effects of vegetables and fruit consumption (Steinmetz and Potter, 1996; Heber, 2004). Nevertheless, some animal experiments with I3C showed an increase in tumor formation after initiation with a carcinogen (Dashwood, 1998) and ICZ showed a TCDD-like inhibition of gap junction intercellular communication *in vitro*, indicative of a tumor promotive effect (Herrmann *et al.*, 2002). Also epidemiological studies are inconsistent as to the overall healthy effects of foods containing NAhRAs or vegetables and fruit in general (Verhoeven *et al.*, 1996; van Gils *et al.*, 2005; Lynn *et al.*, 2006). The growing market of dietary supplements, containing pure NAhRAs in very high concentrations compared to the foods of origin (Zeligs, 2002; Noonan and Noonan, 2006), makes it even more important to study this kind of compounds for possible unperceived health risks.

Because the mechanisms behind TCDD-toxicity are not well understood (Steenland *et al.*, 2004), examination of the effect of AhR-activation on gene expression may serve as a first step in the elucidation of the molecular pathways involved (Heijne *et al.*, 2005; Boverhof and Zacharewski, 2006). By using microarray technology, many dioxin-responsive genes in animal tissue and human cell lines have been determined after exposure to TCDD, and links with toxic properties of dioxins have been made (Puga *et al.*, 2000; Frueh *et al.*, 2001; Fletcher *et al.*, 2005). Previously we compared the gene expression profiles in human Caco-2 colon cancer cells after exposure to ICZ or a grapefruit juice extract with those induced by TCDD and benzo[*a*]pyrene (B[*a*]P). We found a remarkable

resemblance between the gene expression profiles caused by xenobiotic and by natural AhR-agonists and many of the commonly expressed genes were related to cell proliferation and tumor promotion (De Waard *et al.*, 2007). At the same time, it is likely that differences in kinetics and metabolism will change this picture in the intact organism.

In order to study the effects *in vivo*, the typical AhR responsive gene expression profile can possibly be used as a biomarker for AhR-activation. Since gene expression profiling in peripheral lymphocytes has been shown to be a promising approach to identify candidate biomarkers for AhR activation (Lampe *et al.*, 2004; McHale *et al.*, 2007), we first studied the effects on gene expression of TCDD and NAhRAs in *ex vivo* exposed human lymphocytes using microarrays. These cells were also exposed to the AhR antagonist α -naphthoflavone (ANF) to check whether affected genes were AhR-regulated. Subsequently, the expression of some prototypical AhR-regulated genes was examined by RT-PCR in both human total blood cells and lymphocytes, obtained from volunteers consuming a NAhRA-enriched diet, based on grapefruit juice and the cruciferous vegetables broccoli and Brussels sprouts. The effects of the diet intervention on blood plasma AhR agonist activity was measured by use of the DR CALUX[®] reporter gene bioassay, and on CYP1A2 enzyme activity by studying the metabolism of caffeine, which is a CYP1A2 substrate.

MATERIALS AND METHODS

Preparation of the grapefruit juice extract

Ten ml of grapefruit juice, obtained from a local supermarket, was mixed with 20 ml of methanol/water 85/15, and extracted twice with 20 ml of n-hexane/diethyl ether 97/3 by mixing for 1 h. Hexane-layers were collected, evaporated and the residue dissolved in 40 μ l of DMSO (Merck, Darmstadt, Germany) for analysis. Because the grapefruit juice extract (GJE) contains a mixture of compounds, the concentration is expressed as gram extracted sample per litre medium, used to expose the cells. Also a blank extraction of 10 ml water has been performed (BLEX).

Lymphocyte isolation

Blood was sampled from volunteers and lymphocytes were isolated through gradient centrifugation using Lymphoprep in Leucosep filter tubes (Greiner Bio-One, Frickenhausen, Germany) according to the manufacturer's protocol. Cells were resuspended in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), and penicillin 100 U/ml / streptomycin 100 μ g/ml at 1×10^6 cells/ml. No mitotic stimulation was applied to the quiescent cells.

Cell exposures

Freshly isolated lymphocytes were *ex vivo* exposed to solvent or 5 nM TCDD (Schmidt BV, Amsterdam, The Netherlands) for 24 and 72 h for gene expression analysis. Chemicals and extracts were dissolved in DMSO, of which the final concentration was kept at 0.5% in the growth medium. Freshly isolated lymphocytes from volunteers for the microarray study were exposed *ex vivo* for 48 h to 500 pM TCDD (n=5), 100 nM ICZ (n=4, purity >95%, synthesized as described by Bergman

(Bergman, 1970) and checked for quality by HPLC, NMR, IR and MS, and with a reference sample (A kind gift of prof. J. Bergman, Department of Chemistry, Royal Institute of Technology, and Department of Biosciences at Novum, Huddinge, Sweden), 417 g/L GJE and BLEX (n=4), and 3 μ M ANF (n=3, Sigma, St. Louis, USA).

Human intervention study

Three male and three female healthy non-smoking volunteers, aged 21–46 years, were recruited for a NAhRA-enriched dietary intervention study. No medication or vitamin supplementation was used by the subjects during the intervention (oral contraceptives were permitted). The protocol was approved by the Medical Ethical Committee of Maastricht University and the Academic Hospital Maastricht and the subjects were fully informed about the details of the study and gave their written informed consent. After a three day wash-out period, without consumption of cruciferous vegetables and citrus fruits, the participants received on three consecutive days 600 ml of grapefruit juice, which was divided in three portions of 200 ml distributed over the day. Again after a three week wash out period, the participants consumed on three consecutive days a soup containing 150 g broccoli per portion and a meal with 300 g Brussels sprouts. Blood samples were taken before, and 3 h and 24 h after the last NAhRA-enriched meal. Two types of blood samples were taken, one using Li-heparin containing tubes for isolation of lymphocytes (VenoSafe, Terumo, Leuven, Belgium), and another using PAXgene Blood RNA Tubes for rapid mRNA conservation in whole blood (PreAnalytiX, Qiagen, Crawley, UK).

RNA isolation

Total RNA was isolated from freshly isolated lymphocytes using Trizol (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. Total RNA was isolated from the PAXgene Blood RNA Tubes also according to the manufacturer's protocol. In short, tubes were centrifuged at 4000g, after removal of the supernatant, the pellet was resolved in 4 ml of RNase-free water, centrifuged again, and the pellet was dissolved in 350 μ l resuspension buffer. The sample was transferred to a microcentrifuge tube and incubated with 300 μ l binding buffer and 40 μ l proteinase K for 10 min at 55°C. The lysate was transferred to a PAXgene Shredder spin column placed in a 2 ml processing tube and centrifuged for 3 min at 12000 g. The flow-through fraction was mixed with 350 μ l ethanol and pipetted into the PAXgene RNA spin column placed in a 2 ml processing tube and centrifuged for 1 min at 12000 g. The column was washed with 350 μ l wash buffer 1, centrifuged at 12000 g and 80 μ l of DNase incubation mix was added. After 15 min the sample was washed with wash buffers 1 and 2 respectively, eluted twice with 40 μ l elution buffer and incubated for 5 min at 65°C. Purity and integrity of the RNA were checked by gel electrophoresis and UV-spectrometry.

Quantitative real-time RT-PCR

One µg of total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in a final volume of 20 µl. For amplification of β -actin, CYP1A1, CYP1B1, and NQO1 the following primers were used:

β -Actin: forward 5'- CCTGGCACCCAGCACAAT-3';

reverse 5'-GCCGATCCACACGGAGTACT-3';

CYP1A1: forward 5'-TCCTGGAGACCTTCCGACACT-3';

reverse 5'-CTTTCAAACCTGTGTCTCTTGTGT-3';

CYP1B1: forward 5'-AGTGCAGGCAGAATTGGATCA-3';

reverse 5'-GCGCATGGCTTCATAAAGGA-3';

NQO1: forward 5'-GGTGGAGTCGGACCTCTATGC-3';

reverse 5'- CCTTCAGTTTACCTGTGATGTCCTT-3'.

β -Actin was used to normalize for differences in RNA input and efficiency during the reverse transcription. Samples were diluted 15 times and duplicates were mixed with primers and iQ™SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA) and amplification took place in the iCycler PCR machine with the MyiQ Single Color Real-Time PCR Detector System (Bio-Rad, Veenendaal, The Netherlands). The specificity of the PCR products was checked by melt curve peak analysis and by gel electrophoresis. Gene expression levels were calculated from the threshold cycle values and plotted as the ratio with the β -actin expression level.

Microarray hybridisation

Hybridisation of the samples was performed using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA). To a 1.5 ml microcentrifuge tube, 400 ng RNA in 10.3 µl and 1.2 µl of T7 Promotor Primer were added and incubated for 10 min at 65°C. After cooling on ice, 8.5 µl cDNA Master Mix was added and samples were first incubated at 40°C for 2 h and then at 65°C for 15 min. To each sample, 1.2 µl cyanine-3-CTP or cyanine-5-CTP (5 mM, Perkin Elmer, Boston, MA, USA) and 28.8 µl of Transcription Master Mix were added, mixed and incubated at 40°C for 2 h. Next, 50 µl nuclease-free water, 350 µl Buffer RLT, and 250 µl ethanol were added, and 700 µl of the cRNA sample was transferred to an RNeasy mini column in a 2 ml collection tube. The sample was centrifuged for 30 sec at 13000 rpm, washed twice with 500 µl buffer RPE and eluted twice with 30 µl of RNase-free water. Yield and build-in of label were measured by use of the NanoDrop ND-1000 Spectrophotometer (Isogen Life Sciences, IJsselstein, The Netherlands). Of the linearly amplified Cy3- and Cy5-labeled cRNA, 1 µg (or 0.5 µg of 2 samples of GJE and 2 corresponding samples of BLEX, because their yield was too low) was mixed with 50 µl 10x control targets solution and nuclease-free water up to 240 µl, 10 µl 25x fragmentation buffer was added and samples were incubated at 60°C for 30 min in the dark. Afterwards, 250 µl 2x hybridization buffer was added, mixed and the solution was dispensed onto a gasket slide in a chamber base (In situ Hybridization Kit Plus, Agilent Technologies). The 22K Human 1A (V2) Oligo Microarray slide (Agilent Technologies) was gently placed on the gasket slide, the chamber cover

was assembled and loaded into a hybridization rotator rack. Samples were hybridized at 60°C for 17 h at 4 rpm, washed with 6x SSPE/0.005% N-lauroylsarcosine, 0.06x SSPE, 0.005% N-lauroylsarcosine and Stabilization and Drying Solution (Agilent Technologies) respectively. Slides were scanned with the Scan Array Express HT (Packerd BioScience, Boston, USA).

Microarray data analysis

The fluorescence signals were quantified using ImaGene 5.0 (BioDiscovery, Marina del Rey, CA, USA), poor spots, and spots with low expression levels were flagged, and background subtracted data were exported to Microsoft Excel. All flagged spots and spots with a lower signal than twice the background were removed and the remaining 4268 spots were normalized first by correcting the Cy5 signals with the Cy3 internal standard signals. Subsequently, the median of the adjusted Cy5 values was used to correct for possible differences between experiments with respect to the efficiency of labelling and the amount of label. Ratios of differential gene expression were calculated by dividing the normalized values of cells treated with test-sample by those treated with the solvent DMSO, and significance was calculated with the Student's t-test. Principle Component Analysis (PCA) was carried out with GeneMath 2.1 (Applied Maths, St-Martens-Latem, Belgium).

Determination of enzyme activities by measuring caffeine metabolites in urine

Caffeine is a substrate for CYP1A2, and also for CYP2A6, N-acetyltransferase 2 (NAT2) and xanthine oxidase (XO), all of which can be determined simultaneously by use of HPLC. The enzyme activities were determined by measuring the caffeine metabolites in urine as described before (Krul and Hageman, 1998). In short, the subjects were instructed to refrain from consumption of products containing caffeine for 24h, after which at least one cup of coffee or two cups of tea were consumed. Four hours after consumption, urine samples were collected and frozen immediately. This procedure was performed before and after the grapefruit juice and vegetable interventions. After addition of 120 mg of ammonium sulphate, 200 µl of urine and 200 µl of internal standard (N-acetaminophen 150 mg/l) were pipetted into a glass tube, and extracted with 6 ml of chloroform/isopropanol 4:1 (v/v). After centrifugation at 1000 g for 5 min, the organic phase was evaporated at 40°C under a gentle stream of nitrogen, and the residue was dissolved in 1 ml of 0.05% acetic acid. A 20 µl aliquot of sample was injected onto a Hypersil 5 µm ODS HPLC column (15 cm x 4.6 mm i.d.) from Phase Separations (Deeside, UK), eluted with 33% acetic acid:tetrahydrofuran:acetonitrile:milli-Q-water (1:2.5:44:952.5, v/v) at a flow-rate of 1 ml/min and detected at a wavelength of 280 nm (UV-Vis SPD-6AV detector, Shimadzu, 's Hertogenbosch, The Netherlands). Identities of the caffeine metabolites 1-methylxanthine (1X), 1-methyluric acid (1U), 1,7 dimethylxanthine (17X), 1,7-dimethyluric acid (17U, Sigma Aldrich, Steinheim, Germany), and 5-acetylamino-6-formylamino-3-methyluracil (AFMU, a kind gift from Dr. R. Fumeaux, Nestlé Research Centre, Lausanne, Switzerland) were determined by co injection. CYP1A2 activity was assessed by calculating the molar ratio $(AFMU+1X+1U)/17U$, CYP2A6 by $17U/(17U+17X+1U+1X+AFMU)$, NAT2 by $AFMU/1X$ and XO by $1U/(1U+1X)$.

Blood plasma extraction

Of human blood plasma samples, 1.5 ml was mixed with 2 ml of 2-propanol (Biosolve BV, Valkenswaard, The Netherlands) and extracted twice with 4 ml hexane/diethyl ether (97/3 v/v, Biosolve BV). The hexane/diethyl ether layer was evaporated by a nitrogen flow and the residue was dissolved in 10 µl DMSO (Merck, Darmstadt, Germany), and subsequently diluted in 1 ml cell exposure medium. For control, blank chemicals were extracted in the same way.

DR CALUX® assay

The Dioxin Receptor - Chemical-Activated Luciferase gene eXpression assay was performed as described previously (Bovee *et al.*, 1998). DR CALUX® cells (rat H4IIE hepatoma cells stably transfected with pGudluc1.1, an AhR-regulated luciferase reporter plasmid) (Aarts *et al.*, 1995) were grown in α -MEM (Sigma, St-Louis, USA), containing 10% fetal bovine serum (GIBCO, Breda, The Netherlands) and 1% penicillin/streptomycin (Sigma), at 37°C and 5% CO₂. Cells were seeded in 48 well plates 24 h before exposure. Blood plasma samples from the donors of each sampling moment were pooled and diluted with cell medium to 15% v/v of the exposure medium (experiment 1), or extracted with hexane/diethyl ether (experiment 2, see 2.10). After 24 h exposure of the cells, the medium was removed and cells were washed with PBS. Cells were lysed using Cell Culture Lysis Reagent (Promega), centrifuged and 20 µl of lysed solution of each well was transferred to a 96 wells plate. Luminescence was measured with a Luminoskan Ascent (Thermo Labsystems, Finland) upon addition of 0.1 ml of assay mixture (20 mM Tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄·7H₂O, 0.1 mM EDTA, 33.3 mM DTT, 261 µM Coenzyme A, 470 µM Luciferin, 530 µM ATP, pH 7.8). TCDD-equivalent (TEQ) values were calculated by use of the program SlideWrite V6 and the formula $a0/(1+(x/a1)^{a2})$, which describes the AhR activation process suitably (Hoogenboom *et al.*, 2006).

RESULTS

Effects of TCDD and NAhRAs in isolated human lymphocytes

Induction of AhR responsive genes in isolated human lymphocytes by TCDD

Lymphocytes can be cultured either stimulated or non-stimulated. In the present study it was decided to use non-stimulated cells. The freshly isolated lymphocytes were used to determine the sensitivity of human lymphocytes to the activation of the AhR. Figure 1 shows that after exposure to a high, but non-cytotoxic amount of 5 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the freshly isolated lymphocytes showed a highly significant up-regulation of CYP1B1 mRNA of 2.4 times after both 24h and 72h. CYP1A1 mRNA was only markedly up-regulated in these cells at 72h, but could not be tested for significance (n=1) (Fig. 1). These findings are in line with the results obtained with mitogen stimulated lymphocytes exposed to 10 nM TCDD (van Duursen *et al.*, 2005)

After 48h of exposure to a ten-fold lower concentration of 500 pM TCDD, freshly isolated lymphocytes of two other donors showed an up-regulation of CYP1A1 mRNA (14.3 and 3.3-fold

resp. The large variation was caused by very low amounts of mRNA), CYP1B1 mRNA (1.5 and 1.3-fold resp.), and NQO1 mRNA (1.8 and 2.0-fold resp.) (Fig. 2). It was decided to use these exposure conditions for TCDD and equivalent amounts of NAhRAs in the following experiments.

Modulation of gene expression by TCDD, NAhRAs and the AhR antagonist ANF

Freshly isolated lymphocytes from volunteers were exposed *in vitro* for 48 h to 500 pM TCDD, 100 nM indolo[3,2-*b*]carbazole (ICZ), 417 geq./L grapefruit juice extract (GJE), an extraction blank (BLEX), and 3 μ M α -naphthoflavone (ANF). Principal Component Analysis (PCA) with discriminant analyses of all 4268 selected genes showed a clustering of TCDD and ICZ treatments and separate clusters of GJE, BLEX and ANF treatments (Fig. 3). The genes which contributed most clearly to the effect of TCDD are listed in Table 1. A fold change limit of 1.5-2.0 and a *t*-test *P*-value of 0.05 would be preferable (Guo *et al.*, 2006), but not many genes met these criteria. Therefore we applied the less stringent criteria of a fold change of 1.3 and a *P*-value of 0.2 (Table 1). Of the 24 genes listed in Table 1, only CYP1B1 and TIPARP (TCDD-inducible poly(ADP-ribose) polymerase) have been described as AhR responsive in literature (Hankinson, 1995; Ma *et al.*, 2001). Several of the well-known AhR-responsive genes, including CYP1A1, CYP1A2 and MMP1, were below the detection limit in our microarray experiment. The expression changes of CYP1B1, TIPARP and two other known AhR-responsive genes, NQO1 and GSTP1 upon exposure to TCDD, ICZ, GJE, BLEX

Fig.1. Fold change in amount of CYP1A1 mRNA and CYP1B1 mRNA in freshly isolated human lymphocytes after exposure to 5000 pM TCDD as measured by RT-PCR. Mean of four samples from one donor (except for CYP1A1, 72h: one sample) $\pm$ SD, ** $p < 0.01$ vs DMSO control.

Fig. 2. Fold change in amount of CYP1A1 mRNA, CYP1B1 mRNA and NQO1 mRNA in freshly isolated human lymphocytes of two donors after exposure for 48h to 500 pM TCDD as measured by RT-PCR. Mean of 3 (CYP1A1) or 4 samples (CYP1B1 and NQO1) $\pm$ SD, * $p < 0.1$ and ** $p < 0.05$ vs DMSO control.

Fig. 3. PCA-plot with discriminant analyses of the fold changes of 4268 genes in human lymphocytes exposed ex vivo during 48 h. TCDD + ICZ, 500 pM TCDD (dark dots) and 100 nM ICZ (light dots); GJE, grapefruit juice extract; BLEX, blank extract; ANF, 3μM α-naphtoflavone. The variance can be explained for resp. 43.9%, 24.8% and 17.2% by the first three components.

Table 1A. Mean fold changes in expression of 24 genes which were most prominently affected (fold change > 1.3 up or down; $p < 0.2$) after exposure of freshly isolated human lymphocytes to 500 pM TCDD. Also listed is the fold change of same 24 genes after exposure to 100 nM indolo[3,2-b]carbazole (ICZ), grapefruit juice extract (GJE), blank extraction (BLEX) and 3 μ M α -naphthoflavone (ANF). Fold changes were determined by microarray analysis and are the average of *ex vivo* exposed lymphocytes obtained from 5 (TCDD), 4 (ICZ), 3 (ANF), respectively 2 donors (GJE and BLEX).

Gene symbol	Acc. No.	Fold change				GJE	BLEX	ANF	
		TCDD		ICZ					
CYP1B1	NM_000104	1.73	**	1.76	**	0.80	0.99	0.52	‡
RPL39	NM_001000	1.44	*	1.03		0.97	1.01	0.99	
YPEL5	NM_016061	1.40	**	1.39	*	0.87	1.29	1.12	
TIPARP	NM_015508	1.40	*	1.46	**	0.98	1.27	0.54	‡
MTCO2	NM_173705	1.37	**	1.06		1.25	0.90	1.06	
ERBP	NM_014597	1.36	***	1.23	*	1.05	1.16	1.14	*
S100A8	NM_002964	1.35	**	1.51	*	1.45	0.66	1.20	
TTC11	NM_016068	1.35	*	1.09		0.93	0.90	1.25	
RPS4X	NM_001007	1.35	**	1.21		0.85	1.25	0.91	
GRSF1	NM_002092	1.35	**	0.94		1.00	1.05	1.30	
S100A8	NM_002964	1.34	*	1.48	*	1.47	0.61	0.77	
EVI2A	NM_014210	1.34	*	1.26	*	0.84	1.19	0.98	
COX7B	NM_001866	1.34	*	1.07		0.96	1.04	1.11	
ALOX5AP	NM_001629	1.33	***	1.29	***	1.36	0.63	0.73	‡
TCF7	NM_003202	1.32	*	1.05		0.97	1.25	1.48	***
AF241539	AF241539	1.31	**	0.89		0.87	1.01	0.94	
ATP5C1	NM_005174	1.30	**	1.42		0.99	0.73	0.96	
HIST1H4C	NM_003542	1.30	*	1.31		1.03	1.56	1.31	
TNFRSF25	NM_148965	0.77	***	1.05		1.19	1.42	1.23	
XRCC1	NM_006297	0.77	***	0.99		0.96	1.17	0.98	
FLJ13769	AK023831	0.76	*	0.76	***	1.74	0.72	0.54	***
ANKRD11	NM_013275	0.75	**	0.78	***	0.94	1.08	0.90	
HERC1	NM_003922	0.75	***	0.85		0.98	0.99	0.83	
MGC4796	NM_032017	0.74	***	1.05		1.17	0.87	0.94	

Note: Significance of fold change in gene expression of samples compared to control blank:

* $p < 0.2$, ** $p < 0.1$, *** $p < 0.05$, ‡ direction reversed with $p < 0.2$

and ANF as analyzed by microarray are plotted in Fig. 4. No stringently significant up- or down-regulation was observed, except for the down-regulation of TIPARP by ANF ($p < 0.05$). A tendency of up-regulation of CYP1B1 and TIPARP by TCDD and ICZ ($p < 0.1$), and down-regulation of these genes by the antagonist ANF ($p = 0.15$ and < 0.05 resp.) are noticeable. This tendency is consistent with the clustering of TCDD and ICZ shown in the PCA-plot (Fig.3).

Human intervention study with cruciferous vegetables and grapefruit juice

Human volunteers received cruciferous vegetables or grapefruit juice on three consecutive days, following a period without these products. Based on the results of a former study on DR CALUX[®] measurements in foods (de Waard *et al*, *in press*), we calculated the amount of AhR agonists as

Table 1B. Description of the Gene symbols.

Gene symbol	Description
CYP1B1	Homo sapiens cytochrome P450, family 1, subfamily B, polypeptide 1
RPL39	Homo sapiens ribosomal protein L39
YPEL5	Homo sapiens yippee protein (CGI-127)
TIPARP	Homo sapiens TCDD-inducible poly(ADP-ribose) polymerase
MTCO2	Homo sapiens cytochrome c oxidase II
ERBP	Homo sapiens acidic 82 kDa protein mRNA (HSU15552)
S100A8	Homo sapiens S100 calcium binding protein A8 (calgranulin A)
TTC11	Homo sapiens tetratricopeptide repeat domain 11
RPS4X	Homo sapiens ribosomal protein S4, X-linked
GRSF1	Homo sapiens G-rich RNA sequence binding factor 1
S100A8	Homo sapiens S100 calcium binding protein A8 (calgranulin A)
EVI2A	Homo sapiens ecotropic viral integration site 2A
COX7B	Homo sapiens cytochrome c oxidase subunit VIIb (COX7B)
ALOX5AP	Homo sapiens arachidonate 5-lipoxygenase-activating protein
TCF7	Homo sapiens transcription factor 7 (T-cell specific, HMG-box)
AF241539	Homo sapiens ncaml mRNA, complete cds
ATP5C1	Homo sapiens ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1
HIST1H4C	Homo sapiens histone 1, H4c (HIST1H4C)
TNFRSF25	Homo sapiens tumor necrosis factor receptor superfamily, member 25
XRCC1	Homo sapiens X-ray repair complementing defective repair in Chinese hamster cells 1
FLJ13769	Homo sapiens cDNA FLJ13769 fis, clone PLACE4000222.
ANKRD11	Homo sapiens ankyrin repeat domain 11 (ANKRD11)
HERC1	Homo sapiens hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain
MGC4796	Homo sapiens Ser/Thr-like kinase

measured by the DR CALUX[®] assay in the Brussels sprouts intervention to be an extra 0.9-1.6 ng TCDD equivalents/day and in the grapefruit juice intervention to be an extra 14-20 ng TCDD equivalents/day. Blood samples were taken before, and 3 and 24 h after the last NAhRA-enriched diet.

AhR related gene expression in human blood cells

The mRNA levels of the prototypically AhR-responsive genes coding for CYP1A1, CYP1B1 and NQO1 before and after consumption of grapefruit juice and cruciferous vegetables, were determined in blood cells by quantitative RT-PCR (Fig.5). No significant up- or down-regulation of the three investigated genes, or differences between samples taken 3 h or 24 h after exposure was observed in the isolated lymphocytes or in the whole blood cells, both for the intervention with grapefruit juice and with cruciferous vegetables. CYP1A1 mRNA could not be detected in the PAX gene obtained total RNA of blood cells because of the very low amount of CYP1A1 mRNA.

Fig. 4. Fold change in four AhR-responsive gene products as determined by microarray, after 48h exposure of freshly isolated human lymphocytes to 500 pM TCDD, 100 nM ICZ, grapefruit juice extract (GJE), blank extract (BLEX) and 3 μ M α -naphthoflavone (ANF). Mean $\pm$ SD, * $p < 0.1$, ** $p < 0.05$ vs DMSO control.

Biotransformation enzyme activities as determined by urinary caffeine metabolites

Before and after the interventions with grapefruit juice and cruciferous vegetables the activities of the four biotransformation enzymes CYP1A2, CYP2A6, NAT2 and XO were determined by calculating the ratios of caffeine metabolites in urine. Changes in enzyme activity are shown as ratios and plotted in Fig. 6. Consumption of grapefruit juice slightly reduced the activity of CYP1A2 and significantly reduced the activity of NAT2 down to 70%. Consumption of cruciferous vegetables did not show any significant changes in enzyme activity (Fig. 6).

AhR activity in blood plasma as determined by DR CALUX®

During the three day NAhRA-enriched diet intervention study, blood plasma samples were collected before the first consumption, and 3 h and 24 h after the last consumption of grapefruit juice or cruciferous vegetables. The presence of AhR agonists, as measured in pooled plasma samples by use of the DR CALUX® assay, showed a significant decrease after 3 h and a smaller, non-significant decrease after 24 h of the last grapefruit juice consumption. No significant effects were found after the intervention with cruciferous vegetables (results not shown). In this case, the blood plasma was directly mixed with the incubation medium of the cells, which was limited to 15% (v/v), and the signal levels were relatively low. In order to obtain higher signal levels and so decreasing the detection limit, we concentrated the plasma samples by extraction with hexane/diethyl ether (97/3), which resulted in similar findings, but as expected, higher signal levels with an overall lesser deviation (Fig. 7). Both time intervals showed a small but significant 12% increase of DR CALUX® activity after the

Fig. 5. Fold change in three prototypical AhR-responsive mRNAs (CYP1A1, CYP1B1, and NQO1 mRNA) in human blood cells from 5 to 6 volunteers as determined by RT-PCR. Samples were taken before, 3h and 24h after the last consumption of grapefruit juice or cruciferous vegetables of a three day intervention. Data expressed in comparison to the sample taken before the intervention. Mean $\pm$ SD; ND, not detectable; PAX, blood from PreAnalytiX RNA tubes; lymph, freshly isolated peripheral blood lymphocytes.

Fig. 6. Relative biotransformation enzyme activities in 5 to 6 volunteers before and after consumption of grapefruit juice or cruciferous vegetables, determined by HPLC analysis of urinary caffeine metabolites. Mean $\pm$ SD, * $p < 0.05$ vs before intervention.

Fig. 7. AhR activation by extracts of blood plasma samples from human volunteers before and after (3h and 24h) three days of intervention with grapefruit juice (GJ) or cruciferous vegetables (C), determined as luciferase induction in DR-CALUX® cells. Control is blank extraction with the chemicals, but without plasma. Data were corrected for the response obtained with the solvent DMSO. Mean $\pm$ SD; * $p < 0.05$ for difference with the response measured before the intervention.

cruciferous vegetable intervention, and an insignificant 12% decrease 24h after the grapefruit juice intervention. All plasma samples showed a significantly higher signal than the blank extraction control, with levels between those of the 1 and 1.5 pM TCDD-standards (Fig.7).

DISCUSSION

In contrast to the AhR-mediated toxic responses induced by environmental contaminants such as TCDD, exposure to natural AhR-agonists by consumption of citrus fruits and vegetables is generally associated with beneficial health effects. Insight in the molecular pathways modulated by NAhRAs after binding to the AhR, and detection of possible AhR-related modulation of these pathways in human tissues may be of great value for the risk-benefit analysis of these dietary constituents. In order to identify AhR related pathways in human blood cells, we analyzed gene expression modulation in peripheral lymphocytes *ex vivo* exposed to TCDD and NAhRAs, and analyzed blood samples from human volunteers intervened with a NAhRA-enriched diet.

The well-known AhR-regulated gene products CYP1A1, and CYP1B1 have been determined previously in blood cells from workers accidentally exposed to dioxins and related AhR agonists, but there is no consensus about the reliability of these two genes as biomarkers for AhR agonist exposure (Spencer *et al.*, 1999; Toide *et al.*, 2003; Landi *et al.*, 2005; van Duursen *et al.*, 2005). Therefore, it was of interest to establish if other genes, preferentially or specifically expressed in blood cells, are also affected by exposure to AhR agonists, potentially showing higher levels of up- or down-regulation than CYP1A1 and CYP1B1. By using quantitative RT-PCR the up-regulation of the genes coding for CYP1A1, CYP1B1 and NQO1 could be reproducibly detected in *ex vivo* exposed human lymphocytes (Fig 1 and 2). However, the response of these genes was relatively moderate as compared to the induction levels reported for other human cells, like hepatoma, lung and colon cells (Puga *et al.*, 2000; Martinez *et al.*, 2002). The 22 K gene microarray used in this study did not reveal genes significantly more affected by TCDD than CYP1B1 (Table 1).

ICZ showed some overall TCDD-like gene expression modulation in lymphocytes, whereas GJE and ANF showed no TCDD-like effect whatsoever (Table 1). Interestingly, the two genes that are known to be responsive to AhR activation, CYP1B1 and TIPARP, are both linked to tumor formation (Spink *et al.*, 1998; Ma *et al.*, 2001; Katoh, 2003; Peter Guengerich *et al.*, 2003) and CYP1B1 was already proposed as a biomarker for AhR activation in human lymphocytes (Spencer *et al.*, 1999). As expected, the AhR antagonist ANF does not affect these genes in the same way. Also the fold change in mRNA of NQO1 caused by ICZ resembles the up-regulation by TCDD more than those caused by GJE and ANF (Fig. 4). The established AhR-responsive gene for GSTP1 was neither affected by TCDD, nor by ICZ, suggesting that up-regulation of this gene is possibly cell type-specific. Together with the PCA results (Fig. 3) all this shows that there is a resemblance in gene expression between exposure to TCDD and ICZ, which is in agreement with our results from a microarray experiment with human colon Caco-2 cells (De Waard *et al.*, 2007). In contrast, GJE does not induce a TCDD-like gene expression modulation in this study (Table 1, Figs. 3 and 4). This may also be a cell specific effect, or, in view of the longer exposure time of 48 h compared to the 24 h in our previous study,

this difference may possibly be caused by instability of the AhR activating compounds in GJE, most likely furocoumarins like bergamottin.

In view of the apparent TCDD-like response in lymphocytes induced by ICZ, and the AhR activation by GJE observed in the former Caco-2 study, we performed a human intervention study with NAhRA-containing foods, to investigate if a response of AhR-controlled genes could be detected *in vivo*. With quantitative RT-PCR the gene expression of CYP1A1, CYP1B1 and NQO1 was determined before, shortly after (3 h) and longer after (24 h) the intervention. However, no significant effect on the regulation of these genes could be detected, neither in total blood cells collected by the PAX gene system, nor in freshly isolated lymphocytes, both shortly and longer after the 3-day intervention period (Fig. 5). Possibly the doses of NAhRAs were too low, or the exposure time was too short, to measure any effects. In other studies comparable amounts and exposure times of cruciferous vegetables produced AhR related effects in humans, e.g. induction of AhR responsive glutathione-S-transferase isozymes, or CYP1A2 induction measured by caffeine metabolism after consumption of 300 to 500 g of cruciferous vegetables (Nijhoff *et al.*, 1995; Kall *et al.*, 1996; Lampe *et al.*, 2000). However, by using the caffeine metabolism assay, we could not detect an up-regulation of CYP1A2 enzyme activity (Fig. 6). Moreover, we did observe a down-regulation of the phase II enzyme NAT2 by the grapefruit juice intervention (Fig. 6), which has not yet been described in literature as an AhR-mediated effect. Because grapefruit components can inhibit the catalytic activity of many biotransformation enzymes, especially by its constituent bergamottin (Goosen *et al.*, 2004), possibly these components can also inhibit the enzyme activity of NAT2. Relatively small doses of grapefruit juice, once or a few times repeatedly, showed a clear CYP3A4 inhibition in human studies, which is ascribed to bergamottin (Goosen *et al.*, 2004). Furthermore, the low micromolar range concentrations of bergamottin applied here are within the concentration range reported to result in AhR-mediated induction of CYP1A1 gene expression (Baumgart *et al.*, 2005). This implies that theoretically, the amount of grapefruit juice used in our intervention study should be high enough to activate the AhR.

The estimated amounts of AhR agonists as measured by the DR CALUX[®] assay of 0.9-20 ng TCDD-equivalents/day extra in the intervention diets seems considerably high in comparison with the estimated intake of the sum of dioxins and dioxin-like PCBs in The Netherlands, namely 1.2 pg WHO-TEQ (Toxic Equivalents) per kg body weight (bw) per day, which would be for a person weighing 70 kg 84 pg WHO-TEQ (Baars *et al.*, 2004). However, measurements of AhR-activity in blood plasma by use of the very sensitive reporter gene assay DR CALUX[®] detected a low level of activity after consuming cruciferous vegetables. On the contrary, after the intervention with grapefruit juice, the activity even decreased (Fig. 7), which suggests that there is more AhR-antagonistic activity in these vegetables than agonistic activity. An explanation for the absence of AhR-response in blood cells may be the presence of high amounts of other sources of AhR agonists or antagonists in other foods, which mask the effects of grapefruit juice and cruciferous vegetables (Amakura *et al.*, 2003a; Amakura *et al.*, 2003b; Jeuken *et al.*, 2003). The response observed in the DR CALUX[®] assay corresponds with a level of 0.5-1.5 pg TCDD/g blood plasma, which is relatively high compared to the reported average

of 0.12-0.25 pg TEQ/g plasma (based on an average of 25.4 pg TEQ/g lipid and 0.5-1% serum lipid) (Warner *et al.*, 2005). Connor *et al.* found even higher TCDD-equivalents in blood measured by DR CALUX[®] in an intervention study, comparable with ours. Blood collected during a baseline diet contained 13-64 pg TCDD-equivalents/g, and after consumption of cruciferous vegetables 20-133 pg TCDD-equivalents/g, a significant increment. Consumption of pure I3C-supplements showed even levels of 62-218 pg TCDD-equivalents/g whole blood (Connor *et al.*, 2004). The difference with our results may be partly explained by their CALUX measurement. Connor *et al.* used a mouse cell line, extracted the samples with tert-butyl-methyl ether and used a very short exposure time of 4h. Shorter exposure times result often in a relatively high response from compounds other than TCDD because of the degradation of compounds by the cells and the relatively slow kinetics of the response to TCDD, which influences the outcome of the TEQ-calculations considerably. With a comparable CALUX method as used by Connor *et al.*, Schecter *et al.* found a background level of 136 pg TCDD induction equivalents (IEQ)/g blood in humans, using the term IEQ instead of TEQ, for the latter is in principle specific for dioxin-like compounds. These pooled samples contained 0.25 pg TEQ/g blood when examined by using a clean-up procedure designed for detecting dioxin-like chemicals and GC-MS (Schecter, 1999). The 136 pg/g IEQ is about the average found in the I3C-supplement study of Connor *et al.*, which suggests that the normal exposure to non-dioxin-like AhR agonists may be considerable.

We conclude that, although TCDD and the NAhRA ICZ do show AhR-mediated effects in *ex vivo* exposed human lymphocytes, the response of AhR related genes seems too low to use it as a sensitive biomarker for exposure to NAhRA-containing foods added to the normal human diet. More reliable, but also more invasive and much less readily available, would be obtaining bioptic material from target tissues like liver and colon.

Also other ways of detection of AhR-activity (DR CALUX[®] and caffeine metabolism) in the human body after consumption of significant amounts of NAhRA-containing foods failed to reveal clear effects related to the AhR. This indicates that the AhR-agonistic effects of NAhRAs in food may be very subtle, or influenced by a high background of AhR-agonists or -antagonists in food. A possible normal occurrence of relatively high amounts of AhR-mediating compounds in the human diet should therefore be taken into consideration for the risk/benefit evaluation of both NAhRAs and dioxin-like compounds.

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5

Repeated exposure of green-fluorescent protein-based reporter cells (DRE-CAFLUX) improved detection of non-persistent aryl hydrocarbon (Ah) receptor agonists in low concentrations in human blood plasma

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AhR) is thought to be the major toxicity mechanism of dioxins and dioxin-like compounds. To screen environmental and food samples for the presence of AhR-agonists, the DR CALUX[®] assay has been developed, which uses the luciferase reporter gene encoding a relatively unstable product. In this study, we investigated if the use of the DRE-CAFLUX bioassay, based on enhanced green-fluorescent protein (EGFP) as a reporter gene encoding a more stable product, would improve the detection limit of non-persistent AhR-agonists as compared to the DR CALUX[®] assay. We hypothesized that repeated exposure of the DRE-CAFLUX cells would lower the limit of detection for unstable AhR-activating compounds, while this would not be the case for the DR CALUX[®] assay. After two extra exposures of the same cells to the biodegradable AhR-agonist benzo[*a*]pyrene, the detection limit in the DRE-CAFLUX was lowered by 10 times. Additionally, we measured the AhR-activity in human blood plasma samples from volunteers who consumed grapefruit juice or cruciferous vegetables containing natural AhR-agonists (NAhRAs). The bioassay cells were directly exposed to the samples and no differences before and after the intervention were shown in the DR CALUX[®] assay. However, in the DRE-CAFLUX assay using repeated exposure, a significant increment of the signal was shown in plasma samples after consumption of cruciferous vegetables. We conclude that by use of repeated exposures, the DRE-CAFLUX assay can be a relatively sensitive, cheap and powerful tool for screening of large numbers of complex samples with low amounts of non-persistent AhR-agonists.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) acts as a ligand-activated transcription factor and is present in most types of mammalian cells. The activated AhR complex translocates to the nucleus and binds to dioxin-responsive elements (DREs) occurring in the regulatory sequences of AhR-regulated genes resulting in the induction of, among others, the biotransformation enzymes CYP1A1, CYP1A2, CYP1B1, GSTP1 and UDPGT1A6. The dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the strongest AhR activators and the prototypical AhR-agonist. The activation of the AhR is considered to be the first step in the mechanism behind the toxic effects of dioxins and dioxin-like environmental pollutants (Hankinson, 1995; Safe, 2001). Also other types of compounds, like the polycyclic aromatic hydrocarbon benz[*a*]pyrene (BaP) and natural Ah-receptor agonists (NAHRAs), such as indoles originating from cruciferous vegetables and furanocoumarins in citrus fruits, can bind to and activate the AhR (Denison and Nagy, 2003; Van Ede *et al.*, 2008). Whether these types of compounds may have a dioxin-like toxic potency is still under investigation.

The DR CALUX[®] (Dioxin Receptor Chemically-Activated LUCiferase eXpression) bioassay has been developed as a high throughput screening assay for AhR agonist activity. Rat hepatoma H4IIE cells have been stably transfected with the reporter vector pGudLuc1.1 containing a dioxin-responsive element-driven firefly (*Photinus pyralis*) luciferase gene. The induction of luciferase by AhR-agonists can be detected by addition of luciferin and measurement of the resulting bioluminescence (Aarts *et al.*, 1995; Garrison *et al.*, 1996). However, native firefly luciferase, the reporter protein used in the DR-CALUX[®], has a C-terminal signal peptide targeting the expressed reporter protein to the peroxisomes of the cells (Keller *et al.*, 1987) and displays relatively pronounced protein instability (Thompson *et al.*, 1991) resulting in rapid inactivation. In addition, DR CALUX[®] (H4IIE rat hepatoma) cells are able to metabolize certain AhR agonists into inactive metabolites. As a result, the bioluminescence signal from the luciferase reporter protein induced by a rapidly metabolised AhR agonist, such as benzo[*a*]pyrene (Baird *et al.*, 2005), will be only transiently detectable, and will rapidly extinguish after the inducer has been metabolised (Aarts *et al.*, 1998; Hoogenboom *et al.*, 1999). This short half-life of luciferase makes it a less suitable reporter for the detection of especially low levels of labile or biodegradable AhR-active compounds. As an alternative bioassay, the Dioxin Responsive Element-driven Chemical-Activated FLUorescent protein eXpression (DRE-CAFLUX) assay was developed, using the same H4IIE rat hepatoma cells and the same dioxin-responsive regulatory sequence as applied in the DR CALUX[®] assay (Aarts *et al.*, 1998). However, a more stable reporter protein was used. The DRE-CAFLUX cells contain a dioxin-responsive element-driven *egfp* reporter gene which encodes the enhanced version of a jellyfish (*Aequoria Victoria*) green-fluorescent protein (EGFP). This will remain stable and continue to be detectable by its characteristic fluorescence for many days (Chalfie *et al.*, 1994). An additional advantage of the EGFP reporter protein is, that it can be measured non-destructively and real-time in living cells using a fluorimetric plate reader (Aarts *et al.*, 1998).

Recently, we performed a human intervention study with cruciferous vegetables and grapefruit juice, both food items containing relatively high levels of NAhRAs (De Waard *et al.*, 2008), to investigate the levels of AhR-agonist activity attained in human blood plasma upon consumption of a NAhRA-rich diet (De Waard *et al.*, *submitted*). The analysis of blood samples obtained from participants of this intervention study using the DR-CALUX^{*} bioassay did not reveal a clear difference between samples taken before and after the intervention. However, the response obtained in the test was rather low, indicating the possibility that the sensitivity of the DR-CALUX was too low to detect potential differences due to the low input of NAhRAs into the assay resulting from direct measurement in blood plasma.

In the present study, we investigated whether the use of the DRE-CAFLUX, based on a more stable reporter protein, would improve the detection limit for low amounts of non-persistent AhR-agonists as compared to the DR CALUX^{*}. In addition, we investigated whether repeated exposure of the DRE-CAFLUX cells, which is supposed to result in a stepwise accumulation of EGFP reporter protein after each consecutive exposure, would even further improve the detection limit of the DRE-CAFLUX. The polycyclic hydrocarbon benzo[*a*]pyrene (BaP) was used as a model compound for rapidly metabolized AhR-agonists, and the blood plasma samples originating from the above mentioned NAhRA-intervention study were used as model samples with very low amounts of natural non-persistent AhR-agonists.

MATERIALS AND METHODS

Materials

HBSS, α -MEM medium, trypsin, Foetal Calf Serum (FCS) and gentamicin, were purchased from Invitrogen (Breda, The Netherlands), standard chemicals from Merck (Darmstadt, Germany), and TCDD from Schmidt B.V. (Amsterdam, The Netherlands). The TCDD stocks were checked by gas chromatography-mass spectrometry (GC-MS) for accurate concentrations. Molecular-biological reagents were from Bethesda Research Laboratories (Gaithersburg, MD, USA) and Roche (Mannheim, Germany). All other chemicals used were p.a. grade.

Human NAhRA-enriched food intervention study

This study is described in De Waard *et al.*, *submitted* (see Chapter 4 in this thesis). In short: after a three day wash-out period, without consumption of cruciferous vegetables and citrus fruits, three male and three female healthy non-smoking volunteers received during three consecutive days 600 ml of grapefruit juice, which was divided in three portions of 200 ml distributed over the day. Three weeks later and again after a three day wash-out period, the same participants consumed during three consecutive days a soup containing 150 g broccoli per portion and a meal with 300 g Brussels sprouts. During the intervention, no medication or vitamin supplementation was used by the subjects. Blood plasma samples were taken before, and 3 h and 24 h after the last NAhRA-enriched meal. The protocol was approved by the Medical-Ethical Committee of Maastricht University and

the Academic Hospital Maastricht. The subjects were fully informed about the details of the study and gave their written informed consent.

Construction of the DRE-CAFLUX reporter cell line

The DRE-CAFLUX cell line was generated as briefly described in Aarts *et al.* (1998) by stable transfection of the H4IIE cell line purchased from ATCC with an EGFP reporter plasmid constructed analogous to pGudLuc1.1 used to generate the DR CALUX⁺ cells (Aarts *et al.*, 1995). A 1829 bp *Hind* III fragment was excised from the reporter construct pGudLuc1.1 containing a 1330 bp portion of the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) promoter sequences, and in addition, four canonical DRE sequences derived from the mouse *cyp1a1* upstream gene regulatory region (Garrison *et al.*, 1996). The promoter- and enhancer-less EGFP reporter vector pEGFP-1 (Clontech) was digested with *Hind* III and the 1810 bp vector fragment was gel-purified and ligated to the gel-purified *Hind* III fragment excised from pGudLuc1.1. The resulting pMDREegfp1.1 construct (Aarts *et al.*, 1998), similar to pGreen1.1 (Nagy *et al.*, 2002), was propagated in *E. coli* using standard recombinant DNA techniques (Sambrook *et al.*, 1989). Subsequently, H4IIE cells were stably transfected with the pMDREegfp1.1 construct using DOTAP (N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate) liposomal transfection reagent as prescribed by the manufacturer (Roche Diagnostics Nederland B.V., Almere, The Netherlands). The transfected cell population was diluted and plated in five tissue culture petri dishes (diameter 10 cm) in α -MEM culture medium containing 1 mg/ml G418 (Geneticin, Gibco). Separate and rounded cell colonies were isolated from the petri dishes using cloning rings and propagated. To obtain the DRE-CAFLUX cell line, three consecutive rounds were applied of selecting the clone with the highest induction factor and absolute level of EGFP expression upon exposure to 1nM TCDD.

DRE-CAFLUX assay

DRE-CAFLUX cells were grown in α -MEM (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), containing 10% heat-inactivated fetal calf serum (Gibco, Invitrogen, Breda, The Netherlands), at 37°C, 5% CO₂, and 100% humidity. Cells were seeded in 96-well plates (Greiner Bio-One cat. no. 655180, Alphen a/d Rijn, Netherlands) 24 h before exposure at 95-100% confluence in 100 μ l of medium per well and allowed to attach overnight. Concentration series of TCDD and BaP were made dissolved in DMSO. From these stocks, exposure medium was prepared by diluting the stocks 200 times into the required volume of culture medium without gentamicin and geneticin (final concentration of DMSO 0.5% v/v). The resulting exposure medium was mixed on a plate shaker for at least 5 min. before addition to the cells. Human blood plasma was spiked using the same TCDD working stocks by addition of 10 μ l from the TCDD stock with the appropriate concentration into 1 ml plasma. To prepare exposure medium from (spiked) plasma samples, plasma was 10 times diluted in culture medium. Both TCDD and spiked plasma stocks were stored at 4°C to avoid precipitation of TCDD; plasma samples from the intervention study were stored at -80°C. All stocks were warmed in the incubator at least one hour and vortexed before addition to

the medium. To expose the cells the culture medium in which they had been seeded was replaced by the appropriate exposure medium. Medium containing DMSO, respectively medium with 10% v/v human plasma and DMSO were used as a control. Cells were exposed in a tissue culture incubator at 37°C, 5% CO₂, and 100% humidity. Fluorescence was measured real-time using the Cytofluor 2350 fluorescent plate reader (Millipore) with 485/530 nm excitation/emission filters. To preserve sterility and to allow repeated measurements, the lid was left on the plates during the measurements.

DR CALUX® assay

DR CALUX® cells (rat H4IIE hepatoma cells stably transfected with the AhR-regulated luciferase reporter plasmid pGudLuc1.1) (Aarts et al., 1995) were grown in α -MEM (Sigma, St-Louis, USA), containing 10% heat-inactivated fetal calf serum (Gibco, Invitrogen, Breda, The Netherlands), at 37°C, 5% CO₂, and 100% humidity. Cells were seeded in 96-well plates (Greiner Bio-One cat. no. 655180, Alphen a/d Rijn, Netherlands) 24 h before exposure at 95-100% confluence in 100 μ l of medium per well and allowed to attach overnight. Exposure medium containing test chemicals, human plasma and controls were prepared as described above for the DRE-CAFLUX. To expose the cells the culture medium above the cells was exchanged for 100 μ l of exposure medium. After the desired exposure time, the exposure medium was removed and cells were washed twice with diluted phosphate-buffered saline (PBS) (50% demineralised water/50% PBS v/v) and subsequently 30 μ l of Low Salt Buffer (10 mM Tris pH 7.8, 2 mM DTT) was added to each well and the plate was frozen in a freezer at -80 °C during at least 30 min. and thawed again to lyse the cells. Luciferase activity was measured using a Luminoskan luminometer (LabSystems) equipped with two injectors used to first inject 100 μ l of Flash Mix (20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂•5H₂O, 2.67 mM MgSO₄•7H₂O, 0.1 mM Na₂EDTA•2H₂O, 2.0 mM DTT, 0.47 mM luciferine, 5.0 mM ATP) into each well. The resulting light production was quantified during 2 sec and then the remaining luciferase activity was quenched by injecting 50 μ l of 0.2 M NaOH using the second injector. Light production was expressed as relative light units (RLUs).

Data analysis

Significance of the induction of reporter gene expression was tested against the vehicle control using Student's t-test. Dose-response curves for induction of reporter protein expression by TCDD were constructed by fitting the data to the one-site ligand binding equation [response = (maximal response level $\times$ concentration TCDD) / (concentration TCDD + EC₅₀)] describing a response controlled by binding of ligand to a receptor with one ligand-binding site in relation to ligand concentration.

RESULTS

Construction and characterization of the DRE-CAFLUX reporter cell line

A reporter construct analogous to pGudLuc1.1 (Aarts et al., 1995) was generated, carrying an enhanced green-fluorescent protein (EGFP), instead of a firefly luciferase (LUC) reporter gene, under transcriptional control of the same regulatory sequences as applied to generate the AhR-regulated

luciferase reporter gene cell line DR-CALUX (Aarts *et al.*, 1993; Aarts *et al.*, 1995). This construct was called pMDREegfp1.1 and was stably transfected into rat H4IIE hepatoma cells as described above to produce a dioxin-responsive element (DRE)-mediated AhR-controlled EGFP reporter cell line, to be applied as a Dioxin-Responsive Element-driven Chemical-Activated FLUorescent protein eXpression assay (DRE-CAFLUX) for measuring AhR-mediated biological effects.

The validation of the induction of enhanced green-fluorescent protein (EGFP) expression in DRE-CAFLUX cells as a true AhR-mediated response and the basic comparison to the analogous firefly luciferase-based DR CALUX[®] reporter gene assay has been described elsewhere (Aarts *et al.*, 1998). The major difference between these reporter gene systems is the prolonged reporter protein expression due to the much higher stability of EGFP as compared to luciferase, resulting in intracellular accumulation.

Induction of EGFP expression in DRE-CAFLUX cells by TCDD

Firstly, DRE-CAFLUX cells were exposed to TCDD. TCDD is a compound that can be considered as a constant inducer because it is very slowly metabolized and due to its persistency should lead to an accumulated signal over time. Indeed, when DRE-CAFLUX cells are exposed to a stable inducer such as TCDD the production of EGFP turns out to be sustained resulting in gradual accumulation of EGFP up to at least 96 hours after exposure start (Fig. 1). The EC₅₀ value observed with different exposure times varies from 9.7 to 24.7 pM TCDD, which is highly comparable with the variation in EC₅₀ found using the DR CALUX[®] assay, which is on average 15 pM at 24 h exposure (Murk *et al.*, 1996; Hoogenboom *et al.*, 1999). Moreover, as with the DR CALUX[®] based on luciferase, the dose-response relation for induction of the EGFP reporter protein by 24 hours of exposure to TCDD is very accurately described by the 1-site ligand equation describing occupation of a receptor with one ligand-binding site in relation to ligand concentration, and this also holds for the longer exposure times up to 96 hours. Therefore, we conclude that the DRE-CAFLUX and DR CALUX[®] assays are highly comparable test systems suitable for high throughput screening of (human plasma) samples for AhR agonists. We also determined whether the quantification of AhR agonists in human blood plasma is influenced by the presence of plasma matrix components. A human blood plasma sample was spiked with different concentrations of TCDD and analysed by singular and repeated DRE-CAFLUX assays. Comparison of the response to the same concentration of TCDD in the presence and absence of plasma, showed neither upon singular, nor upon repeated exposure, a significant difference on the EGFP induction in DRE-CAFLUX cells, except for a higher background level (results not shown).

Induction of EGFP expression in DRE-CAFLUX cells by benzo[a]pyrene

Fig. 2 shows the difference in response upon exposure to the readily biodegradable AhR agonist benzo[a]pyrene (BaP) in DR CALUX[®] cells and DRE-CAFLUX cells. Up to a concentration of 100 nM, the response to BaP is significantly higher after 8 h than after 24 h of exposure (Fig. 2A), because BaP is readily metabolised in H4IIE cells, and the induced luciferase relatively unstable. Above 100

Fig. 1. Dose-response relationship for the induction and accumulation of EGFP in DRE-CAFLUX cells by TCDD. Measurements were performed at 24h (■), 48h (▼), 72h (●) and 96 h (▲) after single exposures to TCDD. Means of six exposures $\pm$ SD.

nM, the effect is reversed, probably due to saturated kinetics with respect to the metabolism of BaP. However, in the analogous DRE-CAFLUX cells, the EGFP reporter protein induced by exposure to BaP is stable and the expression level maintained in time (Fig. 2B), although the concentration of BaP must have decreased likewise after 8 hours of exposure as was observed in the DR CALUX⁺ (Fig. 2A), because both reporter cell variants originate from the same H4IIE parent cell strain.

Repeated exposure to benzo[a]pyrene

Subsequently, we investigated whether repeated exposure to the non-persistent AhR agonist BaP, which would result in repeated transient activation of reporter gene expression, would lead to accumulation of EGFP, and as such in an increased sensitivity of the DRE-CAFLUX assay for BaP as compared to the DR CALUX⁺ (Fig. 3). A second and third exposure to the same concentration of BaP, 24 respectively 48 hours after the first exposure, was each found to result in a further increase in the level of EGFP expression (Fig. 3A). In the DR CALUX⁺, a second exposure 24 hours after the first one, did produce a second pulse of luciferase expression which was clearly detectable 8 hours after the second exposure, but was found to be transient, as expected, and to have disappeared completely, or largely, 24 hours later (Fig. 3B). The higher signals found by DR CALUX⁺ after the second exposure are probably caused by the freshly added medium, an unexplained effect seen often in our and others' lab. The detection limit of the DR CALUX⁺ assay, 8 h after changing the medium, remained at 0.1 nM BaP (Fig. 3B), so this effect does not seem to decrease the detection limit, for it concerns especially the higher concentrations of BaP. Because the same result was expected

Fig. 2 A

Fig. 2 B

Fig. 2. Time course of the induction by BaP of (A) luciferase expression in DR CALUX[®] cells, and (B) EGFP expression in DRE-CAFLUX cells. Cells were once exposed to samples for 8 h (●), and 24 h (○) (DR CALUX[®]), and for 24 h (●), 48 h (○), and 72 h (▲) (DRE-CAFLUX). Means of triplicate exposures ± SD.

Fig. 3 A

Fig. 3 B

Fig. 3. Response generated by repeated exposure to 0.03 – 3 nM BaP (A) in the DRE-CAFLUX, and (B), the DR CALUX® cell line. Measurements were performed after 8, 24, 32, 48, 56 and 72 h after the first exposure. Second and third exposures were started respectively 24 h and 48h after the start of the first exposure, after the fluorescence resulting from the previous exposure had been determined at those time points. Data were corrected for the blank DMSO. Means of nine exposures $\pm$ SD. Responses significantly different from background are marked * $p < 0.05$, and ** $p < 0.01$.

again, the exposure was not repeated a third time at 48 hours in case of the DR CALUX[®]. In the DRE-CAFLUX the detection limit for BaP (Fig. 3A) decreased with repeated exposure (1 nM, 0.3 nM, and 0.1 nM for single (24 h), double (2×24 h) and triple (3×24 h) exposure, respectively, and a non-significant but distinct response was observed at 0.03 nM after triple exposure). After a single 24 h period of exposure the DR CALUX[®] started to show a non-significant response at 0.3 nM BaP, becoming significant only at higher concentrations of 1 nM and 3 nM BaP (Fig. 3B), which is similar to the performance observed for the DRE-CAFLUX after a single 24 h exposure. At time point 32 h a significant response is observed in the DR-CALUX[®] due to the second exposure already at 0.1 nM and 0.3 nM BaP, but these responses appear to be transient and therefore present the practical difficulty to catch them the moment of peak induction. When the result is measured at 48 h when the response has stabilised, the second exposure appears not to have improved the detection limit in the DR CALUX[®].

DR CALUX[®] in plasma samples from a human intervention study

AhR agonist activity in human blood plasma samples (10% v/v, 24 h exposure time) from a food intervention study with AhR agonist-containing vegetables or grapefruit juice was first analysed using the luciferase-based DR CALUX[®] assay. Using the standard period of 24 hours of exposure, AhR activity turned out to be only marginally detectable by direct measurement of these plasma samples without any extraction and concentration step (Fig. 4). A low induction was measured with responses at the level found for approximately 0.3 pM TCDD, and no differences were seen between blood plasma samples taken before and after the food intervention.

DRE-CAFLUX in plasma samples from a human intervention study

We tested if the EGFP-based DRE-CAFLUX with repeated exposure would allow direct measurement of AhR agonist activity in the human blood plasma samples from the intervention study with NAhRA-enriched diets with improved sensitivity. Figure 5 shows the response of DRE-CAFLUX cells after repeated exposure to culture medium with 10% human plasma sample during three consecutive periods of 24 hours, and EGFP expression being measured 24, 32, 48 and 72 hours after the first exposure. All samples of the intervention with grapefruit juice showed a similar low response at all measured time points (Fig. 5), which was in the range of the response generated by 1 pM TCDD (24 and 32 hours of exposure), or even substantially below that level (48 and 72 hours of exposure). No effect of the intervention was found. However, after 48 h and a second round of exposure, blood plasma from the intervention with cruciferous vegetables showed a tendency of a higher response 3h after the last consumption ($p = 0.11$), and significantly ($p = 0.04$) at 24 h after the last intervention (Fig. 5C). After 72 h and a third round of exposure, the response of the samples taken at 3 h ($p = 0.08$) and 24 h ($p = 0.02$) after the intervention with cruciferous vegetables was significantly elevated. Using the grapefruit juice intervention as a reference, the effect of the intervention with cruciferous vegetables was even significant after both the second and the third round of exposure (respectively 48 and 72 hours after the first exposure), and this holds for both the

Fig. 4. Luciferase induction in DR CALUX* cells after 24h exposure to human plasma samples (10% v/v in medium) from an intervention study with cruciferous vegetables or grapefruit juice. The average of the sample signal minus the mean DMSO control signal from five (cruciferous vegetables) or six (grapefruit juice) study participants $\pm$ SD was plotted. No significant differences were measured between samples from before the intervention and 3 h or 24 h after the intervention. The bar of TCDD is shown to indicate the level of the signals.

3 h and 24 h time points (Fig. 5C and 5D). As expected, the response to 1 pM TCDD increased after each extra exposure, but the responses of all plasma samples, except for the 3 h and 24 h cruciferous samples, remained as low as the first measurement after 24 h. Also, the response level of all blood samples taken after the intervention with cruciferous vegetables did not increase as much as that of 1 pM TCDD after three consecutive 24 h periods of exposure, and the response level attained by these blood samples appeared to be about half that level (estimated to correspond to approximately 0.5 pM TCDD).

DISCUSSION

In this study, we investigated if the DRE-CAFLUX bioassay, which is based on the stable reporter protein enhanced green-fluorescent protein (EGFP), would enable to detect lower concentrations of non-persistent AhR agonists than the DR CALUX* assay, especially when repeated exposures are performed in order to achieve accumulation of EGFP. Because of the great stability of EGFP, repeated exposure increases the signal in the DRE-CAFLUX assay, while there is no such effect in the analogous DR CALUX* assay, for its reporter gene encodes the relatively unstable luciferase (Aarts *et al.*, 1998). For use in large human intervention studies concerning AhR-agonist exposure,

Fig. 5. Induced EGFP expression in DRE-CALUX cells after 24 h (A), 32 h (B), 48 h (C) and 72 h (D) exposure to human plasma samples (10% v/v in medium) from an intervention study with cruciferous vegetables or grapefruit juice. A second and third exposure of the DRE-CALUX cells were started immediately after the measurements at 24 h respectively 48h after the start of the first exposure. The average of the sample signal minus the mean DMSO control signal from five (cruciferous vegetables) or six (grapefruit juice) study participants $\pm$ SD was plotted. *, $p < 0.05$ for differences between before and after intervention. †, ‡, †‡, $p = 0.05$, resp. $p = 0.01$ for differences between cruciferous vegetables and grapefruit juice interventions.

but also for many other research fields concerning AhR-active components, such as in food and environmental toxicology and in medical research, a sensitive, specific, simple and cheap detection system for AhR-mediated biological effects would offer great advantages.

In a pilot study, we detected by use of the DR CALUX[®] assay marginally elevated levels of AhR-active compounds in human blood plasma samples after a dietary intervention with cruciferous vegetables (de Waard *et al.*, *submitted*). However, this required relatively large plasma amounts and an extraction and concentration procedure. AhR-activity in blood samples after consumption of cruciferous vegetables was reported by Connor *et al.*, also using an extraction procedure and a reporter gene bioassay based on the mouse hepatoma H1L6.1c3 cell line stably transfected with the dioxin-inducible pGudLuc6.1 reporter plasmid (Connor *et al.*, 2007). In our study, measurements involving direct addition of crude test plasma to the cells up to 15% v/v did not show responses well above the detection limit of the DR CALUX[®] and did not reveal effects of the intervention (de Waard *et al.*, *submitted*). Extraction of the samples, however, is costly, time-consuming and may

compromise the recovery and integrity of the compounds of interest and produce artefacts, since the AhR-agonists we study do originate from natural compounds, and may be unstable (Pohjanvirta *et al.*, 2002).

Our present study with the rapidly metabolised AhR-agonist model compound benzo[a]pyrene (BaP) (Machala *et al.*, 2001) showed indeed, that repeated exposure in the DRE-CAFLUX assay decreased the lowest detectable concentration from 1 nM at 24 h exposure to 0.1 nM BaP at 72 h with two repeated exposures (Fig. 3).

It appears that both for the DR CALUX⁺ and DRE-CAFLUX all blood plasma samples show a response just above the detection limit upon a single exposure during 24 hours. However, the samples taken at 3 h and 24 h after the intervention with cruciferous vegetables produce an increased response in the DRE-CAFLUX after repeated exposure as compared to the samples taken before the intervention (Fig. 5). This response was statistically significant and estimated to correspond to approximately 0.5 pM TCDD. Also in our previous study we detected a basal response of blood plasma samples from study subjects on an AhR agonist-enriched diet, and in a blank fetal bovine serum sample (de Waard *et al.*, submitted). This low response in the background range was also found by others (N'Guyen *et al.*, 2002), and a possible explanation is that the presence of corticosteroids in the plasma or serum may have a stimulating effect on the reporter assay signal. For instance, the glucocorticoid receptor agonist dexamethasone causes a small response in the DR CALUX⁺ assay, but also increases the signal of TCDD significantly (Hoogenboom *et al.*, 1999), possibly by upregulation of the AhR (Sonneveld *et al.*, 2007). However, the increased response observed after repeated exposure to the samples from the crucifer intervention only occurring in the DRE-CAFLUX strongly suggests that this response is due to the unstable NAhRAs originating from the cruciferous vegetables, which are apparently found in an AhR-active form in blood plasma. Cruciferous vegetables contain glucobrassicine, which is converted by the enzyme myrosinase into indole-3-carbinol after crushing of the vegetables. Upon consumption, indole-3-carbinol undergoes self-condensation reactions in the acid environment of the stomach, and some of the reaction products, like indolo[3,2-*b*]carbazole (Bjeldanes *et al.*, 1991), are well-known AhR-agonists. If a 10% solution of plasma induces an activity comparable with 0.5 - 1 pM TCDD, the bioactivity in non-diluted blood plasma will be comparable to 5 - 10 pM TCDD. This is near the EC₅₀ in the DR CALUX⁺ (Murk *et al.*, 1996) and therefore likely to be sufficiently high to cause considerable AhR-agonistic effects in the human body. The reason why the EGFP-accumulation effect is stronger for TCDD is probably due to the fact that NAhRAs and BaP are more biodegradable. Another possible explanation is that TCDD accumulates more readily in the cells than the other compounds, and thus more of the previous dose is still remaining during repeated exposure.

The blood plasma samples from the grapefruit juice intervention did not produce a clear response in the DRE-CAFLUX after repeated exposure. As grapefruit contains furanocoumarins, like bergamottin, which can bind to and activate the AhR (Baumgart *et al.*, 2005; Van Ede *et al.*, 2008), increased AhR activity was expected in the blood samples taken after the intervention. Moreover, the calculated amount of TCDD/CALUX-equivalents from NAhRAs in grapefruit juice was much

higher than in the cruciferous vegetables administered in the intervention studies (de Waard *et al.*, *submitted*). Since grapefruit juice as such is known to display AhR-activating bioactivity in the DR CALUX⁺ assay (De Waard *et al.*, 2008), most of the NAhRAs from the grapefruit juice are apparently metabolized into compounds which do not activate the AhR, before they enter the bloodstream, In conclusion, the DRE-CAFLUX bioassay performed with repeated exposure to the test samples can exceed the DR CALUX⁺ in sensitivity and furthermore is capable of real-time and repeated measurements allowing to take measurements when the response has become optimal, is less critical to the time point the measurements are taken because peaks in reporter gene induction are “memorised”, is reasonably fast, and very well amenable to high throughput screening applications for the detection of very low levels of unstable AhR-agonists in blood plasma and other biocompatible complex matrices.

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6

Influence of TCDD and natural Ah receptor agonists on benzo[*a*]pyrene-DNA adduct formation in the Caco-2 human colon cell line

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ABSTRACT

Several compounds originating from cruciferous vegetables and citrus fruits bind to, and activate the aryl hydrocarbon receptor (AhR). This receptor plays an important role in the toxicity of the known tumour promoter and potent AhR-agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). However, vegetables and fruits are generally considered as healthy. Therefore, besides the AhR-activation, the natural AhR agonists (NAhRAs) are assumed to show other health concerning effects. AhR-activation induces several cytochrome P450 phase I enzymes involved e.g. in the bioactivation of carcinogenic polycyclic aromatic hydrocarbons (PAHs), like benzo[*a*]pyrene (BaP), and may as such stimulate DNA-adduct formation of those compounds. Therefore, the influence of TCDD, indolo[3,2-*b*]carbazole (ICZ, a NAhRA originating from cruciferous vegetables) and a NAhRA-containing extract of grapefruit juice (GJE) on BaP-DNA adduct formation in the human Caco-2 cell line was studied. Also we investigated if different effects of TCDD, ICZ and GJE on adduct formation could be related to the modulation of transcription of biotransformation- and DNA-repair enzymes. Co-exposure to high AhR-activating concentrations of both TCDD and ICZ significantly reduced the amount of BaP-DNA adducts at 0.1 μ M BaP, while at higher concentrations of BaP no influence was observed. In contrast, exposure to 0.1 μ M BaP combined with GJE showed a significant increase in BaP-DNA adducts, and a significant decrease at 0.3 and 1 μ M BaP. These differences could not be related to transcription of the phase I and II enzymes CYP1A1, CYP1B1, NQO1, GSTP1 and UGT1A6, nor to transcription of the nucleotide excision repair (NER) enzymes ERCC1, XPA, XPC, XPF and XPG. We conclude that ICZ showed a similar effect on BaP-DNA adduct formation than TCDD, while GJE influenced the adduct formation in a different way. The difference in the influence on adduct formation may be due to effects at the level of enzyme activity, rather than gene expression.

INTRODUCTION

Cruciferous vegetables and citrus fruits have been shown to contain significant amounts of compounds that can act as natural aryl hydrocarbon receptor (AhR) agonists (NAhRAs) (Hoogenboom, 2002; Denison and Nagy, 2003; Jeuken *et al.*, 2003). Binding of an agonist to the AhR activates it as a transcription factor, which is considered the main pathway by which dioxins, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exert their toxicity (Hankinson, 1995; Mimura and Fujii-Kuriyama, 2003; Steenland *et al.*, 2004). Nevertheless, NAhRA-containing vegetables and fruits were reported to possess several health promoting properties, including cancer-preventive potential (Steinmetz and Potter, 1996; Verhoeven *et al.*, 1996; Heber, 2004; Yu *et al.*, 2005), although not all epidemiological studies are consistent in this respect (Verhoeven *et al.*, 1996; van Gils *et al.*, 2005; Lynn *et al.*, 2006). Benzo[*a*]pyrene (BaP), a well known carcinogenic compound originating from incomplete combustion of organic material, for instance by cigarette smoking, is also an AhR agonist. Phase I biotransformation enzymes like the P450 cytochromes CYP1A1 and CYP1B1 can convert BaP into reactive epoxide metabolites, and in the presence of epoxide hydrolase activity also dihydrodiol epoxides are formed. These electrophilic compounds can bind to the DNA and form BaP-DNA adducts, which can cause mutations, and finally may lead to cancer (Baird *et al.*, 2005; Rundle, 2006). Several detoxifying mechanisms in the cell may prevent these injurious effects; Phase II biotransformation enzymes such as glutathione-S-transferase transform the electrophilic compounds into products which can be eliminated rapidly from the cell, and DNA repair enzymes, like the nucleotide excision repair (NER) system, can remove the bulky BaP-DNA adducts from the DNA (Hanawalt *et al.*, 2003; Baird *et al.*, 2005). Thus there appears to be a dynamic balance between these possible metabolic routes of BaP. Since both TCDD and NAhRAs activate the AhR, they theoretically can stimulate the BaP-DNA adduct formation by induction of CYP1A1 and CYP1B1. It has indeed been reported that TCDD can promote the BaP-adduct formation by AhR activation in rats (Harrigan *et al.*, 2004), and that mice lacking the AhR did not suffer from BaP carcinogenicity (Shimizu *et al.*, 2000).

In this study, we investigated the influence of NAhRAs on BaP-DNA adduct formation in a human colon cancer cell line, and compared it with the effects of TCDD. The Caco-2 cell line was chosen since it is generally used as a model system to study the effect and absorption of compounds in the small intestine, and it's known to show AhR-activity and BaP-DNA adduct formation (Shah *et al.*, 2006). We examined the pure NAhRA indolo[3,2-*b*]carbazole, a metabolite from cruciferous vegetables, and a hexane extract of grapefruit juice, containing the suspected NAhRAs bergamottin and certain polymethoxy flavones (Canivenc-Lavier *et al.*, 1996; Baumgart *et al.*, 2005; De Waard *et al.*, 2007). Based on the above mentioned tumour-promoting effects of TCDD on the one hand, and the health-protective properties of vegetables and fruits on the other hand, we hypothesized that addition of TCDD to cells exposed to BaP will show an increase in BaP-DNA adduct formation, while addition of NAhRAs will result in a decrease in BaP-DNA adduct formation. The influence on adduct formation was subsequently related to the effects on bioactivation, elimination and DNA-

repair as examined at the transcription level by measuring mRNA of phase I and II enzymes and NER enzymes.

MATERIALS AND METHODS

Chemicals

Methanol, ethanol, n-hexane, diethyl ether, DMSO, NaCl, EDTA, isoamylalcohol, sodiumacetate, NaH_2PO_4 , formic acid and LiOH were purchased from Merck (Darmstadt, Germany), phenol, tris, endonuclease, spleen phosphodiesterase and urea from Sigma-Aldrich (Steinheim, Germany), fetal bovine serum, penicillin/streptomycin and trypsin from GIBCO (Paisley, UK), SDS, nuclease P1, [γ - ^{32}P]-ATP and LiCl from MP Biomedicals (Irvine, California, USA), chloroform from Biosolve (Valkenswaard, The Netherlands) and T4 polynucleotide kinase from Fermentas (Hanover, USA). ICZ (purity >95%) was synthesized as described by Bergman (Bergman, 1970) and checked for quality by HPLC, NMR, IR and MS, using a reference sample which was a kind gift of prof. J. Bergman, Department of Chemistry, Royal Institute of Technology, and Department of Biosciences at Novum, Huddinge, Sweden).

Preparation of the grapefruit juice extract

Ten ml of grapefruit juice, obtained from the local supermarket, was mixed with 20 ml of methanol/water 85/15, and extracted twice with 20 ml of n-hexane/diethyl ether 97/3 by mixing for 1 h. Hexane-layers were collected, evaporated and the residue dissolved in 40 μl of DMSO for analysis. In a recent study (De Waard *et al.*, 2007) the grapefruit juice extract (GJE) was analysed by HPLC and found to contain a mixture of suspected NAHRAs, like polymethoxy flavones and a furocoumarin. The grapefruit juice extract contained the polymethoxy flavones tangeretin (48.5 μM), sinensetin (3.7 μM), tetramethylscutellarein, nobiletin and heptamethoxyflavone (all three < 0.1 μM), and the furocoumarin bergamottin (4150 μM). Because bergamottin showed the highest concentration, we express in this study the amount of extract as μM bergamottin. Also a blank extraction with 10 ml water has been performed.

Cell culture and exposure

The Caco-2 human colorectal adenocarcinoma cell-line (ATCC, Manassas, USA) was grown in DMEM (Sigma, St-Louis, USA), containing 10% fetal bovine serum and 1% penicillin/streptomycin, at 37°C and 5% CO_2 . Cells were seeded 24 h before exposure in 6-well plates (5.10⁵ cells/well) and exposed for 24 h to sample solutions in DMSO added to the culture medium. Concentration ranges were made of 1.5-1500 pM TCDD (purity 98%, Schmidt BV, Amsterdam, The Netherlands), 1-1000 nM ICZ, 0.008-25 μM benzo[a]pyrene (purity >98%, Aldrich, Steinheim, Germany), and 0.03-6.9 μM bergamottin in grapefruit juice extract for the CYP1A1 mRNA induction experiments. For the adduct analysis, cells were exposed to 0.1 (duplicate), 0.3 or 1 μM BaP (triplicate) together with a very low, low or high AhR-activating concentration of TCDD, ICZ or GJE. In two additional experiments, 0.1 μM BaP was added 6 h (duplicate), or 0.3 μM BaP was added 15 h (triplicate) after

treatment with TCDD, ICZ and GJE, to investigate if pre-treatment with a CYP1A1 inducer could result in a more pronounced effect. The final concentration of DMSO in all exposures was 0.5% (v/v). Cytotoxicity of the mixtures was evaluated using the MTT-colorimetric assay according to Mosmann (Mosmann, 1983), with modifications as described previously (Borm *et al.*, 1997). The number of replicates was four.

RNA and DNA isolation

After exposure total RNA was isolated from the cells using Trizol (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. Purity and integrity of the RNA were checked by UV-absorption and gel electrophoresis, respectively. For DNA isolation, cells were washed twice with ice-cold PBS, trypsinized and suspended in 1 ml medium. After centrifugation (1 min, 1500 rpm) liquid was removed and cells were incubated with 400 µl SET/SDS (100 mM NaCl, 20 mM EDTA, 50 mM Tris, 0.5% SDS, pH 8.0) for 2 h at 37°C, followed by treatment with RNase A/T1 and proteinase K (DNase-free, Boehringer Mannheim). The DNA was purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction and precipitation with 3 M sodiumacetate pH=5.2, and 100% ethanol. After washing with 70% ethanol (v/v) the DNA was dried under a mild nitrogen flow and dissolved in 2 mM Tris (pH 7.4). Concentration and purity of the DNA were determined spectroscopically at 230, 260, and 280 nm.

Quantitative Real-time RT-PCR

One µg of total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in a final volume of 20 µl. Table 1 lists the primer sequences used. β-Actin was used to normalize for differences in RNA input and efficiency during the reverse transcription. Samples were diluted 15 times and duplicates were mixed with primers and iQ™SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA) and amplification took place in an iCycler PCR machine with a MyiQ Single Color Real-Time PCR Detector System (Bio-Rad, Veenendaal, The Netherlands). The specificity of the PCR products was checked by melt curve peak analysis and

Table 1. Primer sequences used for quantitative real-time PCR measurements.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
β-actin	CCT GGC ACC CAG CAC AAT	GCC GAT CCA CAC GGA GTA CT
CYP1A1	TCC TGG AGA CCT TCC GAC ACT	CTT TCA AAC TTG TGT CTC TTG TTG T
CYP1B1	AGT GCA GGC AGA ATT GGA TCA	GCG CAT GGC TTC ATA AAG GA
GSTP1	TGC AAA TAC ATC TCC CTC ATC TAC A	CGG GCA GTG CCT TCA CAT
UGT1A6	GGA ACC CGA CCA TCG AAT C	TCG GGT GAC CAA GCA GAT C
NQO1	GGT GGA GTC GGA CCT CTA TGC	CCT TCA GTT TAC CTG TGA TGT CCT T
ERCC1	ACC CCT CGA CGA GGA TGA G	CAG TGG GAA GGC TCT GTG TAG A
XPA	CCG ACA GGA AAA CCG AGA AA	TTC CAC ACG CTG CTT CTT ACT G
XPC	CCC AGC CCG CTT TAC CA	TGC ATT AAC TGT AAA TGT TCC AAT GA
XPF (ERCC4)	CAC CTC CCT CGC CGT GTA	CGC AAA TAT AAC ACC ACC TTG
XPG (ERCC5)	GCA TGA AAT CTT GAC TGA TAT GAA AGA	TAA GCA AGC CTT TGA GTT GGT ACT G

by gel electrophoresis. Gene expression levels were calculated from the threshold cycle values and normalised by calculating the ratio with the β -actin expression level.

³²P-Postlabeling of BaP-DNA Adducts

The ³²P-postlabeling assay for BaP-DNA adducts was performed as described earlier (Van Schooten *et al.*, 2004). In short, 10 μ g DNA was digested into deoxyribonucleoside 3'-monophosphates by incubation with micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37°C. 75% of the digest was treated with nuclease P1 for 30 min at 37°C and the reaction was terminated by adding 0.5 M Tris. The labeling was performed by using [γ -³²P]-ATP (50 μ Ci) in the presence of T4 polynucleotide kinase for 30 min at 37°C, followed by 2-dimensional chromatography using polyethylene-imine cellulose-sheets (Macherey Nagel, Düren, Germany) using the following solvent systems: S1, 1 M NaH₂PO₄ (pH 6.5); S2, 5.3 M lithium formate 8.5 M urea (pH 3.5); S3, 1.2 M lithium chloride, 0.5 M Tris, 8.5 M urea (pH 8.0); S4, 1.7 M NaH₂PO₄ (pH 6.0). For calibration, three standards of [³H]-BaP-diol-epoxide modified DNA with known modification levels (1 per 10⁶, 10⁷ and 10⁸ nucleotides) were run in parallel in each experiment. Quantification was performed using a PhosphorImager (Raytest-Fujifilm FLA-3000, Germany) with a detection limit of 1 adduct/10⁹ nucleotides. The other 25% of the digest was used for the determination of the amount of normal nucleotides by HPLC using UV-detection.

Statistics

Differences in DNA-adduct levels and gene expression levels were statistically evaluated using the Student's t-test.

RESULTS

CYP1A1 and CYP1B1 mRNA induction

To estimate the onset and progress of the AhR response in Caco-2 cells, different exposure times and concentration ranges have been applied for TCDD, BaP, ICZ and GJE and the amount of mRNA of CYP1A1 was measured by use of quantitative RT-PCR (Fig. 1). Exposure to TCDD resulted in increased gene expression in the low picomolar range and a maximum of about 400 fold change in mRNA amount was reached after 15 h of incubation with 1.5 nM. Six h of exposure showed a considerable lower induction of CYP1A1 mRNA. BaP induced CYP1A1 in a comparable way as TCDD, however at concentrations a thousand times higher (Fig. 1A). The maximum induction of CYP1A1 by ICZ and GJE was about a 100 fold change, both after 15 and 24 h. The effective concentration of ICZ was in the same range as of BaP, and GJE, expressed as μ M bergamottin, induced CYP1A1 to the same level in a concentration range about a factor 10 higher (Fig. 1B). CYP1B1 mRNA determination resulted in similar induction properties of the samples, although the maximum levels were much lower (10-30 fold change, results not shown). The extraction procedure blank (water subjected to the extraction procedure applied to extract the grapefruit juice) did not induce CYP1A1 or CYP1B1 mRNA significantly (results not shown).

Fig. 1 A

Fig. 1 B

Fig. 1. Dose-response curves for CYP1A1 mRNA induction in human Caco-2 cells upon 24 h of exposure to TCDD and BaP (A), and to ICZ and GJE (B). CYP1A1 mRNA was measured by Q-RT-PCR. Means of duplicate measurements. ICZ, indolo[3,2-b]carbazole; GJE, grapefruit juice extract.

The concentrations chosen for BaP, TCDD, ICZ and GJE in the following studies were based on the level of induction of CYP1A1 mRNA after 24h of exposure, a marker for AhR-activation. Based on figure 1A, we estimated a low, medium and high effective concentration of BaP and a low and high effective concentration for TCDD, ICZ and GJE. Low is defined as around 10% of the maximum activation, and high is the maximum activation.

BaP-DNA adducts

In order to determine the effects of TCDD and NAhRAs on the DNA-adduct formation by BaP, human Caco-2 cells were exposed to BaP in combination with TCDD or NAhRAs, and the adduct formation was measured by ^{32}P -postlabelling (Fig. 2). Exposure to 0.1, 0.3 and 1 μM BaP resulted in a dose-dependent formation of DNA-adducts of resp. 3.7, 38, and 244 adducts/10E8 nucleotides. Two concentration levels of TCDD and NAhRAs were tested, resulting in either a relatively high or low level of AhR-activation, as judged from the CYP1A gene expression (Fig. 1A). The concentration of TCDD producing a high level of CYP1A1 gene expression (500 pM) showed nearly a four-fold decrease in BaP-DNA adduct formation ($P = 0.11$, $n = 2$), but only at the lowest concentration of BaP tested (0.1 μM) (Fig. 2A). The low 15 pM TCDD concentration reduced the adduct formation significantly to 64% at 1 μM BaP, but had no significant effect at 0.1 and 0.3 μM BaP. Both the low and high concentration of ICZ caused a strong reduction of BaP-DNA adduct levels (100 nM ICZ: $P = 0.11$, $n = 2$), down to one third of the adducts induced by 0.1 μM BaP, but, like TCDD, ICZ showed no effect at the higher concentrations of BaP (Fig. 2B), with the exception of a small but significant increase at 0.3 μM BaP. In contrast with TCDD and ICZ, both concentrations of GJE showed an approximately two-fold increase in adduct formation at 0.1 μM BaP (Fig. 2C). However, both at 0.3 and 1 μM BaP, the highest concentration of GJE decreased the amount of adducts significantly by five times (Fig. 2C). TCDD, ICZ and GJE without co-exposure with BaP, just as the solvent DMSO had no effect on DNA adduct formation (results not shown).

We also tested whether pre-treatment with a CYP1A1 inducer would produce a more pronounced effect on BaP-DNA adduct formation as compared to simultaneous exposure. Overall, both after 6 h (0.1 μM BaP) and 15 h (0.3 μM BaP) of pre-treatment of the cells with TCDD or NAhRAs, the adduct levels induced by BaP were not significantly different from those induced by simultaneous exposure to these compounds (results not shown). The only small, but statistically significant, difference was observed between simultaneous incubation with 0.3 μM BaP and 100 nM ICZ and 15h pre-incubation with 100 nM ICZ followed by simultaneous incubation. Overall, this indicates that a 6 to 15h earlier onset of AhR-induction does not produce a more pronounced effect on adduct formation.

For statistical purpose, and because the pre-incubation step was found to have mostly no significant effect at all, the results of the duplicate experiments with 0.1 μM BaP simultaneously exposed with (N)AhRAs, and with 6h pre-incubation with (N)AhRAs were pooled to obtain $n=4$. In this way, the decrease in BaP-DNA adduct formation by 500 pM TCDD ($P < 0.05$), 3 nM ICZ ($P < 0.05$) and 100

Fig. 2 A **Fig. 2 B** **Fig. 2 C**

Fig. 2. BaP-DNA adduct formation as measured by 32 P-postlabelling in Caco-2 cells after combined 24h exposure to BaP with TCDD (A), ICZ (B) or GJE (C). White, exposure to BaP alone (0.1 μ M BaP shows 3.7 adducts/10E8 nucleotides, 0.3 μ M BaP shows 38 adducts/10E8 nucleotides, and 1 μ M BaP shows 244 adducts/10E8 nucleotides), light grey, BaP with low concentration of (N)AhRA, dark grey, BaP with high concentration of (N)AhRA. Means of duplicate (0.1 μ M BaP) or triplicate exposures $\pm$ SD, * $P < 0.05$ and ** $P < 0.01$ for the difference between combined exposure and BaP alone. ICZ, indolo[3,2-b]carbazole; GJE, grapefruit juice extract.

nM ICZ ($P < 0.01$), and the increase of adduct formation by 0.15 μM GJE ($P < 0.05$) and 2.3 μM GJE ($P < 0.01$) were found significant (results not shown).

Gene expression modulation

To examine whether the effects of (N)AhRAs on BaP-DNA adduct formation could be related to modulation of the expression of biotransformation- or DNA-repair genes, the mRNA levels of several genes involved in these cellular processes have been measured by Q-RT-PCR (Fig. 3). Of the biotransformation enzymes, it is known that they are effected by AhR-activation (Hankinson, 1995; Denison and Nagy, 2003), for the DNA repair enzymes it is not and therefore adding new information.

CYP1A1. BaP increased the mRNA levels of the phase I biotransformation enzyme CYP1A1 by 12 (0.1 μM BaP) to 84 (1 μM BaP) times (Fig. 3A). Addition of the relatively high (in terms of AhR activation) concentrations of TCDD, ICZ and GJE significantly increased the amount of CYP1A1 mRNA at all BaP concentrations, except for TCDD at 1 μM BaP.

CYP1B1. BaP increased the mRNA levels of the phase I biotransformation enzyme CYP1B1 by 2 (0.1 μM BaP) to 7 (1 μM BaP) times (Fig. 3B). Addition of TCDD and GJE significantly increased the amount of CYP1B1 mRNA caused by BaP (Fig. 3B). ICZ showed only a minor increase at 0.1 μM BaP. The induction by ICZ at 1 μM BaP was not determined.

NQO1. No up or down regulation of the phase II biotransformation enzyme NQO1 was observed after exposure to BaP, TCDD and NAhRAs (Results not shown).

GSTP1. BaP caused a 2.5-3 fold increase of the phase II biotransformation enzyme GSTP1 but the response was not dose-related. Although TCDD, ICZ and GJE alone showed significantly a 1.6-2.5 fold increase, they inhibited the GSTP1 induction by BaP at 0.1 and 0.3 μM BaP (except for GJE at 0.1 μM BaP), whereas they had no effect at 1 μM BaP (Fig. 3C).

UGT1A6. BaP induced this phase II biotransformation enzyme mRNA significantly about twofold. TCDD and the NAhRAs showed a similar induction, and did not have a significant effect in the mixture with 0.1 μM BaP (Fig. 3D). The UGT1A6 mRNA induction at higher levels of BaP has not been determined.

ERCC1. BaP had no significant effect on the gene expression of ERCC1 (Fig. 3E). ICZ down-regulated the ERCC1-mRNA expression 1.7 times. Co-exposure to TCDD, ICZ and GJE significantly decreased the ERCC1 expression levels found at 0.3 μM BaP nearly two-fold but had no or minimal effects at 0.1 and 1 μM BaP.

XPF (ERCC4). The NER enzyme XPF tended to be up-regulated 1.4-2.6 times by TCDD and the NAhRAs and 0.1 μM BaP, however not significantly (Fig. 3F). Simultaneous exposure to TCDD had no effect at 0.1 μM BaP, but ICZ and GJE decreased the induction at 0.1 μM BaP two-fold, but again not significantly. No influence was observed at 0.3 μM BaP. No determinations were carried out at 1 μM BaP, and for ICZ at 0.3 μM BaP.

XPG (ERCC5). TCDD and the NAhRAs had no significant influence on the induction of the NER enzyme XPG mRNA. BaP showed a 1.6 fold increase at 0.1 μM BaP, which was diminished by

Fig. 3 A

Fig. 3 B

Fig. 3 C

Fig. 3 D

Fig. 3 E

Fig. 3 F

Fig. 3. Induction of mRNA from phase I (A and B) and phase II (C and D) biotransformation enzyme genes and nucleotide excision repair (NER) enzyme genes (E-H) in Caco-2 cells after 24 h of exposure to 0, 0.1, 0.3 and 1 μM BaP alone (white), or these concentrations of BaP in combination with 500 pM TCDD (light grey), 100 nM ICZ (grey), or 2.3 μM GJE (dark grey). At least duplicate exposures $\pm$ SD, * $P < 0.05$ for the difference between combined exposure and BaP alone. ICZ, indolo[3,2-b]carbazole; GJE, grapefruit juice extract. ND, not determined.

addition of TCDD significantly ($P < 0.05$). GJE decreased the XPG mRNA found at 0.3 μM BaP (results not shown). No analyses were performed at 1 μM BaP, and for ICZ at 0.3 μM BaP.

XPA and XPC. No significant effects of BaP alone and in combinations with TCDD or GJE on the NER enzymes XPA and XPC mRNA were observed at 0.3 μM BaP (results not shown). Therefore, no other concentrations of BaP were tested any more.

DISCUSSION

In this study we investigated the influence of Natural AhR-activating compounds in fruits and vegetables on BaP-DNA adduct formation in relation to the effect of the prototypical AhR agonist TCDD. Possible differences observed might be explained by differences between TCDD and NAhRAs with respect to the induction of biotransformation or DNA repair activity. As a first step in this complicated process, we evaluated the differences between the effects of TCDD and NAhRAs on BaP-DNA adduct formation in relation to the modulation of expression of genes for several biotransformation and DNA-repair enzymes.

The influence of TCDD and NAhRAs on adduct formation appeared highly dependent on the concentration of BaP. Both TCDD and ICZ appear to reduce the amount of adducts considerable at 0.1 μM BaP, while they showed no clear effect at higher concentrations of BaP. This suggests that at low concentrations of BaP, which has a relatively low effect on e.g. CYP1A1 and 1B1 mRNA levels by itself, the extra induction of CYP1A1 and CYP1B1 by the added AhR agonists can accelerate the elimination of the electrophilic BaP-metabolites out of the cells; at higher concentrations of BaP this effect already seems to be saturated by BaP itself. This detoxification process by TCDD was not hypothesized, but for ICZ it is in agreement with the findings of Ebert *et al.*, who found that the NAhRAs indole-3-carbinol (I3C, a precursor of ICZ) and flavone induced CYP1A1 in Caco-2 cells, and that pre-treatment with these compounds accelerated metabolism and clearance of BaP (Ebert *et al.*, 2005b). They also found that I3C, ICZ and also TCDD AhR-dependently induced breast cancer resistance protein (BCRP), which is involved in the transport of phase II metabolites of BaP (Ebert *et al.*, 2005a). Arif *et al.* showed that gavage treatment of I3C to rats exposed to cigarette smoke, inhibited the DNA adduct formation in lung, trachea, bladder and heart, which is also indicative for I3C stimulated BaP removal (Arif *et al.*, 2000). Furthermore, the apparent absence of BaP-metabolization by CYP1A1 and/or CYP1B1 and the subsequent lack of BaP clearance apparently resulting from it, could explain the increase in BaP-DNA adducts in BaP-exposed CYP1A1/1B1 deficient mice (Uno *et al.*, 2004; Uno *et al.*, 2006). In contrast to these findings, the results from some other studies do not support the hypothesis that induction of CYP1A1 and 1B1 decreases the formation of reactive BaP metabolites. For instance, liver and lung tissues of rats, pretreated with TCDD 48 h before sacrifice, showed a significant increase in BaP-DNA adducts after *in vitro* incubation with 10 μM BaP, which was related to a higher induction of CYP1A1 and CYP1B1 (Harrigan *et al.*, 2004). Also Shimizu *et al.* reported that BaP-exposed AhR-/- mice, which lacked the CYP1A1 induction, showed no BaP-related tumour formation (Shimizu *et al.*, 2000). A potential other explanation may be that the induction of CYP1A1 and 1B1 results in the accelerated activation

of BaP, initially resulting in higher DNA-adduct levels, which are subsequently removed by DNA-repair. As a result the levels are lower due to the longer time available for the repair enzymes.

The grapefruit juice extract (GJE) showed a completely different effect on BaP-DNA formation than TCDD and ICZ (an increase at 0.1 μM BaP instead of a decrease; and a decrease at 0.3 and 1 μM BaP instead of no effect or a slight increase), although it also strongly induced CYP1A1 and CYP1B1 mRNA. GJE can inhibit the CYP1A1-related 7-ethoxyresorufin-O-deethylase-activity (EROD, (De Waard *et al.*, 2007)) and it has been reported that constituents in grapefruit, e.g. bergamottin, inhibit the enzyme activity of CYP1A1 and CYP1B1 (Baumgart *et al.*, 2005). Inhibition of these enzymes could hamper the rapid clearance of BaP, and thereby cause an increase in DNA-adducts. This would be in agreement with the adduct reducing effects at 0.1 μM BaP of TCDD and ICZ, which do not inhibit the CYP enzymes. Additionally, the formation of reactive BaP-metabolites could be reduced by GJE-mediated inhibition of enzyme activity. This might explain why at higher levels of BaP, where the detoxification process seems also saturated according to our TCDD and ICZ results, GJE has a strong reducing effect on the BaP-DNA adduct formation.

Modulating effects on the transcription of the phase II enzymes NQO1, GSTP1 and UGT1A6 and the nucleotide excision repair (NER) enzymes were moderate and could not be related to the different effects on adduct formation by the AhR agonists. Both the expression of GSTP1 and UGT1A6 was slightly induced by TCDD and NAhRAs, but the mixtures with BaP did not show an additive effect, as was seen for the induction of CYP1A1 and CYP1B1, but at 0.1 and 0.3 μM BaP even an inhibition of GSTP1 gene expression. Although the influence on gene expression of the NER enzymes fluctuated considerably between the several BaP, TCDD and NAhRA exposures, presumably the opposite effects on adduct formation by GJE and the other compounds tested are not related to NER enzyme transcription.

We conclude that the influence of TCDD and NAhRAs on BaP-DNA adduct formation is not linearly related to AhR activation, and that ICZ as a single NAhRA-compound shows considerably more resemblance to TCDD, than GJE as a complex mixture containing NAhRAs, at high AhR-activating concentrations. In the lower, and therefore more physiological BaP-concentration range, TCDD and ICZ showed an apparent inhibiting effect on BaP-DNA adduct formation, while the grapefruit juice extract showed a stimulating effect. This seems to contradict our hypothesis based on the presumed toxic properties of TCDD and the healthy properties of citrus fruits. Differences between the effects of TCDD/ICZ and GJE on BaP genotoxicity could not be attributed to phase I and II enzymes and NER enzymes at the transcription level. Therefore, the influences on the dynamic balance between BaP elimination, bioactivation/DNA-adduct formation and DNA-repair should further also be examined at the level of enzyme activity. Our results emphasize the importance of testing mixtures at realistic concentrations for risk-benefit evaluations. In view of the complex mechanisms our *in vitro* findings should be confirmed with *in vivo* studies.

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7

Summary and general discussion

SUMMARY

Although contrasting observations have been done during the last decade, there is no doubt that the high consumption of vegetables and fruits can be linked to health effects in humans (Wattenberg, 1971; Steinmetz and Potter, 1996; Verhoeven *et al.*, 1996; Heber, 2004; WCRF/AICR, 2007). Yet, various vegetables and fruits contain natural compounds, or their precursors, which can bind and activate the aryl hydrocarbon receptor (AhR). Because the activation of this receptor is thought to be essential in the toxicity of dioxins, a well-known group of environmental pollutants, it is of great importance to investigate whether these natural AhR-agonists (NAhRAs) may also exert similar toxic effects as dioxins, or dioxin-like compounds (Fernandez-Salguero *et al.*, 1996; Safe, 2001; Mimura and Fujii-Kuriyama, 2003; Steenland *et al.*, 2004). The importance of investigating such a potential health concern is even more evident in view of the fact that the intake of high doses of purified NAhRAs is being promoted as healthy food supplements. The interest in this type of food supplements is based on the observation that some identified NAhRAs from vegetables and fruits, like indole-3-carbinol (I3C) in cruciferous vegetables, furocoumarins in citrus fruits and flavonoids such as chrysin, baicalein and cantharidin in vegetables and herbs, can show health promoting effects, especially as tumor suppressors, and are thought to be an important cause for the observed beneficial effects of vegetable and fruit enriched diets (Zhang *et al.*, 2003; Aggarwal and Ichikawa, 2005; Chlouchi *et al.*, 2007; WCRF/AICR, 2007). It is remarkable that the proposed mechanism of anti-tumor activity is also based on the AhR activation, and the subsequent induction of phase I and II biotransformation enzymes like CYP1A1, CYP1A2 and UDPGT1A6 (Broadbent and Broadbent, 1998a; Broadbent and Broadbent, 1998b). This seemingly contradiction of both toxic and health beneficial effects initiated by the same receptor may be caused by differences down-stream the AhR-activation or alternative mechanisms besides the AhR-activation.

In this thesis we investigated to what extent the effects of NAhRAs are comparable with the effects of the most potent dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at the transcriptomic and genotoxic level in human cells. Therefore, we examined the gene expression profiles in human colon cancer cells and lymphocytes after exposure to some specific NAhRAs and the xenobiotic AhR-agonists TCDD and benzo[*a*]pyrene (BaP). Based on the transcriptome of these compounds or extracts we attempted to determine relevant NAhRA-specific biomarkers in the human body after consumption of foods rich in NAhRAs. As interspecies differences of dioxin toxicity can be huge (Panteleyev and Bickers, 2006), it appears of great importance that human *in vitro* and *in vivo* models were used.

In order to identify the major types of NAhRA-containing foods and to estimate the overall dietary intake of NAhRAs, we measured the AhR-activity of extracts of a large number of frequently consumed food items using the DR CALUX[®] -reporter gene bioassay (**chapter 3**). The CALUX-induction of the food extracts was compared with the induction by the prototypical dioxin AhR-agonist TCDD. Besides extracts of cruciferous vegetables and citrus products, also extracts of potatoes, bread and hamburgers contained a relatively high AhR-activity. Estimation of TCDD-induction equivalents

demonstrated that the amount of NAhRAs present in food is significantly higher than the amount of xenobiotic AhR-agonists as dioxins and dioxin-like compounds. Although the calculation of TCDD-induction equivalents strongly depends on the exposure time of the CALUX-cells to the test samples, we conclude that the exposure of humans to NAhRAs in foods is quite substantial (possibly in the order of thousands of pg TCDD-IEQ/day).

In **chapter 2**, the effect of NAhRAs, TCDD and BaP on the gene expression is described. These effects have been established in human colon Caco-2 cells exposed to concentrations of near maximum AhR-activation as measured by the DR CALUX' assay. Using the microarray technique, the expression of more than 1000 different genes was measured, and more than 20 genes were found to be up- or down-regulated by at least 1.5 times by TCDD. Most of these by TCDD affected genes were in the same way and to about the same extent up- or down-regulated by BaP and the NAhRA samples. The same phase I and II biotransformation enzymes like CYP1A1, CYP1B1 and UDPGT1A6, and a number of genes involved in cell proliferation were elevated to the same magnitude. Although not extensively investigated, for it was not the scope of this study, no important differences in gene regulation could be detected between NAhRAs and TCDD. The large overlap of gene expression profiles in Caco-2 cells after exposure to NAhRAs and the xenobiotic AhR agonists was not expected. However, although significant differences have been shown in other studies between the effects on gene expression of TCDD, BaP and some NAhRAs such as indirubin and diindolylmethane (DIM) in both several human cell lines, animal models and tissues, all studies show a similar very high induction of CYP1A1 and substantial induction of a number of other genes for xenobiotic metabolism. Sometimes, also comparable effects are shown for cell proliferation, cell cycle regulation and estrogen metabolism in these studies (Puga *et al.*, 2000; Frueh *et al.*, 2001; Sanderson *et al.*, 2001; Martinez *et al.*, 2002; Adachi *et al.*, 2004; Hanlon *et al.*, 2005; Boverhof *et al.*, 2006; Frericks *et al.*, 2007; Hockley *et al.*, 2007). Possibly, for some NAhRAs the presumed beneficial effects may be caused by a difference with TCDD at the protein-activity level. We found that one of the NAhRA samples tested, an extract of grapefruit juice, induced the AhR-related gene expression strongly, but inhibited the enzymatic activity of the gene product CYP1A1 as measured by the EROD-assay, in contrast with TCDD and indolo[3,2-b]carbazol (ICZ, an acid reaction product of I3C and one of the most potent NAhRAs). The TCDD-like gene expression profiles of the NAhRAs suggest that NAhRAs may indeed induce TCDD-like toxicity, and therefore we determined whether the expression of the most important AhR-responsive genes could also be detected in *ex vivo* exposed human lymphocytes. Blood is a relatively easy obtainable human tissue, and finding genomics biomarkers for exposure or effects in human blood cells would enable toxicological evaluation of NAhRA exposure in human populations. For that purpose, in **chapter 4** we tested if a same kind of gene expression profile as in Caco-2 cells could be shown in freshly isolated human lymphocytes after *in vitro* exposure to NAhRAs and TCDD. Although the lymphocytes appeared to be less sensitive than the Caco-2 cells, a small number of AhR-specific genes were significantly up-regulated, including CYP1A1, CYP1B1 and NQO1 (NAD(P)H quinone oxidoreductase 1, a phase II biotransformation enzyme). As the elevated expression of these three genes was considered to be

a promising biomarker for human exposure to AhR-agonists, a pilot study was initiated to verify the up-regulation of these three genes in human blood cells after consumption of NAhRA-rich food items. The screening of foods using the DR CALUX[®] assay had shown that most NAhRA-activity was found in grapefruit juice and cruciferous vegetables (**chapter 3**), so these were used for a three-day intervention study. Although the level of NAhRA intake was considerable, no up-regulation of gene expression of CYP1A1, CYP1B1 or NQO1 could be detected both 3 and 24 hours after the dietary intervention. Two alternative measurements were performed to establish AhR-related activity after the dietary intervention. Firstly, the effect of the intervention on CYP1A2 induction was determined by measuring caffeine-metabolite ratios in urine samples, and secondly, the DR CALUX[®]-activity was measured in plasma samples. No effect on CYP1A2 induction could be detected, although studies with comparable amounts of cruciferous vegetables showed CYP1A2 induction by way of altered caffeine metabolism (Kall *et al.*, 1996; Lampe *et al.*, 2000), but the DR CALUX[®]-assay showed a slight increase in activity after consumption of cruciferous vegetables. A very sensitive, newly developed gene reporter assay, the Dioxin Responsive Element-driven Chemical Activated FLUorescent protein eXpression (DRE-CAFLUX) assay confirmed the slight elevation in AhR-activity found in the human plasma samples from the intervention with cruciferous vegetables (**chapter 5**). Although the AhR-activity in grapefruit juice extract (GJE) was much higher than in the cruciferous vegetables, the NAhRAs in GJE do not appear to reach the bloodstream in high amounts. Therefore it can be concluded that gene expression profiling in human lymphocytes is not a very sensitive method for the detection for NAhRA-exposure or AhR-related effects in humans, and does not seem suitable for the screening of human populations.

To investigate the effects of the presumed tumor promoter TCDD and the presumed tumor suppressing NAhRAs on the genotoxicity of BaP *in vitro*, we exposed human Caco-2 colon cancer cells to mixtures of BaP and either TCDD or NAhRAs (**chapter 6**). We hypothesized that the induction of CYP1A1 and CYP1B1 phase I biotransformation enzymes by the activation of the AhR would result in a faster metabolic conversion of BaP into the electrophilic epoxide benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) and the increased induction of BaP-DNA adducts. On the contrary, we found that at low BaP concentration both TCDD and ICZ decreased the amount of BaP-DNA adducts in a dose-dependent manner. At this low, and thus physiologically more relevant concentration of BaP, GJE showed the opposite effect, namely a doubling in the formation of BaP-DNA adducts. At higher concentrations of BaP, where TCDD and ICZ both did not show an effect, GJE could decrease the amount of BaP-DNA adduct formation significantly. These results could not be related to the gene expression of a number of phase I and II enzymes, and nucleotide excision repair enzymes (NER). This indicates, again, that important health related effects of NAhRAs may be induced at the protein level and not at the transcriptome level. Interestingly, this model suggests that under certain conditions dioxin-like compounds can show a beneficial effect, while some NAhRAs can show a potential toxic effect.

BENEFICIAL FOOD COMPONENTS...?

Although certainly not all epidemiological studies show a reduced cancer risk as a result of high intake of vegetables and fruits (van Gils *et al.*, 2005; Lynn *et al.*, 2006), there is ample evidence that a relatively high amount of vegetables and fruits in the human diet has more beneficial than detrimental effects on human health (WCRF/AICR, 2007). However, the beneficial effects ascribed to phytochemicals present in the diet do not necessarily have to be found after intake of such compounds as food supplements. E.g., a recent systematic review and meta-analysis of extensively studied use of antioxidant supplements, does not show a beneficial effect on human cancer and cardiovascular mortality (Bjelakovic *et al.*, 2007). Thus, thinking of chemopreventive or medicinal use of phytochemicals, one should be cautious for high doses of pure compounds, because it may cause adverse side effects (Verschoyle *et al.*, 2007). The NAhRA I3C, also considered to be an antioxidant (Shertzer and Sainsbury, 1991), is now sold on the market as a food supplement to be taken in relatively high doses (Zeligs, 2002). Extensive animal studies showed a significant anticancer property of I3C (Broadbent and Broadbent, 1998b; Aggarwal and Ichikawa, 2005) and clinical trials in women have shown chemopreventive effects against breast and cervical cancer (Aggarwal and Ichikawa, 2005; Reed *et al.*, 2005). However, these chemopreventive effects are related to an increased hormonal 2-hydroxyestrone/16 α -hydroxyestrone ratio caused by the induced enzyme activity of CYP1A1/1A2 (Bradlow *et al.*, 1996; Aggarwal and Ichikawa, 2005), which is AhR-dependent and also affected in the same way by TCDD (Gierthy *et al.*, 1988; Bertazzi *et al.*, 1993; Greenlee *et al.*, 2001). Besides, an inhibitory AhR-estrogen receptor cross-talk is considered to be even more important in the anti-estrogenic effects of AhR-agonists like TCDD (Safe and Wormke, 2003; Matthews and Gustafsson, 2006). Because of these chemoprotective effects, selective AhR-agonists, like 2,3,7,8-tetrachlorophenothiazine (TCPT) and various substituted diindolylmethanes (DIMs), are under investigation for possible anticancer agents (Safe *et al.*, 1999; Morrow *et al.*, 2004; Fried *et al.*, 2007).

Interestingly, although TCDD can be toxic at relatively low doses for a number of animals, epidemiological studies can not clearly prove this for humans yet (Steenland *et al.*, 2004; Connor and Aylward, 2006; Guzelian *et al.*, 2006). Besides the above mentioned anti-estrogenic effects of TCDD, a case-control study of a normal human population in Finland showed that the risk of soft tissue sarcoma was highest in the lowest dioxin exposed group, whereas similarly as in rat studies the risk was smallest at the high-end range of dioxin concentrations (Tuomisto, 2005), also suggesting some health beneficial effects from low amounts of TCDD.

From this point of view, NAhRAs in a complex food environment can have health beneficial effects, probably by a subtle induction of a number of phase I and phase II enzymes, which may be a mechanism for tumor suppression of certain cancers.

... OR UNPERCEIVED RISK FACTORS?

On the other hand, many animal experiments showed a high toxicity for TCDD at low levels, which makes it a severe environmental pollutant, and several human epidemiological findings suggest a

possible increment of certain cancers after exposure to high dioxin concentrations, e.g. at the Seveso accident in Italy in 1976 (Bertazzi *et al.*, 1993; Warner *et al.*, 2005). Also the effect of chloracne is well-known for industrial workers employed in the production of various chlorinated chemicals, industrial accidents, American veterans from the Vietnam war, exposed to TCDD-containing defoliants (like Agent Orange) and recently, the TCDD-poisoning of the Ukrainian president Yushchenko in 2004 (Sterling and Hanke, 2005; Panteleyev and Bickers, 2006). Because these toxic effects are thought to be mediated by the AhR (Fernandez-Salguero *et al.*, 1996; Mimura and Fujii-Kuriyama, 2003; Steenland *et al.*, 2004), it is important to evaluate whether this toxicity can also be induced by high doses of NAhRAs, for instance as a result of dietary intake or prolonged use of supplements.

In **Chapter 2** it is demonstrated that the global gene expression in a human colon cell line after exposure to NAhRAs shows a quite similar profile as after exposure to TCDD, and most of the affected genes are theoretically involved in dioxin toxicity. Other similarities in expression of genes related to NAhRAs and TCDD were determined in a number of *in vitro* and *in vivo* models. Besides the induction of the prototypical AhR responsive genes for CYP1A1, CYP1A2, CYP1B1, GST, UDPGT and NQO (Broadbent and Broadbent, 1998a; Broadbent and Broadbent, 1998b), I3C or ICZ can cause, like TCDD, weak estrogenic effects like binding to an estrogen responsive element (Liu *et al.*, 1994; Ohtake *et al.*, 2003), and reproductive abnormalities in male rat offspring (Wilker *et al.*, 1996). ICZ caused TCDD-like gap-junction intercellular communication inhibition, which is considered to play a role in tumor promotion (Herrmann *et al.*, 2002) and increased COX-2 expression leading to enhanced prostaglandin E2 production and colon cancer progression (Sherratt *et al.*, 2003). Also, I3C can cause tumor promotion in several animal models (Dashwood, 1998). However, unlike TCDD, the strong NAhRA 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE, a hypothetical endogenous AhR ligand) did not induce cleft palate or hydronephrosis in the mouse, a typical teratogenic effect of TCDD (Henry *et al.*, 2006).

Overall, these similarities and differences in effects of NAhRAs and TCDD suggest a delicate balance in mechanistic effects. Besides, some toxic effects of TCDD can not be clearly related to the AhR-activation, like the ARNT-independent activation of the epidermal growth factor receptor (EGFR) and activation of c-Src kinase which are probably involved in loss of body weight ("wasting syndrome") and epithelial hyperplasia (Panteleyev and Bickers, 2006), an alternative way to explain differences in effects between dioxins and NAhRAs.

Some important problems concerning the risk assessment of AhR agonists are the still unrevealed mechanisms of dioxin toxicity (Steenland *et al.*, 2004; Connor and Aylward, 2006), the animal species specificity, and the difficulties in using the TEQ-concept in a proper way for non-dioxin-like agonists (Safe, 1998; Windal *et al.*, 2005). The latter is discussed in **chapter 3**. It is suggested that the difference between the AhR-related potential toxic effects of dioxins and NAhRAs probably depends highly on the persistent properties of the dioxins in the body (Bock and Kohle, 2006). The half-life of TCDD is much longer than that of the known NAhRAs (Pohjanvirta *et al.*, 2002), which implies that the dioxins can accumulate in tissue and cause a continuous over-activation of the AhR. This may

eventually result in increased toxicological risk. On the other hand, one could argue that, because of the continuous exposure to dietary NAhRAs (**Chapter 3**), the AhR may also be continuously activated, comparable with dioxin exposure.

Different approaches have been used to estimate the possible toxic dose of dioxin-like compounds. Both the EC Scientific Committee for Food (SCF) and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) derived a tolerable weekly intake (TWI) of 14 pg WHO-TEQ/kg bw (Larsen, 2006). This level is based on the most sensitive adverse effects reported for TCDD, namely developmental and reproductive effects in rats. The US Environmental Protection Agency (US EPA) calculated the human body burdens of dioxin and dioxin-like substances leading to an estimated 1% increase (ED_{01}) in the lifetime risk of cancer in three occupational studies with the best exposure information, and the ED_{01} ranged from 6 to 62 ng TCDD/kg bw (Larsen, 2006). Animal data fell in the middle of this range. For the pivotal noncancer hazards, the EPA used the margin of exposure (MOE), which is the ratio of effect level in the comparison species (ED_{01} or LOEL, i.e. lowest observed effect level) to the human body burden. For the most sensitive endpoints, the MOE ranged from less than 1 for enzyme induction in mice and rats, 4 for developmental neurotoxicity and endometriosis in non-human primates, 5-8 for developmental/reproductive toxicity in rats, and 12 for developmental immunotoxicity in rats (Larsen, 2006). Connor and Aylward reviewed a number of human and animal studies on TCDD exposures which showed that significant CYP1A enzyme activity or CYP1A gene expression in humans occurred at the level above 750 ng WHO-TEQ/kg/day (no such effect below 250 ng/kg/day, lacking data in between) and in marmoset monkey, mouse and rat above ca. 50 ng/kg/day (Connor and Aylward, 2006). This was correlated with a suggested “impaired” human AhR with a receptor binding affinity of TCDD about 10 times less than in the concerned animal models, which may imply that humans are possibly less vulnerable than these animals used for the risk assessment of dioxins and dioxin-like compounds (Connor and Aylward, 2006; Larsen, 2006). In **chapter 3** we estimated the total intake of NAhRA-containing foods at 1545-6235 pg TCDD-IEQ/day, which corresponds with a daily body burden, based on a person of 70 kg, of 22-89 pg TCDD-IEQ/kg bw. Taken into account that NAhRAs have, in contrast with dioxins, probably a short half-life, it could be concluded that the exposure to NAhRAs is too low to be able to induce CYP1A activity and so exhibiting the dioxin-like toxicity related to this enzyme induction. However, according to the WHO-TWI and the EPA-MOE, the NAhRA-intake is far above the safety margins. But using the dioxin TEQ-concept for non-dioxin-like compounds or mixtures is highly controversial, and probably unrealistic. In **chapter 3** we discussed that the calculations of TCDD-IEQs made by Jeuken *et al* (Jeuken *et al.*, 2003) were three orders of magnitude higher than ours, because of a shorter exposure time in the DR CALUX[®] bioassay. This makes it very complicated to accurately estimate the dose of AhR-activating compounds. Furthermore, total body burden may not be a very useful parameter, because NAhRAs probably concentrate in relatively high amounts in certain target tissues like the liver (Stresser *et al.*, 1995; Anderton *et al.*, 2004).

CONCLUSION

In **chapter 4** it is seen that a relatively high intake of NAhRA-containing foods (900 – 20000 pg TCDD-IEQ/day) could not be clearly related to AhR effects in human lymphocytes. Either this effect is absent, or alternatively the used biomarkers in lymphocytes are not sufficiently sensitive for detection of the aimed AhR-related effects and are also probably not related to the most sensitive adverse effects of TCDD, such as endocrine disruption and developmental and reproductive toxicity. Together with the relatively high amounts of NAhRAs given to laboratory animals to cause AhR-related effects, it can be argued that AhR activation by consumption of NAhRA-containing foods will probably not reach the toxic level as induced by a high dose of TCDD. Also the CYP-enzyme inhibiting properties of some NAhRAs, like bergamottin in grapefruit juice as seen in **chapter 2** and demonstrated by Baumgart *et al.* (Baumgart *et al.*, 2005), and the complex composition of foods with many possible inhibitors of AhR-activity (Amakura *et al.*, 2003; Zhang *et al.*, 2003), support this conclusion. Also other compounds in foods, like the isothiocyanates in cruciferous vegetables (Higdon *et al.*, 2007), can compensate the hypothetical adverse effects of NAhRAs by selectively inducing phase II enzymes, which may efficiently eliminate electrophilic metabolites generated by upregulated phase I enzymes. More caution may be necessary for the intake of NAhRAs as food supplements. It is often recommended to take such supplements in a pure form on a daily basis and in doses which are often hundreds of times higher as compared to normal dietary intake (Zeligs, 2002; Bagchi, 2006; Verschoyle *et al.*, 2007; WCRF/AICR, 2007). In view of the unpredictable influence on DNA-adduct formation by environmental carcinogens such as PAHs, as seen in **chapter 6**, these supplements may result e.g. in an increased genotoxic risk under certain conditions and eventually promote tumor formation in the longer run.

FUTURE RESEARCH

The use of genomics in food and toxicology research appears to be a promising approach to establish mechanistic pathways involved in both chemopreventive and toxic effects of dietary compounds. At this moment, transcriptomics has shown to be a powerful technique for screening genome-wide gene expression. However, as seen in this thesis, the sensitivity of this technique may sometimes be too low to establish gene expression changes in certain cell types. More importantly, not all the essential steps in the mechanistic pathways are represented at the transcriptomic level. Therefore, an integrated systems biology approach for risk/benefit analysis of specific foods, by using the combination of transcriptomics with proteomics and metabolomics, may be necessary to find useful biomarkers. (Gatzidou *et al.*, 2007; Collings and Vaidya, 2008; Decristofaro and Daniels, 2008). Much attention should be paid on tissue specificity and time of sampling, and probably pathway analysis will be a promising tool for obtaining biomarkers, instead of the determination of a number of affected genes or proteins. With regard to the research on the effects of AhR-activation, we recommend to use both the persistent xenobiotics like TCDD and some of the more rapidly metabolized NAhRAs in the same test models, to observe where these types of AhR-agonists show their different effects. Also attention should be paid to antagonistic effects of compounds e.g. in food. Preferably human *in vitro*

and *in vivo* (for NAhRAs) models should be used, because of the large interspecies differences in AhR-related effects. Because the necessary amount of sample for -omics studies is still decreasing, very small biopsies of more relevant human tissues, such as epithelial cells or colon cells, may replace the relatively non-invasive blood sampling. However, to obtain insight in the mechanisms of the most sensitive toxic effects such as reproductive and developmental toxicity, the systems biology approach should probably first be applied to the most suitable animal models. All this implicates a complex and costly, but very promising, research, which may be more appropriate for environmental risk assessment, and also potential pharmaceutical purposes, than for developing food supplements intended for healthy humans (for: “An apple a day keeps cancer away”).

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Een samenvatting voor de leek

Het eten van groente en fruit wordt als zeer gezond gezien en een aantal epidemiologische studies lijken hier ook op te wijzen. Hoewel de chemische samenstelling van deze voedingsmiddelen buitengewoon complex is, heeft men veel onderzoek verricht naar afzonderlijke stoffen in groenten en fruit die verantwoordelijk zouden kunnen zijn voor de effecten op de gezondheid. Een voorbeeld van zo'n stof is indol-3-carbinol (I3C), afkomstig uit spruitjes en andere koolsoorten, waarvan uit dierstudies bekend is dat het bepaalde vormen van kanker kan tegengaan. Het gevolg is dat deze stof in pillen of capsules met een zeer hoge dosis, tot honderden malen de hoeveelheid in een bord vol spruitjes, commercieel verkrijgbaar is. Omdat het om een "natuurlijke" stof gaat, zou een uitputtend toxicologisch onderzoek zoals bij medicijnen niet nodig zijn. De vraag is echter of dit wel zo verstandig is, aangezien deze stof en een aantal in het lichaam gevormde omzettingsproducten (metabolieten) binden aan de zg. Ah-receptor (aryl hydrocarbon receptor), een eiwit dat voorkomt in bijna alle cellen van dieren en dus ook mensen. Deze Ah-receptor wordt ook wel de dioxine receptor genoemd, omdat het beruchte milieuverontreinigende dioxine, bekend van vele krantenberichten over milieuproblemen, via binding aan en activering van dit eiwit zijn giftige effecten veroorzaakt. Hierdoor rijst de vraag of die natuurlijke Ah-receptor activatoren ook giftige effecten kunnen veroorzaken. Intussen worden er steeds meer natuurlijke stoffen ontdekt die aan de Ah-receptor binden en deze kunnen activeren, voor het gemak aangeduid met NAhRAs (Natuurlijke Ah-receptor agonisten), waaronder bergamottin in grapefruit.

Dit proefschrift probeert meer licht te werpen op de overeenkomsten en verschillen tussen natuurlijke en milieugevaarlijke Ah-receptor agonisten. Dit is onder meer gedaan op het onderzoeksgebied van genexpressie en gentoxiciteit in cellen, dus op DNA niveau.

In **hoofdstuk 2** wordt het effect beschreven op genexpressie na blootstelling van gekweekte menselijke darmcellen (Caco-2) aan dioxine (TCDD), benzo[a]pyreen (BaP, een kankerverwekkende stof in o.a. sigarettenrook en aangebrand voedsel) en diverse NAhRAs (ICZ, een metaboliet van I3C, en extracten van grapefruitsap en citruspulp). Middels de microarray techniek, waarbij de relatieve hoeveelheid in de cellen gevormd mRNA wordt gemeten (het product van de zogeheten expressie van de genen, de dragers van de erfelijke eigenschappen gecodeerd door het DNA), werd de expressie van duizenden verschillende genen bepaald. Verwacht werd dat de natuurlijke stoffen met effecten op de AhR een (fors) aantal andere genen hoger of lager tot expressie zouden laten komen dan de niet-natuurlijke. Echter, van de duizenden genen werden er maar ongeveer twintig duidelijk beïnvloed en wel op ongeveer gelijke wijze bij zowel de natuurlijke als de niet-natuurlijke Ah-receptor agonisten. Van een aantal genen was al in de literatuur bekend dat ze beïnvloed werden door dioxine, en de meeste van deze genen kunnen betrokken zijn bij het ontstaan van kanker. Op genexpressie-niveau in darmcellen bleek er dus geen duidelijk onderscheid te zijn tussen dioxine, BaP en NAhRAs. Van één van de tot expressie gekomen genen is de werkzaamheid van het bijbehorende eiwit bepaald (het enzym CYP1A1). De activiteit van dit enzym blijkt door TCDD, BaP, ICZ en het citruspulp extract verhoogd te worden maar door het grapefruitsap extract sterk geremd te kunnen worden. Dit duidt

op een verschil van sommige NAhRAs ten opzichte van dioxine in effecten op de werking van de eiwitten die gevormd worden (dit zijn op hun beurt de producten van het eerder genoemde mRNA dat wordt afgelezen van de genen.).

In **hoofdstuk 3** is het onderzoek beschreven naar de mate van voorkomen van NAhRAs in belangrijke voedingsmiddelen waaruit het gemiddelde Nederlandse menu bestaat. Dit is uitgevoerd met behulp van de CALUX-methode. Deze methode behelst het blootstellen van genetisch gemodificeerde rattencellen aan extracten van voedingsmiddelen en aan dioxine als standaardstof. De cellen zijn zo bewerkt dat bioluminescentie optreedt als NAhRAs of dioxines de cel binnenkomen en binden aan de Ah-receptor. Dit signaal wordt met een luminometer gemeten en hoe hoger het signaal, des te meer NAhRA of dioxine er aanwezig is. Op deze manier kan worden bepaald hoeveel NAhRAs er in totaal in de voeding zitten ten opzichte van dioxine. Het blijkt dat de hoeveelheid NAhRAs in de Nederlandse voeding vele malen hoger is dan die van dioxine. Deze methode zegt echter nog niets over de eventuele giftigheid van NAhRAs.

Hoofdstuk 4 beschrijft het onderzoek naar de genexpressie in menselijke bloedcellen na het consumeren van spruitjes en grapefruitsap, die beide relatief veel NAhRAs bevatten. Eerst is gekeken naar de genexpressie in uit mensenbloed geïsoleerde en gekweekte cellen (lymfocyten) nadat deze cellen tijdens het kweken waren blootgesteld aan dioxine en NAhRAs. Ditmaal werd een microarray-techniek gebruikt waarbij de expressie van tienduizenden genen kan worden bepaald. Hierbij bleek dat de expressie in deze cellen veel minder gevoelig is voor Ah-receptor agonisten dan in de darmcellen. Ook bleek dat het expressieprofiel van het grapefruitsap extract verschilde van dat van dioxine, terwijl ICZ (uit spruitjes) wel bijna hetzelfde profiel vertoonde. Consumptie van grote hoeveelheden spruitjes en grapefruitsap deed de expressie van een aantal kenmerkende genen voor Ah-receptor activiteit in bloedcellen niet veranderen. Wel was een zeer kleine verhoging van het CALUX-sigitaal in bloed meetbaar na de consumptie van spruitjes. Het lijkt er dus op dat het consumeren van grote hoeveelheden NAhRA bevattende groenten of fruitdrank de Ah-receptor niet veel extra activeert.

In **hoofdstuk 5** wordt een techniek besproken die wat gevoeliger dan de CALUX zou kunnen zijn. Met behulp van een vergelijkbare reporter-gen methode, de CAFLUX, werden opnieuw de bloedmonsters doorgemeten die verzameld waren na de interventies met spruitjes en grapefruitsap. Hiermee werd bevestigd dat het gehalte aan via de Ah-receptor actieve stoffen in bloed na het eten van spruitjes iets hoger is dan er voor, en dat dit niet bij het drinken van grapefruitsap het geval is.

Ten slotte is in **hoofdstuk 6** beschreven welke invloed dioxine en NAhRAs kunnen hebben op de DNA-schade die veroorzaakt kan worden door BaP. De genotoxische stof BaP is alomtegenwoordig in o.a. sigarettenrook, uitlaatgassen en aangebrand vlees en kan kanker veroorzaken door aan DNA te binden (DNA adducten) en de normale genexpressie daarmee te ontregelen. De effecten van

dioxine en NAhRAs op BaP-DNA adducten in gekweekte menselijke darmcellen blijken in hoge mate afhankelijk te zijn van de concentraties BaP in die cellen. Bij een lage concentratie BaP in de cellen hebben zowel dioxine als de NAhRA ICZ een remmende invloed op de adductvorming (gunstig), terwijl bij een hoge concentratie BaP er geen effect is. Bij de blootstelling van darmcellen aan BaP en het NAhRA bevattende grapefruitsapextract treden er heel andere effecten op. Bij een lage concentratie BaP verhoogt het grapefruitsap de adductvorming (ongunstig), terwijl bij een hoge BaP concentratie de adductvorming juist sterk wordt verminderd (gunstig). Ook zijn de expressies van een aantal genen bepaald die met dit adductvormings proces te maken kunnen hebben, maar hiermee werd geen correlatie gevonden.

Conclusie

In voeding zijn grote hoeveelheden via de Ah-receptor actieve stoffen aanwezig. Op genexpressieniveau kunnen deze grote gelijkenis vertonen met het giftige dioxine. Bij het eten van relatief grote hoeveelheden NAhRA bevattende voeding is een verhoogde Ah-receptor activiteit in bloed zeer moeilijk waar te nemen. Het effect lijkt dus gering en men hoeft kennelijk niet bang te zijn voor dioxineachtige vergiftigingsverschijnselen. Tevens bevat voeding ook stoffen die door Ah-receptoractivering gevormde eiwitten kunnen remmen. Of de zeer hoge dosis aan zuivere NAhRAs in voedingssupplementen wel dioxineachtige effecten teweeg kan brengen moet nog nader worden onderzocht. Ondertussen blijkt uit de literatuur dat dioxine zelf voor mensen niet zo extreem giftig lijkt te zijn als decennia terug werd verondersteld en in bepaalde modellen, zoals bij de bovengenoemde adductvormingstest, zelfs gezondheidsbevorderende effecten lijkt te kunnen veroorzaken. Op basis van bevindingen beschreven in dit proefschrift en in combinatie met een groeiende hoeveelheid wetenschappelijke literatuur, kan worden vastgesteld dat inname van groente en fruit niet onder alle omstandigheden resulteert in duidelijk gezondheidsbevorderende effecten.

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(Met dank aan Lou en vrij naar Heinrich Heine)

In de straote vaan Mestreech aon de Maos

Weurt Vastelaovend gevierd

Dao flikkere de bougies, dao späölt de meziek

Dao danse de lui, kŕeurriek verseerd

Curriculum vitae

Pim de Waard was born on the 8th of June 1959 in Rhoon, Zuid-Holland. In 1975 he finished Secondary School MAVO-4 at Meyenhage, Rotterdam, after which he started the Higher Professional Education in Analytical Chemistry at Van't Hoff Institute, Rotterdam. After finishing in 1979 he worked as a Chemical Engineer at Hercules Inc, Zwijndrecht until 1989. From then till 2000 he worked as a Pharmaceutical Engineer at the Hospital Pharmacy of Gelderse Vallei, Bennekom. Besides this appointment he performed the Master study Food and Toxicology at the Open University, getting his Drs. degree (MSc.) in 1999. In 2000 he became a Researcher Biotechnological Pharmaceuticals at RIVM (National Institute of Public Health and the Environment), Bilthoven. In 2003 he started his PhD project at three locations: RIKILT Institute of Food Safety (Wageningen), the Division of Toxicology, Wageningen University, and the Department of Health Risk Analysis and Toxicology, Maastricht University. Besides, he participated in a number of Postgraduate Education in Toxicology courses, including Laboratory Animal Science (art.9), in order to obtain the certification as Toxicologist at graduation. In 2007 he returned to the RIVM as a Preclinical Assessor at the Centre of Biological Medicines and Medical Technology.

List of publications

PEER REVIEWED PAPERS

Pim de Waard, Jac Aarts, Ad Peijnenburg, Hakan Baykus, Elise Talsma, Ans Punt, Theo de Kok, Frederik-Jan van Schooten, Ron Hoogenboom:

Gene expression profiling in Caco-2 human colon cells exposed to TCDD, benzo[a]pyrene, and natural Ah receptor agonists from cruciferous vegetables and citrus fruits.

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Pim de Waard, Jac Aarts, Ad Peijnenburg, Theo de Kok, Frederik-Jan van Schooten, Ron Hoogenboom:

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In preparation.

ABSTRACTS

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