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Chemoprotection against cancer by Phase 2 enzyme induction

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Abstract

Mammalian cells have evolved elaborate mechanisms for protection against the toxic and neoplastic effects of electrophilic metabolites of carcinogens and reactive oxygen species. Phase 2 enzymes (e.g. glutathione transferase, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases) and high intracellular levels of glutathione play a prominent role in providing such protection. Phase 2 enzymes are transcriptionally induced by low concentrations of a wide variety of chemical agents and such induction blocks chemical carcinogenesis. The inducers belong to many chemical classes including phenolic antioxidants, Michael reaction acceptors, isothiocyanates, 1,2-dithiole-3-thiones, trivalent arsenicals, $HgCl_2$ and organomercurials, hydroperoxides, and vicinal dimercaptans. Induction by all classes of inducers involves the antioxidant/electrophile response element (ARE/EpRE). Inducers are widely, but unequally, distributed among edible plants. Search for such inducer activity in broccoli led to the isolation of sulforaphane, an isothiocyanate that is a very potent Phase 2 enzyme inducer and blocks mammary tumor formation in rats.

Keywords: Glutathione transferases; Quinone reductase; Vegetables; Antioxidant response element (ARE); Electrophile response element (EpRE)

1. Introduction

The initiation of many tumors results from damage to DNA by electrophilic carcinogen metabolites, or by reactive oxygen species that arise during carcinogen metabolism or endogenous cellular processes. Mammalian cells have evolved multiple and elaborate mechanisms for protection against such toxic insults. Phase 2 enzymes (e.g. glutathione transferases (GST), NAD(P)H:quinone reductase (QR), epoxide hydrolase, glucuronosyltransferases, aldehyde reductase, and others) and high cellular levels of glutathione are the primary lines of defense against these reactive chemical species. These protective mechanisms disarm and facilitate the disposal of reactive electrophiles and oxygen species. Much recent evidence indicates that elevation of Phase 2 enzymes and of glutathione levels by inducers results in protection against chemical carcinogens. Indeed, modulation of the metabolism of carcinogens is one of the most effective and well-established strategies for protecting animals and their cells against the toxic and neoplastic effects of carcinogens.

Phase 2 enzymes are transcriptionally reg-

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ulated in animal cells by low concentrations of a wide variety of chemical agents, many of which are already present in the human diet [1–3]. Consequently, specific modification of the human diet to increase the consumption of phytochemicals that induce Phase 2 enzymes is an attractive, safe, and promising strategy for decreasing the risk of developing cancer. Implementation of this strategy requires: (a) identification of effective inducers and determination of their inducer potencies; (b) elucidation of the chemistry and molecular mechanisms of action of inducers; (c) identification of edible plants rich in inducer activity; (d) demonstration that such plants can raise Phase 2 enzymes when administered to animals and to humans; (e) trials in humans with short-term surrogate biomarkers for the protected state; and (f) ultimate demonstration of risk reduction in human populations at high risk of developing cancer. This brief account reviews progress in achieving these objectives.

2. Detection and identification of Phase 2 enzyme inducers and measurement of their potencies

Since Phase 2 enzymes are generally induced coordinately in many tissues and in cells in culture, we have selected a single enzyme as a marker for induction. Quinone reductase (QR) is a convenient representative enzyme because it is widely distributed in mammalian tissues, is easily measured, and shows a large inducer response (as much as 10- to 12-fold in some tissues) [4]. A highly suitable and robust cell line for studying induction of this enzyme is the Hepa 1c1c7 murine hepatoma line [1]. Measurement of QR activity directly (by a coupled tetrazolium dye assay) on digitonin extracts of cells grown in 96-well microtiter plates and exposed to serial dilutions of the inducer (a single chemical compound, a mixture, or a plant extract) provides an accurate assessment of inducer activity [5,6]. The specific activity of the enzyme can then be obtained by relating the activity to cell mass or protein concentration. A convenient index of inducer potency is the concentration required to double (CD) the specific activity of QR. Inducers vary enormously in potency, with CD values ranging from low nanomolar to high millimolar concentrations.

The availability of mutant Hepa 1c1c7 cells that are defective in cytochrome P-450 activity or in *A*ryl *h*ydrocarbon (*Ah*) receptor function provides the means for distinguishing *monofunctional* inducers (that elevate Phase 2 enzymes selectively) from *bifunctional* inducers (that upregulate both Phase 1 and Phase 2 enzymes) [7,8]. This distinction is important because some cytochromes P-450 (e.g. 1A1 and 1A2) are involved in activation of carcinogens, whereas Phase 2 enzymes mostly catalyze detoxication reactions. Monofunctional inducers are therefore preferred as agents for achieving chemoprotection in humans.

3. The chemistry of inducers

The development of detailed understanding of the chemical requirements for inducer activity has been a continuing effort in our laboratory since the demonstration that phenolic antioxidants exerted their major chemoprotective activity by virtue of the induction of glutathione transferases and other Phase 2 enzymes [9,10]. The first insight into the chemistry of inducers was obtained from studies of structural analogues of BHA [11], a chemoprotective antioxidant that is widely used as a food additive. These studies pointed to *tert*-butylhydroquinone, a metabolite of BHA, as probably the active species responsible for the inducer activity of BHA.

Major information on the chemistry of inducers was obtained from studies of analogues of *tert*-butylhydroquinone in which the orientation of the diphenolic hydroxyl groups was changed from 1,4-diphenols to 1,2- or 1,3-diphenols. The results were clear-cut: only the 1,2-diphenols (catechols) or the 1,4-diphenols (hydroquinones) were inducers, whereas the 1,3-diphenols (resorcinols) were inactive, and the presence or absence of other ring substituents was relatively unimportant in specifying inducer activity [12].

These results clearly implicated redox lability in inducer function, since catechols and hydroquinones can be readily oxidized to the corresponding quinones, whereas resorcinols cannot undergo such conversions. These experiments did not, however, reveal whether the quinone products or the redox process itself (perhaps the reactive oxygen species generated) was responsible for induction. This issue was resolved by the finding that many highly electrophilic Michael reaction acceptors (olefins or acetylenes conjugated to electron-withdrawing groups) were inducers, and that their potency generally paralleled their reactivity in the Michael reaction [2]. Since quinones are excellent Michael reaction acceptors, the inducer activity of 1,2- and 1,4-diphenols is therefore dependent upon their oxidation to quinones.

The chemistry of inducers was subsequently greatly expanded [13,14] with the observation that, in addition to oxidizable diphenols (and corresponding phenylenediamines) and Michael reaction acceptors, the following classes of compounds are also efficient inducers: isothiocyanates, 1,2-dithiole-3-thiones, trivalent arsenicals, mercury(II) salts and organic mercurials, and hydroperoxides. These inducers share almost no structural similarities (in the sense of complementarity to a receptor), but are all electrophiles, capable of reacting with sulfhydryl groups. These findings suggested that the signaling of induction involves a primary interaction with a highly reactive sulfhydryl group, or possibly two vicinal sulfhydryl groups, since trivalent (but not pentavalent) arsenicals are excellent inducers. It was therefore somewhat surprising that several compounds carrying vicinal sulfhydryl groups (e.g. 2,3-dimercapto-1-propanol (BAL)), but not monothiols, were also efficient inducers [13,14]. Although these compounds are nucleophiles rather than electrophiles, they can, like all other inducers, modify sulfhydryl groups by redox reactions. We conclude that the following general properties characterize all known inducers: (a) most are electrophiles (including quinones and Michael reaction acceptors); (b) all react with sulfhydryl groups by virtue of their electrophilicity, or by participating in redox reactions; and (c) most inducers are substrates for glutathione transferases [15].

4. Presence of Phase 2 enzyme inducers in edible plants

The extensive evidence that increased consumption of fruit and vegetables is associated with reduced risk to developing cancer [16], naturally raised the issue whether at least some of these effects might be due to the presence of Phase 2 enzyme inducers in edible plants. When the inducer potencies of organic solvent extracts of a variety of commonly-consumed plants were measured, there were marked differences depending on genus, species, and even variety [6]. Cruciferous plants (e.g. broccoli, cabbage, cauliflower, kale) were particularly rich sources of inducer activity. We selected broccoli for detailed study because extracts tended to have high inducer activity and broccoli was already consumed in substantial quantities in the Western world. One cultivar of broccoli (SAGA) was particularly rich in inducer activity. Reverse phase HPLC and other forms of chromatography showed that the majority of the inducer activity of Saga broccoli was attributable to a single compound, an isothiocyanate: sulforaphane (CH_3-S(O)-$(CH_2)_4$-N=C=S) [17]. Sulforaphane, which had been previously isolated from cabbage [18] and had also been synthesized [19], was found to be an exceedingly potent QR inducer in murine hepatoma cells; indeed it is the most potent naturally occurring inducer so far identified. When fed to mice, sulforaphane induced both QR and glutathione transferases in several tissues [17].

Analogues of sulforaphane that differ in the state of oxidation of the methylthio group and the length of the methylene bridge, i.e. $CH_3S(O)_m$ $(CH_2)_n$N=C=S, where $m = 0$, 1, or 2 and $n = 3$, 4 or 5, were prepared and tested for inducer activity in murine hepatoma cells [17]. Sulforaphane was the most potent inducer. The sulfoxides and the sulfones were more potent than the sulfides, and the compounds with four

or five methylene groups were more potent than those with only three methylene groups.

The methyl sulfinyl function of sulforaphane was very important for inducer activity since n-hexyl-N=C=S was a much weaker inducer than sulforaphane [20]. Interestingly, the methyl sulfinyl group (CH_3SO-) could be replaced by an acetyl group (CH_3CO-) without changing the inducer activity significantly. Consequently a number of cyclic analogues were designed in which the distance between the CH_3CO- and the -NCS groups was varied. Among the most potent inducers were certain acetylnorbornyl-NCS analogues, some of which were comparable in inducer potency to sulforaphane [20].

Although the majority of the inducer activity of extracts of SAGA broccoli was attributable to sulforaphane, we have shown recently that such extracts also contain lesser quantities of erucin (the sulfide analogue). Since erucin has only about one-sixth the inducer potency of sulforaphane, it makes only a minor contribution to the total inducer activity of SAGA broccoli extracts. In connection with our conclusion that sulforaphane is the principal Phase 2 enzyme inducer of SAGA broccoli extracts, we now realize that the conditions of isolation of sulforaphane involved the preparation of aqueous homogenates of broccoli that were then lyophilized [17]. More recent experiments indicate that these conditions were favorable for hydrolysis of glucoraphanin (the glucosinolate precursor of sulforaphane) by the coexisting thioglucosidase, myrosinase. It is therefore very likely that in the intact plant a significant proportion of the isolated sulforaphane exists as its glucosinolate.

5. Antitumor effects of sulforaphane

Sulforaphane and its norbornyl-NCS analogues were tested in the single dose DMBA (7,12-dimethylbenzanthracene) mammary tumor model in Sprague–Dawley rats [21]. The chemoprotectors were administered by gavage for 3 days before the DMBA, on the day of carcinogen treatment, and on the following day. Under these circumstances, there was a substantial, dose-dependent reduction in the incidence of mammary tumors that developed. In addition, there was a reduction in both the multiplicity (number of tumors per rat) and the size of the tumors, and tumor appearance was delayed. Sulforaphane and one of the acetylnorbornyl isothiocyanates were of similar potencies in their ability to block tumor development.

The observation of antitumor effects of sulforaphane and its analogues was not altogether surprising, because such properties had already been recognized in other isothiocyanates [22]. However, the finding of the high inducer and anticarcinogenic properties of sulforaphane encouraged us to undertake a systematic search for anticarcinogenic enzyme inducers in edible plants. The results confirmed the validity of the strategy of searching for naturally-occurring chemoprotectors in plants (and designing analogues) based on monitoring inducer potency. We are aware of only three prior examples of this approach: the prediction of the tumor blocking activity of a 1,2-dithiole-3-thione (oltipraz) [23,24], isolation of two terpenoids from green coffee beans [25]; and the demonstration of the blocking activity of the Michael acceptor dimethyl fumarate on hepatic tumor formation in A^{vy}/A mice (Y. Zhang and P. Talalay, unpublished observations).

6. Molecular mechanisms of the regulation of Phase 2 enzyme induction

The rational development of more effective chemoprotective Phase 2 enzyme inducers would be greatly facilitated by a detailed understanding of the molecular mechanisms underlying the regulation of these enzymes. As noted above, the inducers belong to at least eight different chemical families that have few common characteristics apart from their electrophilic reactivity and their ability to interact with sulfhydryl groups either by nucleophilic substitution or oxidoreductions. The proposal that a primary covalent interaction with sulfhydryl groups of a 'target' protein generates the signal for induction is supported not only by the propensity of all inducers to react with

sulfhydryl groups, but also by the finding that inducer potency is related to avidity for sulfhydryl groups. This is illustrated by the general correlation between inducer potency and the reactivity of inducers in the Michael reaction [2]. Furthermore, inducer potency of a series of heavy metals correlates with their affinity for sulfhydryl groups, i.e. $Hg^{2+} > Cd^{2+} > Zn^{2+}$ [26]. The exceptionally high inducer potency of trivalent arsenicals strongly suggests that vicinal sulfhydryl groups that can form cyclic thioarsenites may be involved in the inducer signalling. If we postulate that all inducers must interact with an intracellular 'target protein' in order to initiate the events resulting in enhanced transcription, it seems likely that the cognate sulfhydryl group(s) on this protein must be extremely reactive in comparison to other sulfhydryl groups, otherwise it would be difficult to envisage how the inducers could evade reaction with the very high concentrations of glutathione that prevail in the cytosol (3–8 mM). However, if, as is entirely possible, the ultimate inducing species were the glutathione conjugates of the inducers, these might then be transported to the target protein and there undergo cleavage by the more reactive vicinal sulfhydryl groups of the target protein.

Much more specific information is available on the precise regulatory regions of the genes that are transcriptionally activated by exposure to inducers. Deletion analyses of the upstream regions of the glutathione transferase Ya gene of rat and mouse liver have identified nearly identical 41-bp enhancer regions that respond to the aforementioned inducers [27–31]. When these DNA segments were inserted into plasmids designed for heterologous gene expression, and the resulting plasmids were transfected into hepatoma cells, responses to inducers were observed. The controlling elements have been further narrowed to regions termed the Antioxidant Response Element (ARE) [29] or the Electrophile Response Element (EpRE) [30,31], for which the consensus sequence G(or A)TGACNNNGC has been assigned. The mouse GST Ya gene contains two of these elements separated by five base pairs, whereas the rat GST Ya gene contains only one element. Similar sequences have also been identified in the upstream regions of the human and rat QR genes (reviewed in [32]).

In a recent study [13,14], we inserted the 41-bp enhancer elements derived from the 5′-upstream region of the mouse liver GST Ya gene together with its promoter into plasmids capable of expressing human growth hormone as reporter. These plasmids were transfected into hepatoma cells, and the concentration dependence of growth hormone expression was measured for 28 inducers belonging to all known chemical classes of inducers. The potencies of these compounds in driving heterologous gene expression were then compared with their potencies as inducers of QR in hepatoma cells. Although these potencies spanned nearly four orders of concentration magnitude, they were very similar in the two systems. Furthermore six structurally related compounds were inactive in both systems. These results led to the unequivocal conclusion that the transcriptional activation evoked by all classes of inducers could be fully accounted for by activation of the 41-bp element containing the ARE/EpRE.

The nature of the ARE/EpRE and its transcriptional binding factors has been controversial. The issue is whether the ARE/EpRE has the properties of phorbol ester responsive elements (TRE) (and is regulated by binding of AP-1 factors such as c-*fos* and c-*jun*) [31], or whether ARE/EpRE involves distinctly different mechanisms and transcription factors [33]. Although the consensus TRE sequence [TGAC(or G)TC(or A)A] bears some resemblance to the ARE/EpRE consensus sequence, it lacks the critical 3′-terminal GC bases. This question has been recently examined in several laboratories. In our experiments [34], the behavior to inducers of the above-described growth hormone reporter construct containing the 41-bp ARE/EpRE region derived from the mouse GST Ya gene was compared with the behavior of the same construct in which the ARE/EpRE elements were replaced by one or two consensus phorbol ester response elements (TRE). The wild-type sequence was highly activated by monofunctional inducers of various chemical types, but the con-

structs in which the ARE/EpRE sequences were mutated to TRE were not responsive. Furthermore, transfection of the ARE/EpRE reporter construct into F9 cells, which lack endogenous TRE binding proteins, produced substantial stimulation of growth hormone synthesis by the same inducers that also induced QR enzyme activity in untransfected F9 cells. These results strongly favor the view not only that the ARE/EpRE mediates the induction response to the various types of inducer, but also that this process is independent of phorbol ester responsive elements. This conclusion is supported by independent lines of evidence from other laboratories [35,36].

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