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Commentary

Activation of coupled Ah receptor and Nrf2 gene batteries by dietary phytochemicals in relation to chemoprevention

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Abbreviations:
ALDH, aldehyde dehydrogenase
ARE, antioxidant response element
GCS_H, γ-glutamylcysteine synthetase heavy subunit
GCS_L, γ-glutamylcysteine synthetase light subunit
GSH, glutathione
NQO, NAD(P)H:quinone oxidoreductase
ROS, reactive oxygen species
TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
tBHQ, tert butylhydroquinone
TRE, tetradecanoyl phorbol acetate response element
UGT, UDP-glucuronosyltransferase
XRE, xenobiotic response element

ABSTRACT

The Ah receptor (AhR) is a ligand-activated transcription factor and member of the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family of chemosensors and developmental regulators. It represents a multifunctional molecular switch involved in regulation of endo- and xenobiotic metabolism, in vascular development and in dioxin-mediated toxicities. Recently, the oxidative stress-protecting Nrf2 has been shown to be a downstream target of the AhR [Miao W, Hu L, Scrivens PJ, Batist G. Transcriptional regulation of NF-E2 p45-regulated factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway. J Biol Chem 2005;280:20340–8]. This finding offers the possibility that distinct but partially overlapping AhR and Nrf2 gene batteries of Phase II xenobiotic-metabolizing enzymes can be synergistically activated by a number of phytochemicals, acting as selective or mixed activators of target genes. In addition, it is conceivable that AhR-mediated oxidative/electrophile stress may be attenuated by coupled Nrf2 activation. The commentary discusses potentials and limitations of (i) selective Nrf2 and of (ii) synergistic AhR plus Nrf2 activation by phytochemicals in efforts towards chemoprevention of cancer and degenerative diseases, and describes clinical trials providing the expectation that chemopreventive measures may favorably modulate unavoidable endo- and exogenous toxin exposures in high risk populations.

1. Introduction

The Ah receptor (AhR) is a ligand-activated transcription factor of the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family [1]. It is expressed in most mammalian tissues and represents a multifunctional switch. AhR functions have been classified into 'adaptive' (enhanced metabolism of xenobiotics), 'toxic' (adverse effects of dioxin-like compounds) or 'developmental' (physiologic functions in the absence of

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xenobiotic compounds). Previously, dioxin-mediated toxic events have been discussed as possible deregulated physiologic functions due to sustained activation of the AhR by persistent receptor ligands [2].

The adaptive function of the AhR in detoxification of xenobiotics has been obscured by its known role in bioactivation of carcinogens, in particular by induction of CYP1 enzymes [3,4]. However, studies using CYP1A1-null mice showed that detoxification by inducible CYP1A1 is more important than metabolic activation of orally administered benzo[a]pyrene [5,6]. In addition, evidence was obtained for direct coupling of the AhR with a second transcription factor, Nrf2 (NF-E2-related factor 2) [7,8] which appears to be very effective in protecting against oxidative/electrophile stress [9,10]. Oxidative stress is also required for stabilization of the Nrf2 protein, as discussed later. In the gastrointestinal tract AhR–Nrf2 interaction may facilitate detoxification by efficiently coupling Phase I and II xenobiotic-metabolizing enzymes (XMEs). Interacting networks of ligand-activated transcription factors such as the coupled AhR–Nrf2 system are increasingly being discovered.

Currently, Nrf2-mediated induction of Phase II enzymes (also termed 'indirect antioxidant enzymes') by phytochemicals is intensely studied in efforts towards chemoprevention of cancer and degenerative diseases [11,12]. Careful epidemiologic studies have shown an association between reduced cancer risk and increased intake of vegetables from *Cruciferae* plants (specially within the genus *Brassica*) including cauliflower, cabbage, Brussels sprouts and broccoli ([13,14] and references therein). It has been shown that isothiocyanates such as sulforaphane from cruciferous vegetables activate Nrf2-controlled XMEs, and provide protection against chemical carcinogenesis [11,12]. In addition, indole derivatives originating from tryptophan-derived glucobrassicin, such as indole-3-carbinol has been shown to exert chemopreventive actions in animal models of carcinogenesis [13,14], e.g., in aflatoxin B1-induced rat liver carcinogenesis [15]. Dietary isothiocyanates and indoles activate distinct pathways; their actions were shown to be synergistic in stimulating apoptosis and protection against DNA damage [16]. Chemoprotection efforts are also undertaken with numerous other phytochemicals, in particular with tea polyphenols from the plant *Camellia sinensis* [17,18]. Numerous clinical studies are under way to explore whether chemoprotection may be beneficial to high risk populations [19,20]. The present commentary focuses on Phase II induction by phytochemicals as a major mechanism. However, it has to be emphasized that these phytochemicals also exert other chemoprotective activities, including modulation of inflammation, immune status, and via other mechanisms, for example, modulation of protein kinases [14,21]. Furthermore, numerous findings indicate that AhR and Nrf2 are also modulated by phosphorylation [2,9,22].

Classification of Phase I and II XMEs is used as defined by Paul Talalay's group who principally include CYPs as Phase I enzymes which generally generate non-electrophilic metabolites but also highly reactive electrophiles. The products of Phase I metabolism are effectively detoxified by Phase II enzymes including conjugation enzymes or enzymes converting electrophiles into nucleophiles such as NQO1 [11,12]. It should be noted that the usefulness of Phase I and II enzyme classification is currently debated [23].

The present commentary focuses on the recent finding that Nrf2 is a downstream target of the AhR [8], on the resulting coupling of AhR- and Nrf2-gene batteries of XMEs, and on consequences arising from activation of these gene batteries by selective AhR, Nrf2 and by mixed AhR/Nrf2 phytochemical activators. Application of the new possibilities is discussed in connection with current clinical trials for cancer chemoprevention in high risk populations.

2. Coupled AhR and Nrf2 gene batteries

The AhR is known to be present in the cytoplasm in a latent complex with two Hsp90s and related chaperones. Ligand binding leads to nuclear translocation and the release of the chaperones in exchange for its partner protein Arnt. This AhR-Arnt heterodimer binds to XREs in the regulatory region of target genes (Fig. 1A) [1]. The latter, termed AhR gene battery [24], include Phase I and II XMEs (Table 1). It is increasingly appreciated that, in addition to XMEs, the AhR also controls a heterogeneous group of tissue-specific gene products involved in cell proliferation and differentiation which is not discussed here ([2], for references).

Recent evidence suggests that Nrf2, a CNC (cap'n'collar) basic leucine zipper transcription factor is an AhR target gene [7,8]. Nrf2 is activated by oxidative/electrophile stress. The important role of Nrf in protection against oxidative stress is strengthened by experiments with compound Nrf1- and Nrf2-deficient mice which resulted in early embryonic lethality due to severe oxidative stress, whereas Nrf1-deficient mice showed embryonic lethality at a later time, and Nrf2-deficient mice showed no embryonic lethality [37]. This protective function appears to be evolutionary conserved: *Caenorhabditis elegans* expressing the homologous protein SKN-1 resist oxidative stress [38]. Nrf2 is present in the cytosol in a latent complex with the actin-anchored chaperone Keap1 (Kelch-like ECH-associated protein 1), a sulfhydryl-rich protein which is oxidized by oxidative/electrophile stress, thereby uncoupling the association between Nrf2 and Keap1. Subsequently, Nrf2 translocates to the nucleus, and associates with small Maf proteins (or related basic leucine zipper proteins such as c-jun) [9]. The heterodimers bind to AREs of target genes (Fig. 1B). It is noteworthy that Nrf2 can also be activated by MAP kinases [22]. Complete spectra of AhR target genes are currently being analyzed in many laboratories; for example, hepatic mRNA profiles in AhR-deficient mice versus TCDD-treated or untreated wild-type mice [39]. Similarly, Nrf2 target genes were studied in Nrf2-deficient mice [40,41] and in rats [42], and have also been investigated in human hepatocyte cultures [43]. Of course, in microarray analysis it is hard to distinguish between primary and secondary responses. It is conceivable that coupled AhR and Nrf2 activation should be recognized in global gene expression analysis.

Coupling between AhR and Nrf2 gene batteries (i.e., direct gene targets) became evident by DNA sequence analysis of the mouse Nrf2 promoter: This region contained 3 functional XREs (and 2 AREs) [8]. The study demonstrated that AhR signaling is necessary for inducible Nrf2 expression (Fig. 2). Multi-copy

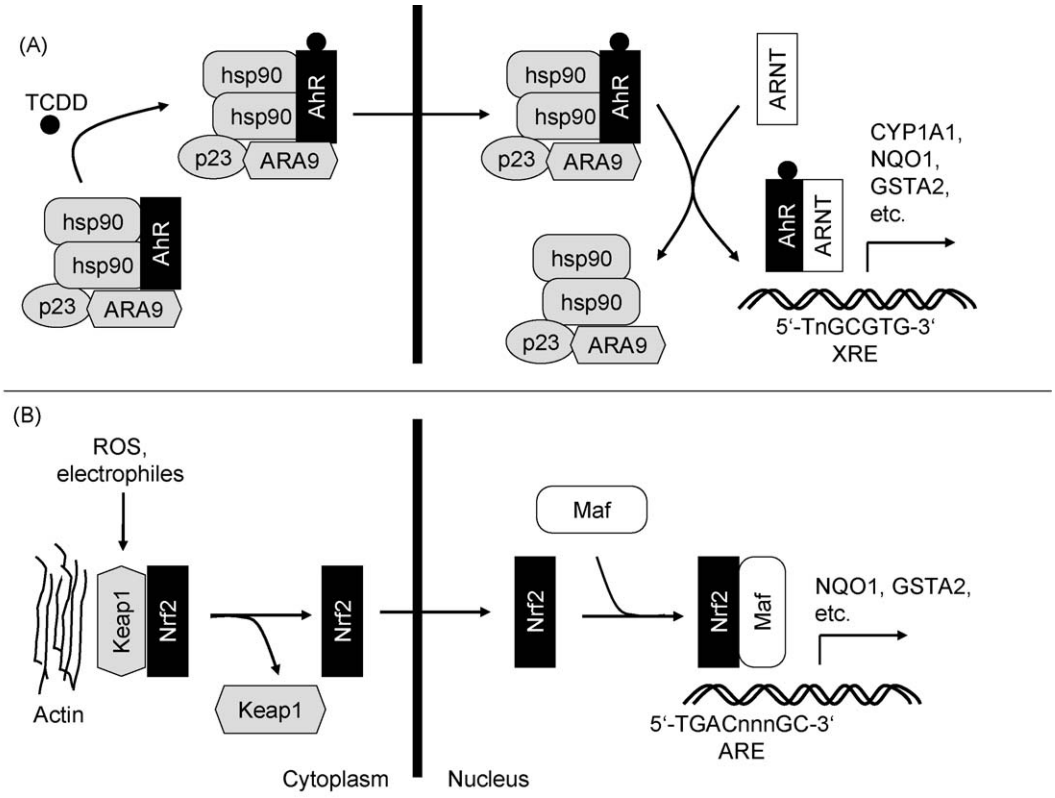

Fig. 1 – Activation and nuclear translocation of Ah receptor (A) and Nrf2 (B), and their binding to XREs and AREs of target genes. (A) The AhR normally is present in cytoplasm in a latent complex with chaperones (Hsp90, ARA9 and p23). On activation by ligands, AhR translocates to the nucleus and exchanges chaperones for its partner Arnt [1]. The AhR-Arnt heterodimer then binds to XREs with the core sequence TnGCGTG and activates downstream target genes. (B) The Nrf2 is present in cytoplasm in complex with the ROS/electrophile sensor Keap1. Upon release by oxidized Keap1, Nrf2 is translocated to the nucleus where it associates with partner proteins, usually small Maf proteins [9,10]. The Nrf2-Maf heterodimer binds to AREs and activates downstream target genes.

Table 1 – Selected AhR and Nrf2 gene battery members

	AhR gene/protein battery		Nrf2 gene/protein battery	
(A)	CYP1A1	[6,24]	–[a]	
	CYP1A2	[6,24]	–	
	CYP1B1	[6,25]	–	
(B)	NQO1	[9,24]	NQO1	[9,10]
	NQO1 (rat)	[10]	NQO1 (rat)	[10]
	NQO1 (mouse)	[10]	NQO1 (mouse)	[9,10,29]
	–		GSTA1(mouse)	[10]
	GSTA2	[9,24]	GSTA2	[10]
	–		GCS_H, GCS_L	[30,31]
	ALDH3A1 (mouse)	[24,26]	ALDH3A1 (mouse)	[26]
	UGT1A1	[27]	n.s.[b]	
	UGT1A6	[24,28]	UGT1A6	[11,28]
(C)	–		Thioredoxin	[32]
	–		Thioredoxin reductase-1	[33]
	–		Metallothionein-1/2	[34]
	–		Heme oxygenase-1	[35]
	–		Ferritin	[36]
(D)	Nrf2	[8]	Nrf2	[8]

Target genes are included for which functional XREs and AREs have been identified. Unless indicated, human target genes are listed.
[a] Not detected.
[b] Not studied.

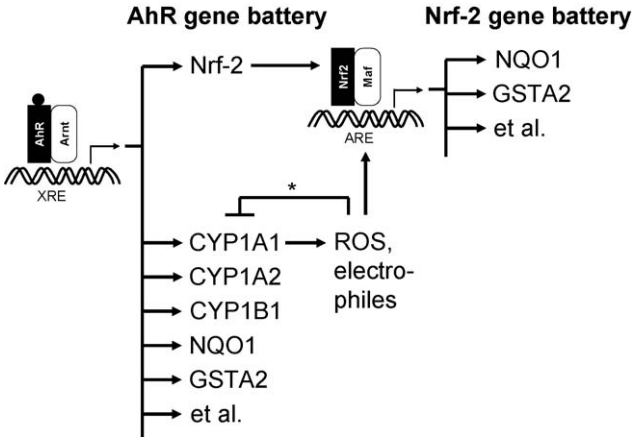

Fig. 2 – Schematic diagram illustrating Nrf2 as downstream target of the AhR. The AhR activates Phase I enzymes such as CYP1A1 at the same time it induces Nrf2 expression. Nrf2 in turn activates Phase II enzymes. In this way, the AhR and Nrf2 gene batteries are coupled and work synergistically in xenobiotic and carcinogen detoxification. *: autoregulatory loop [44].

XREs in Nrf2 promoters have also been found in rats and humans. Hence, Nrf2 appears to be a downstream target of the AhR. However, stabilization of Nrf2 protein by oxidative/electrophile stress appears to be critical for an appropriate Nrf2 response. Coupling of AhR and Nrf2 gene batteries conceivably attenuates accumulation of CYP-generated ROS and electrophiles. Furthermore, synergistic activation of AhR and Nrf2 pathways by phytochemicals may offer new possibilities for chemoprevention, as discussed later.

In the following, AhR and Nrf2 target genes are compared in which functional XREs and AREs have been characterized (Table 1). CYPs as prototypical Phase I genes (grouped under A) are not induced by Nrf2. In fact, CYP1A1 expression appears to be negatively regulated by ROS in an autoregulatory loop [44] (Fig. 2).

Group B in Table 1 includes distinct but overlapping Phase II AhR and Nrf2 gene battery members, including NQO1 and GSTA2 which led to the identification of AREs [9], the GCS_H and GCS_L genes (encoding the rate limiting enzymes of GSH synthesis), as well as Aldh3a4 and UGT1A6. For the latter enzyme functional ARE motifs still need to be identified. UGT1A6 is probably a Nrf2 target gene, based on studies with Nrf2-deficient mice [11,40]. It has to be noted that the list of inducible GSTs [10] and UGTs is not comprehensive. Using 'humanized UGT1 mice', evidence has been obtained recently that most UGT1 genes may be transcriptionally activated by the AhR [45,46].

Targets of AhR and Nrf2 can be activated independently by AhR agonists and by oxidative/electrophile stress, respectively [9]. As discussed before, there is also evidence for coordinate regulation of some AhR/Nrf2 targets. For example, expression of human NQO1 by TCDD appears to be mediated via an ARE mechanism [47]. Coordinate regulation may explain the loss of both TCDD- and tBHQ-induced UGT activity in a rat hepatoma 5L cell clone (BP8) lacking the AhR [28]. Two mechanisms have been proposed to explain coordinate regulation of Phase II

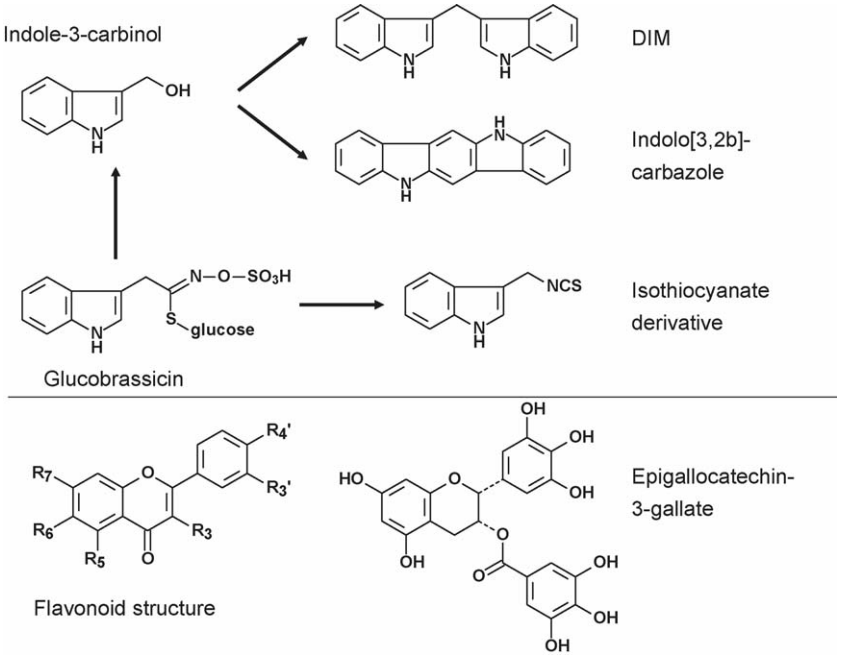

Fig. 3 – Structures of phytochemical AhR and Nrf2 activators. (A) Phytochemical indoles are stored in *Brassica* plants as multiple glucosinolates. Glucobrassicin is a L-tryptophan-derived glucosinolate present in broccoli. Upon damage of the plant by predators or preparation of herbal extracts the conjugates are hydrolyzed, and various phytochemicals are formed: (i) indole-3-carbinol which polymerizes under acid conditions to 3,3′-diindolylmethane (DIM) or indolo-(3,2-b)carbazole, and (ii) multiple isothiocyanate derivatives. In B, the general structure of flavonoids and of epigallocatechin-3-gallate, the major tea polyphenol, is illustrated.

XMEs: (i) control of inducible Nrf2 expression as a downstream target of the AhR [8]. (ii) It is appreciated that Nrf2 is not the only protein factor bound in the neighborhood of AREs. TRE-like sequences are frequently found in proximity to AREs which may be functional [9]. In human NQO1, the consensus TRE sequence TGACTCA is embedded in the ARE core sequence. Hence, ARE responses may be explained in part by the sequence context of AREs outside of the core sequence, as discussed for differential regulation of Nrf2 target genes [9]. In addition, AREs in close proximity to XREs have been described in mouse, rat and human NQO1 [7]. In addition, it is noteworthy that the current ARE consensus sequence (5′-TGACnnnGC-3′) is probably not universally applicable. For example, in the mouse Nqo1 gene the Nrf2 binding motif was identified as 5′-TGAGtcgGC-3′ [29]. Hence, AREs in the regulatory region of different target genes may have differential sequence requirements.

Functionality of putative ARE-like motifs in the 3 kb regulatory region of human UGT1A6 has been investigated in Caco-2 cells in which this isoform is inducible by tBHQ and TCDD (discussed under 3.3) [28]. In the region with high tBHQ induction several ARE-like elements have been found. For example, an ARE-like motif 5′-ttctTGAGcagGCaga-3′ is present at −1733 bp on the reverse strand (numbering is based on the position of the 3′ most nucleotide with respect to the transcriptional start site) [48]. Curiously, one truncated ARE-like sequence (ARE′) is present in proximity to one functional XRE: 5′-aacTcGCGTGccagccagtgtgcaTGACt-aGCtctggg-3′ at −1381 (XRE and ARE′ in capitals and numbering as before), in which one base in the spacer between TGAC and GC is missing [28,49]. As expected, gel-shift analysis suggests that the ARE′ sequence only binds a monomeric protein [28]. In order to test its functionality, we mutated ARE' and analyzed transfectants containing the mutated and wild-type ARE′. Surprisingly, TCDD-mediated AhR responses were attenuated in mutated versus wild-type ARE′ transfectants. If Nrf2 and AhR acted independently, it is expected that the TCDD response would be retained in transfectants containing the mutated ARE′ sequence. The findings suggest cross-interaction between Nrf2 and AhR. Activated Nrf2 (possibly by MAP kinases [21]) may inactivate inhibitory protein(s) bound to ARE′. Hence, the activated Nrf2 may stimulate human UGT1A6 expression by an atypical mechanism using cross-interacting AhR as transcription factor. However, more work is needed to substantiate this proposal. Hence, AREs and mechanisms responsible for tBHQ induction of human UGT1A6 in Caco-2 cells (discussed later) still need to be elucidated.

Group C contains heterogeneous Nrf2 target genes and encoded proteins (not discussed here), including proteins involved in redox control such as thioredoxin and thioredoxin reductase 1. Interestingly, two major redox systems (GSH/GSSG and thioredoxin systems) were shown to differentially regulate Nrf2 activation [50]. Other target gene products are involved in protecting against metal toxicity [34–36].

In group D, Nrf2 is listed as an example for regulatory proteins. In addition to being transcriptionally activated by the AhR, Nrf2 expression may also be controlled by AREs in an autoregulatory loop [8].

Coregulation of AhR and Nrf2 gene batteries may be important for proper detoxification. Efficient coupling between Phase I and II XMEs may largely attenuate accumulation of Phase I-generated ROS and electrophiles when the cell is exposed to low levels of toxins [5,6]. Interestingly, export transporters (included in Phase III of drug metabolism) such as Mrp2 (multidrug resistance protein 2) to Mrp6 may also be coordinately induced by AhR and Nrf2 [51]. Hence, biotransformation by Phase I to III detoxication proteins may be an integral part of a dynamic and interactive defense mechanism.

3. Activation of AhR and Nrf2 by phytochemicals

Activators of AhR and Nrf2 have been termed bi- and monofunctional inducers, respectively, since the AhR induces both Phase I and II enzymes whereas Nrf2 selectively induces Phase II enzymes [52]. Interestingly, abundant phytochemicals, for example glucosinolate-derived compounds from Brassica species, provide a rich source of mono- and bifunctional inducers [13,14]. Many of these phytochemicals activate both the AhR and Nrf2, and may be termed 'mixed AhR/Nrf2 activators'. In the following, three groups of phytoalexins are discussed: (i) glucosinolate-derived indoles as selective AhR activators, (ii) glucosinolate-derived isothiocyanates as selective Nrf2 activators, as well as (iii) flavonoids and dithiolthiones as mixed AhR/Nrf2 activators.

3.1. *Glucosinolate-derived indoles as selective AhR activators*

Indole-3-carbinol found in cruciferous vegetables such as broccoli, cauliflower, cabbage, Brussels sprouts exhibits potent anti-tumor activities in animal models of breast and prostate cancer when given orally [19]. Indole-3-carbinol is formed from multiple tryptophan-derived glucosinolates, such as glucobrassicin from broccoli [13] or brassinin from Chinese cabbage [53] where it is stored in vacuoles of plant cells. Upon plant damage the indole is liberated from the thioglucoside conjugates by myrosinase. Indole-3-carbinol is highly sensitive to aqueous acidic conditions and forms numerous oligomeric products. 3,3′-Diindolylmethane (DIM), a major and stable biologically active oligomer and weak AhR agonist, is probably responsible for the pharmacologic effects of indole-3-carbinol; indolo[3,2-b]carbazole is a potent AhR agonist (Fig. 3) [54]. Indole-3-carbinol and DIM induce Phase I and II XMEs in rat and monkey hepatocyte cultures. However, UGT activity was not induced in monkey hepatocytes [55]. Interestingly, similar tryptophan-derived reactive compounds appear to be enzymatically formed in normal intermediary metabolism of mammalian cells, and may be converted to potent endogenous AhR agonists, for example, in heart tissue [56]. It should be noted that indole-3-carbinol is widely used as nutritional supplement in the USA.

3.2. *Glucosinolate-derived isothiocyanates as selective Nrf2 activators*

Isothiocyanates such as water cress-derived PEITC (β-phenethyl isothiocyanate) and broccoli-derived sulforaphane (4-methylsulfinylbutyl isothiocyanate) are selective Nrf2 activators

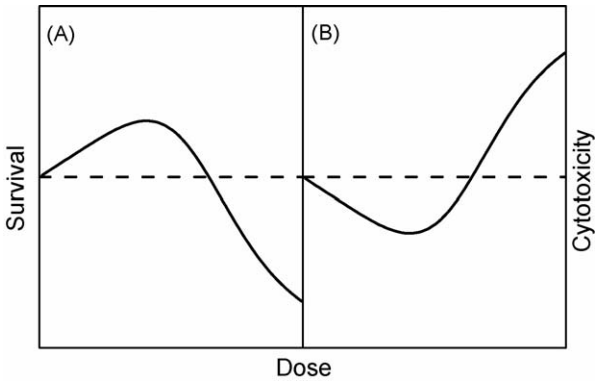

Fig. 4 – Schematic representation of biphasic dose–responses. In A, a dose–response is illustrated, leading to enhanced cell survival in response to prooxidants, as observed in studies of Nrf2 activation by sulforaphane-exposed human retinal pigment epithelial cell cultures [12]. In B, low-dose reduction and high-dose enhancement of adverse effects is depicted, leading to U-shaped dose–response curves.

(Table 2); they have no AhR agonist activity [57]. The moderately toxic sulforaphane is rapidly converted to its GSH conjugate in the human intestinal epithelium [58]. It accumulates in cells and activates multiple Nrf2 target genes, including the Nrf2 gene battery [59]. Following export from cells, the GSH conjugates spontaneously degrade to their isothiocyanates. Thereafter, the isothiocyanates may be taken up again by the cell and reconjugated with GSH. The cyclical process may in some instances result in depletion of intracellular GSH but also assists distribution of isothiocyanates throughout the body.

The antioxidant ethoxyquin has been classified as selective Nrf2 activator [57]. Early studies suggested that this compound exerts distinct inducing properties on UGT activities compared to Ah- or phenobarbital-type inducers [60,61].

Table 2 – Classification of selective and mixed Ah receptor and Nrf2 activators

Class	Compounds
AhR agonists	Indol-3-carbinol*
	3,3′-Diindolylmethane (DIM)*
	Indolo[3,2-b]carbazole*
	TCDD**
Nrf2 activators	Sulforaphane*
	β-Phenethyl isothiocyanate (PEITC)*
	Ethoxyquin
Mixed AhR/Nrf2 activators	Quercetin*
	Luteolin*
	Apigenin*
	Chrysin*
	1,2-Dithiol-3-thione
	Oltipraz
	tert.-Butylhydroquinone (tBHQ)
	β-Naphthoflavone (BNF)

* Phytochemical.
** High-dose exposure to TCDD may generate oxidative stress.

3.3. *Flavonoids and dithiolthiones as mixed AhR/Nrf2 activators*

Fruits, vegetables and plant-derived beverages contain large amounts of structurally diverse flavonoids, such as the flavonol quercetin. This widely studied flavonoid is a weak AhR agonist and moderate Nrf2 activator. Quercetin is rapidly conjugated to various glucuronide and sulfate conjugates in human intestinal epithelium [58].

Bi- or monofunctional inducers have been classified, based on their action as ligand of the AhR or disruptor of the Keap1/Nrf2 complex [52]. Interestingly, many compounds act both as AhR agonists and Keap1 activators. Hence, they represent mixed activators (Table 2). tBHQ has been widely used as monofunctional agent but was recently reclassified as a bifunctional agent [57]. (i) tBHQ undergoes quinone–quinol redox cycling with generation of Keap1 reactants (ROS and tBHQ semiquinones); and (ii) it may be metabolically converted to unidentified AhR ligands. The quantitative contribution of generated ROS/electrophiles or AhR ligands may depend upon the cellular context.

Induction by AhR and Nrf2 activators of human UGT1 family members has been studied in hepatoblastoma HepG2 cells, preferentially expressing UGT1A1 [58], and in colon adenocarcinoma Caco-2 cells, preferentially expressing UGT1A6 [61–63]. In this context, it should be noted that results obtained with tumor cell lines may be influenced by the genotype of the donor patient. For example, Caco-2 cells are particularly sensitive to oxidative stress since the donor exhibited a NQO1 polymorphism (NQO1*2), leading to rapid degradation of the NQO1 protein ([16,65], for references). As predicted from their function as mixed AhR/Nrf2 activators, chrysin [64] or β-naphthoflavone [65] are stronger inducers of UGT1A6 expression and activity than TCDD, a selective AhR activator. In agreement with this proposal, selective inducers such as indoles and isothiocyanates in combination achieve stronger responses than when given alone [16]. Enhanced glucuronide formation was also seen in Caco-2 monolayers treated with the mixed AhR/Nrf2 activator β-naphthoflavone [66], in which coordinate induction of UGTs and Mrp2 is operative [67]. The flavonoids apigenin and luteolin, two intermediates in the oxidative sequence from chrysin to quercetin, can be predicted to be mixed AhR/Nrf2 activators. Flavonoids with more hydroxyl groups have been suggested to possess more potent antioxidant properties; however, further study will be required to characterize the effects of hydroxyl substitution. Flavonoid tea polyphenols are also extensively investigated in chemoprotection trials [17,18]. It appears that the major tea polyphenol epigallocatechin-3-gallate largely exhibits direct antioxidant properties and does not induce Phase II enzymes [57]. 1,2-Dithiol-3-thione and the extensively studied antischistosomal drug oltipraz also represent mixed AhR/Nrf2 activators [11,57].

3.4. *Evolutionary aspects*

Large Phase I and II enzyme families were generated in evolution to detoxify plant phytoalexins [24,68,69]. 'Animal–plant warfare' was proposed as one of the evolutionary driving

forces to generate large XME gene families. In addition, XME regulating transcription factors and xenosensors such as AhR and Nrf2 are known to have an old history in evolution. However, invertebrate AhR homologues (Spineless or Ahr-1, in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively) appear not to be ligand-activated [1], hinting at functions other than detoxication. Nrf2-mediated protection against oxidative stress appears to be conserved in evolution. *C. elegans* SKN-1 is related to Nrf2. Intestinal SKN-1 in *C. elegans* is required for expression of γ-glutamylcysteine synthetase. Based on regulation by glycogen synthase kinase-3, it was concluded that SKN-1 directly integrates multiple independent signals, which combine either to restrain or activate the Phase II response [38]. Adaptive regulation of XME expression by AhR and Nrf2-Keap1 may have shaped their xenosensor binding sites [70]. Interestingly, many phytochemicals represent mixed AhR/Nrf2 activators. It is tempting to speculate that this property may have been a driving force toward the evolution of coupled AhR and Nrf2 gene batteries.

4. Application of selective Nrf2 and mixed AhR/Nrf2 activators for chemoprevention of cancer and degenerative disease

4.1. *Antioxidant activities of flavonoid conjugates*

Despite poor systemic bioavailability, flavonoid conjugates appear to retain antioxidant properties, as clearly shown in studies with rats fed a diet supplemented with 0.2% quercetin [71]. Antioxidant status (measured by generation of Cu^{2+}-catalyzed oxidation of low density lipoproteins) was markedly higher in quercetin-treated versus control rats. These findings suggest that health benefits of flavonoids in foods may be attributed in part to antioxidant properties of their conjugated metabolites. In addition, GSH conjugates of isothiocyanates spontaneously degrade, are taken up, and act intracellularly as Phase II inducers. In this way, they appear to be rapidly distributed throughout the body. Hence, conjugates should not be considered as biologically inactive and readily excretable products.

4.2. *Chemoprevention of cancer and degenerative diseases*

Numerous animal [13–15,17–20] and epidemiologic studies ([14], for references) have shown an association between reduced cancer risk and increased intake of vegetables and tea. Elegant studies indicated that Nrf2 and its activation by oltipraz prevented benzo[a]pyrene-induced tumor of the forestomach by Nrf2-mediated induction of Phase II XMEs [11]. Sulforaphane-containing broccoli extracts were also found to be a promising strategy for protecting against skin tumor formation after exposure to UV radiation [72]. In addition, the AhR/Nrf2 defense system may be useful to prevent degenerative diseases such as macula degeneration, a major vision-impairing disease in the elderly. Human retinal pigment epithelial cell cultures were used as model to investigate indirect antioxidant effects of sulforaphane [12].

4.3. *Biphasic dose–responses and mechanistic aspects*

In contrast to receptor-mediated dose–responses, biphasic dose–responses have to be taken into account with non-receptor Keap1-Nrf2 responses (Fig. 4). A stimulatory response at low dose is followed by adverse responses leading to cytotoxicity at high doses, often resulting in U-shaped dose–response curves. These effects have been clearly described, e.g., in studies with human retinal pigment epithelial cells which were protected against oxidative stress by sulforaphane [12]. Implications of biphasic responses are manifold and complex. Here, it may suffice that there is a dose limit for the application of phytochemicals, representing a pharmacological window above which toxicity sets in. Another severe limitation is given by the observation that effective chemopreventive agents in one experimental setting may enhance carcinogenesis in another setting. For example, indole-3-carbinol was clearly effective in reducing aberrant colon crypt foci and in delaying mammary tumor formation but strongly induced preneoplastic foci in liver carcinogenesis in studies using a multi-organ rat model [73]. A major mechanism responsible for cancer prevention is believed to be the reduction of genotoxic insults due to efficient detoxification. In this context, the Janus face of the AhR (detoxification versus bioactivation) has also to be taken into account. The reason for induced foci formation is assumed to be due to tumor promoting actions of unidentified AhR ligands, generated from indole-3-carbinol, which may trigger sustained AhR signaling and tumor promotion [2]. However, tumor promotion is known to be reversible and dose-dependent. Hence, low-dose application of mixed AhR/Nrf2 activators may be warranted. Enthusiasm in chemoprevention was also tempered by the unexpected outcome of several epidemiologic chemoprevention trials of lung cancer risk in smokers in which the smoker's diet was supplemented with the antioxidant β-carotene. For many years, β-carotene was believed to be a principal candidate for chemopreventive benefit of plant foods. Surprisingly, β-carotene-supplemented smokers showed an increased risk for lung cancer ([14] and references therein). Lung cancer is a leading cause of cancer mortality worldwide. However, there is considerable interindividual variability in disease susceptibility. The reasons for these variations are poorly understood, but there is increasing evidence that various factors (including XMEs) may modify the risk.

Currently, a number of clinical trials with phytochemicals are under way; there is reason for optimism that chemoprevention may be beneficial in some high risk populations [20]. For example, consumption of glucosinolate-rich broccoli sprout extracts was beneficially-modulating Phase II enzymes in a Chinese population highly exposed to environmental carcinogens [74]. Residents of Quidong, People's Republic of China, are at high risk for development of hepatocellular carcinoma due to consumption of aflatoxin B1-contaminated foods coupled with chronic hepatitis B virus infection. In addition, the population was exposed to airborne phenanthrene (the most abundant carcinogenic aromatic hydrocarbon) in this area adjacent to and downwind of Shanghai. A randomized, placebo-controlled chemoprevention trial showed that the excretion patterns of the foodborne and

airborne toxicants (used as biomarkers) were doubled by consumption of the broccoli extracts. The results were consistent with potent action of sulforaphane on GSTs and UGTs, major Phase II enzymes known to be involved in detoxication of aflatoxin B1 and carcinogenic aromatic hydrocarbons [10,49,70,75].

It has to be emphasized that focus on Phase II induction by phytochemicals as the mechanism for chemoprevention should not divert the attention from many other beneficial activities of these compounds. For example, DIM is known to exhibit antiestrogenic activities [76], and has recently been shown to modulate the immune system due to activation of interferon-γ signaling [77]. Sulforaphane and indole-3-carbinol may exhibit nuclear factor kappa B-mediated antiinflammatory mechanisms [78], stimulate apoptosis of colon cancer cells [16,79] and inhibit angiogenesis [80]. These processes may represent major chemopreventive activities under particular pathophysiologic conditions. It was considered appropriate to limit the discussion here on phytochemical-activated, selective Nrf2- and coupled AhR/Nrf2-mediated antioxidant defense.

5. Conclusions

The AhR is increasingly recognized as a multifunctional molecular switch regulating Phase I and II XMEs, controlling vascular development and being responsible for dioxin-mediated toxicities [1,2]. Nrf2 represents another transcription factor orchestrating a major defense system against oxidative/electrophile stress. Recently, this transcription factor was found to be a downstream target of the AhR [8]. These findings suggest that the distinct but overlapping AhR and Nrf2 gene batteries may be coordinately and synergistically regulated. Due to efficient coupling of Phase I and II XMEs, CYP-mediated generation of ROS and electrophiles may be attenuated. Phase I XMEs are negatively regulated by CYP1A1 in an autoregulatory loop, and are not included in the Nrf2 gene battery [44].

The commentary focuses on Phase II XMEs in which functional AREs were identified in their regulatory region, including NQO1 and GSTA2. Many questions about mechanisms responsible for regulation of Phase II genes remain to be answered, for example, mechanisms responsible for regulating human Phase II XMEs. Three groups of phytochemicals activating AhR and Nrf2 are proposed, including glucosinolate-derived indoles as selective AhR activators, glucosinolate-derived isothiocyanates as selective Nrf2 activators, as well as flavonoids and dithiolthiones as mixed AhR/Nrf2 activators. In this context, it is tempting to speculate that the large number of mixed activators among the phytoalexins may have been a driving force for the evolution of coordinate regulation of AhR and Nrf2 gene batteries.

Previously, researchers working on modifiers of XMEs for chemoprevention focused on 'monofunctional' inducers, i.e., selective Nrf2 activators [11,12]. The finding that Nrf2 is a downstream target of the AhR may offer additional possibilities. Mixed AhR/Nrf2 activators may synergistically enhance the effect of monofunctional inducers. It is noteworthy that despite efficient first-pass detoxification in the gastrointestinal tract, conjugation of flavonoids or isothiocyanates does not abolish their antioxidant activities. Potentials and limitations of current clinical chemoprevention trials in high risk populations are discussed. Beneficial effects of phytochemicals in one experimental setting and enhanced carcinogenicity in another setting mandate careful clinical trials [74]. Humans appear to be resistant to most of the adverse health effects of dioxin-like chemicals; a large portion of this resistance may be due to the lower affinity with which the human AhR binds TCDD and other dioxin-like compounds than the rodent AhR ([81], for references). This lower sensitivity of humans to adverse health effects and current results of ongoing trials 'provide an expectation that food-derived chemopreventive agents can be administered in defined, rational, and practical ways to favorably modulate the disposition of unavoidable exposures to environmental carcinogens' [74]. By continuing research to understand mechanisms of pathways of AhR and Nrf2 signaling we will better be able to maximize the protective effects of chemopreventive agents and minimize their adverse effects.

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