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Antioxidant Functions of Sulforaphane: a Potent Inducer of Phase II Detoxication Enzymes

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Abbreviations: ARE = antioxidant response elements; BHA = butylated hydroxyanisole; DMBA = 9,10-dimethyl-1,2-benz[*a*]anthracene; GSH = glutathione; GSSG = oxidized glutathione; GST = glutathione transferase; HO-1 = heme oxygenase-1; NDMA = *N,N*-dimethylnitrosamine; QR = NAD(P)H:quinone reductase.

Introduction

The widespread belief that oxidative damage plays a major role in cancer, ageing, and in a number of chronic diseases has focused scientific and public attention on the possibility that antioxidants could prevent or at least retard these processes. Antioxidants are of two types: *direct* and *indirect*. Direct antioxidants [e.g. glutathione (GSH), tocopherols, ascorbic acid and carotenoids] are substances that can participate in physiological, biochemical or cellular processes that inactivate free radicals or that prevent free-radical initiated chemical reactions. Direct antioxidants may also exhibit prooxidant effects under some experimental conditions, but whether these properties play a significant role *in vivo* remains unclear. In contrast, indirect antioxidants are not able to participate in radical or redox reactions as such, but they boost the antioxidant capacity of cells by a variety of mechanisms described below, and thereby afford protection against oxidative stress.

There is substantial and mounting evidence that Phase II enzymes [e.g. glutathione transferases (GSTs), NAD(P)H:quinone reductase (QR), epoxide hydrolase, heme oxygenase, UDP-glucuronosyltransferase] play important roles in the detoxication of electrophiles and their induction protects animals and their cells against carcinogenesis and mutagenesis (Benson *et al.*, 1978, 1980; Kensler, 1997; Prester *et al.*, 1995; Talalay, 1992). The prevailing

view of the mechanisms responsible for the protective functions of Phase II enzyme inducers has focused on the abilities of these enzymes to detoxify reactive electrophiles, and the inductive process has been termed the “electrophile counterattack response” (Prester *et al.*, 1993).

In this paper, we marshal evidence that a very important but largely ignored consequence of Phase II enzyme induction is the enhancement of cellular antioxidant capacity. There is now considerable information on the chemistry of inducers. Eight classes of chemical agents have been identified as inducers of Phase II enzymes (Prester *et al.*, 1993; Talalay *et al.*, 1988): (1) diphenols, phenylenediamines, and quinones; (2) Michael reaction acceptors—compounds containing olefin or acetylene linkages conjugated to electron-withdrawing groups; (3) isothiocyanates; (4) hydroperoxides and hydrogen peroxide; (5) 1,2-dithiole-3-thiones; (6) vicinal dimercaptans; (7) trivalent arsenicals; (8) divalent heavy metals. These inducers vary greatly in potencies and show little structural similarity, but are all chemically reactive with sulfhydryl groups, and most are also substrates for GSTs (Prester *et al.*, 1993; Spencer *et al.*, 1991). Recently, a ninth class of inducers comprising carotenoids, curcumins and related polyenes has been added (Dinkova-Kostova and Talalay, 1999; Khachik *et al.*, 1999). Inducers of Phase II enzymes also raise the activity of γ -glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis, and thereby elevate tissue GSH levels (Mulcahy *et al.*, 1997). It is of interest that several of the types of aforementioned inducers of

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Phase II enzymes are both indirect and direct antioxidants, for example, phenolic antioxidants, mercaptans, carotenoids and curcumin.

Isothiocyanates are an especially interesting class of anticarcinogenic Phase II enzyme inducers. Many isothiocyanates are present in substantial quantities in the human diet (primarily originating from the ingestion of *Cruciferae*). More than 20 isothiocyanates inhibit the formation of tumours of several animal organ targets elicited by a variety of carcinogens (Hecht, 1995; Zhang and Talalay, 1994). Much evidence suggests that the major mechanisms of chemoprotection by isothiocyanates depend on the induction of Phase II detoxication enzymes and the inhibition of Phase I enzymes that are involved in the activation of certain carcinogens. Isothiocyanates are powerful electrophiles, a property attributable to the central carbon atom of the $-N=C=S$ group, which reacts readily with sulfur-, nitrogen- and oxygen-based nucleophiles. However, there is no known chemical or biochemical evidence that this group can participate in oxidation or reduction reactions as a direct-acting antioxidant under physiological conditions (see Barton and Ollis, 1979). Furthermore, to our knowledge there is no evidence that the isothiocyanate group can behave as a prooxidant, unlike some direct-acting antioxidants.

The isothiocyanate sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane: $CH_3S(O)(CH_2)_4-N=C=S$] has attracted much recent interest since it was isolated in our laboratory from broccoli by monitoring QR inducer activity, and found to be the most potent naturally-occurring inducer of Phase II enzymes (Prochaska *et al.*, 1992; Zhang *et al.*, 1992). Furthermore, sulforaphane inhibits mammary tumour formation in female rats treated with single oral doses of 9,10-dimethyl-1,2-benz[*a*]anthracene (DMBA) (Zhang *et al.*, 1994). Interest in sulforaphane as a chemoprotector has been further stimulated by the recent finding that certain varieties of 3-day-old broccoli sprouts contain remarkably high levels of glucoraphanin, the glucosinolate precursor of sulforaphane and may provide an efficient source of dietary glucoraphanin and consequently of sulforaphane (Fahey *et al.*, 1997).

Isothiocyanates, glucosinolates and myrosinase

Upon disruption of plant cells, isothiocyanates are one of the major products formed from the hydrolysis of glucosinolates (β -thioglucoside *N*-hydroxysulfates) by the action of the co-existing but physically segregated enzyme myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1). Glucosinolates are water-soluble, anionic, non-volatile and heat-stable. They are not believed to possess significant direct biological activity. When plants containing glucosinolates are damaged by predators, or by food prep-

aration or chewing, myrosinase is released and catalyses hydrolysis of the β -thioglucoside linkage. The resultant aglycones then undergo non-enzymatic, intramolecular (Lossen) rearrangement to yield isothiocyanates, thiocyanates, nitriles or epithionitriles. To date, a total of 104 glucosinolates have been reported from about 450 species of higher plants belonging to 16 families. There is a voluminous literature on the glucosinolates from the family *Cruciferae*, particularly those of the genus *Brassica*. This genus contains a large number of commonly consumed vegetables, the glucosinolates of which have been extensively documented and most recently reviewed by Rosa *et al.* (1997). Although there have only been two unconfirmed reports of the presence of glucosinolates in non-vascular plants, myrosinase-like (β -thioglucosidase) activity is quite commonly found in fungi and bacteria, including enteric bacteria. In contrast to their glucosinolate precursors, the isothiocyanates are typically lipophilic and may be highly reactive, volatile, malodorous, taste sharp or bitter, and some of them can be hepatotoxic, antibacterial, antifungal, antiprotozoal, nematocidal, allelopathic, or produce goitrogenic degradation products (Fenwick *et al.*, 1983; Rosa *et al.*, 1997).

Discovery, isolation, chemistry and biosynthesis of sulforaphane

The name glucoraphanin was initially coined for an antibacterial principle isolated from radish (*Raphanus sativus*) (Ivanovics and Horvath, 1947). Close examination of this report suggests that glucoraphenin, the glucosinolate of sulforaphane ($CH_3S(O)CH=CHCH_2CH_2-N=C=S$) may have been the compound actually isolated. Subsequent to this report, glucoraphanin (4-methylsulfinylbutyl glucosinolate) has only been identified in one of 16 higher plant families examined, the *Cruciferae*. In this family it is found in most of the commonly eaten vegetables in the genus *Brassica* (e.g. cole crops such as broccoli, kale, cauliflower, Brussels sprouts, cabbage, collards, turnips) as well as in two other genera containing such widely-consumed vegetables as radish (*Raphanus* sp.) and arugula (*Eruca* sp.), *Arabidopsis* spp. in which much of the genetics of its production has been worked out, and in the following "non-food" genera: *Alyssoides*, *Biscutella*, *Cakile*, *Cardaria*, *Choriopsisora*, *Diploaxis*, *Erysimum*, *Euzomodendron*, *Farestia*, *Fibigia*, *Isatis*, *Lepidium*, *Lesquerella*, *Lunaria*, *Malcomia*, *Physaria*, *Streptanthella* and *Thelypodium*. Kjaer (1960) describes the early isolation of many of these compounds.

Sulforaphane, the cognate isothiocyanate of glucoraphanin, was synthesized in 1948 (Schmid and Karrer, 1948), and subsequently shown to be an abundant component of a weed known as hoary

cross [*Cardaria (Lepidium) draba* L. (Desv.) by Procházka (1959). Sulforaphane was also isolated from savoy and red cabbage by the same group (Procházka *et al.*, 1959). Its antimicrobial activity was first identified by Procházka and Komersová (1959) and was much more extensively characterized by Dornberger *et al.* (1975). Hoary cress is described as a pot herb, and is apparently eaten as a peppery herb in central Europe, although details of its consumption are sketchy. It is also very widely distributed throughout the world as a rangeland weed (McInnis *et al.*, 1993; Miller, 1991). We have found that hoary cress is very rich in glucoraphanin which can be readily converted to sulforaphane by the addition of exogenous myrosinase.

Glucoraphanin biosynthesis probably occurs via elongation of methionine (stepwise addition of methylene groups to form dihomomethionine), followed by addition of the glycoside moiety to provide 4-methylthiobutyl glucosinolate, followed by side-chain modification to produce 4-methylsulfinylbutyl glucosinolate (Faulkner *et al.*, 1998). Many isothiocyanates, including sulforaphane (Schmid and Karrer, 1948) and its closely related sulfonyl isothiocyanate [α -methylsulfonylbutyl isothiocyanate; erysolin (Kjaer and Conti, 1954)] have been synthesized. Further recent improvements in the isolation of sulforaphane and related compounds have been reported (Kore *et al.*, 1993).

We have isolated glucoraphanin from the pods, flowers, stems and leaves of hoary cress (*Cardaria draba*), also known as whitetop or heart-podded hoary cress (McInnis *et al.*, 1993) and from broccoli (*Brassica oleracea* var. *italica* cv. DeCicco) and arugula (*Eruca sativa*) by a combination of chromatographic procedures. Hoary cress was collected from seven locations and extracts were prepared in the same manner as we have previously reported for broccoli sprouts (Fahey *et al.*, 1997). In many of these extracts, glucoraphanin was responsible for most or all of the Phase II enzyme inducer activity as demonstrated by the development of such activity only after hydrolysis by addition of highly purified myrosinase (Shikita *et al.*, 1999). Of the sites sampled, plants harvested from the Antelope and Leona Valleys, North of Los Angeles in 1993 and 1994 were by far the richest sources of detoxication enzyme inducer activity. The flowering tops of these plants rivalled broccoli sprouts (Fahey *et al.*, 1997) in the potency of their inducer activity.

Inhibition of tumour formation

In 1992, after its isolation from broccoli, sulforaphane was shown to be a potent inducer of Phase II enzymes in isolated murine hepatoma cells and in various mouse tissues *in vivo* (Zhang *et al.*, 1994). It was later shown to have potent activity in blocking the formation of mammary tumours in rats treated

with the carcinogen DMBA (Zhang *et al.*, 1994). The effect of the highest doses of sulforaphane tested on tumorigenesis was dramatic, resulting in a reduction in tumor incidence and multiplicity for all animals to 40.3% and 18.2% of control values, respectively, as well as a reduction in tumor weight and a significant delay in tumour development—arguably the most important effect. In subsequent studies with broccoli sprouts containing high levels of glucoraphanin as essentially the only glucosinolate/isothiocyanate, a similar protective effect against tumours was established at oral doses approximately equivalent to those of sulforaphane itself (Fahey *et al.*, 1997). Other laboratories have shown that sulforaphane induces mouse mammary QR and GST activities and at very low concentrations reduces the incidence of preneoplastic lesions in carcinogen-treated mouse mammary glands in organ culture (Gerhauser *et al.*, 1997).

Structure-activity studies (Zhang *et al.*, 1992) have examined the effects of altering the oxidation state of the methylthio group (S, S = O, SO₂) and the length of the methylene chain (n = 3, 4, or 5) separating the methylthio- and the -N = C = S groups of sulforaphane on the inducer potency for QR in murine hepatoma cells. All of these analogues were considerably less potent than the parent sulforaphane. Remarkably, replacement of the S = O by C = O produced an analogue that was equally potent to sulforaphane (Posner *et al.*, 1994). A series of synthetic acetylnorbornyl isothiocyanates varied in inducer potencies depending on positions and steric relations of the acetyl and isothiocyanate groups. Some of these analogues were potent inducers, but in this series also, none was more potent than sulforaphane (Posner *et al.*, 1994).

Cytotoxicity, mutagenicity and metabolism of sulforaphane

Although sulforaphane shows little cytotoxicity, some inhibition of growth of Hepa 1c1c7 cells was observed at concentrations 75-fold greater than those required (0.2 μ M) to double quinone reductase activity (Gerhauser *et al.*, 1997). Barcelo *et al.* (1996) were only able to demonstrate cytotoxicity against mouse hepatocytes at extremely high concentrations (> 500 μ M). Of even more direct relevance, sulforaphane has been shown to exert a dose-dependent cytotoxicity on human colon adenocarcinoma HT29 cells that was irreversible when administered to dividing cells; however, reversible inhibition of DNA synthesis was reported upon treatment of quiescent cells. Moreover, at the same doses (up to 30 μ M), sulforaphane did not affect the viability of differentiated CaCo2 cells (Gamet-Payraastre *et al.*, 1998). Additional evidence for the low toxicity of this compound is embodied in the

demonstration by Barcelo *et al.* (1996) that sulforaphane is not genotoxic to hepatocytes, nor is it mutagenic to *Salmonella typhimurium* strain TA100 even at very high concentrations. Furthermore, sulforaphane does not induce unscheduled DNA synthesis in hepatocytes, but inhibits the mutagenicity of *N,N*-dimethylnitrosamine (NDMA) in the *Salmonella* assay, as well as inhibiting the unscheduled DNA synthesis evoked by this nitrosamine in hepatocytes.

Metabolic investigations of the fate of sulforaphane (administered either as pure compound or as a plant extract) in mammalian systems have shown that: (a) it is a good substrate for glutathione transferases (Kolm *et al.*, 1995; Zhang *et al.*, 1995), (b) it inhibits cytochromes P450 (Maheo *et al.*, 1997; Morel *et al.*, 1997), in particular, CYP2E1 (Barcelo *et al.*, 1996), (c) it is conjugated to GSH and eliminated from mammalian system largely as its *N*-acetylcysteine conjugate (Kassahun *et al.*, 1997); (d) it undergoes some reduction of the sulfoxide group to the sulfide to form erucin (Kassahun *et al.*, 1997); and (e) it is excreted rapidly in humans (Shapiro *et al.*, 1998).

Antioxidant functions of sulforaphane

Sulforaphane is not a direct-acting antioxidant or prooxidant, since it is very unlikely that the isothiocyanate group can participate in oxidation or reduction reactions under physiological conditions (see Barton and Ollis, 1979). There is, however, substantial and growing evidence that sulforaphane administration acts indirectly to increase the antioxidant capacity of animal cells, and their abilities to cope with oxidative stress.

Increased tissue glutathione levels

Many isothiocyanates, in common with other inducers of Phase II enzymes, raise tissue GSH levels (see Zhang and Talalay, 1994), by stimulating the Antioxidant Response Elements (ARE) in the 5'-upstream region of the gene for the heavy subunit of γ -glutamylcysteine synthetase (Mulcahy *et al.*, 1997). This enzyme catalyses the rate-limiting step in GSH synthesis. As GSH is already present in millimolar concentrations in virtually all cells, such increases in GSH presumably augment cellular antioxidant defences.

Effects on enzyme regulation

The major toxicities to cells and their DNA arise from two types of chemical agents: electrophiles and reactive oxygen species. Mammalian cells are endowed with both separate and overlapping mechanisms for dealing with these types of toxicities. One of the central protective mechanisms is the induction of Phase II detoxication enzymes. Much recent evidence supports the view that enzymes

induced by sulforaphane such as glutathione transferases, NAD(P)H:quinone reductase (DT-diaphorase), and heme oxygenase can all function as protectors against oxidative stress.

Antioxidant role of glutathione transferases

Protection against hydroperoxides. Some glutathione transferases have well defined (selenium-independent) glutathione peroxidase activities. They catalyse the reaction of organic peroxides with glutathione (GSH), and thus generate oxidized glutathione (GSSG), which is then reduced to GSH by the very active NADPH-dependent GSSG reductase present in most cells. Thus these enzymes have clear-cut antioxidant functions that contribute to the antioxidant capacity of cells.

Protection against lipid and nucleotide base peroxidation. Ketterer and colleagues have shown that GSTs intervene in lipid peroxidation by efficient reaction with DNA base hydroperoxides and lipid hydroperoxides (see Ketterer *et al.*, 1987). In the case of DNA hydroperoxides, it was shown that 5-hydroperoxymethyl uracil in DNA can be salvaged by the enzymatic action of glutathione peroxidases.

As radiation and hydroxyl radicals can cause the formation of highly toxic DNA base propenals, for example, adenine propenal and thymine propenal, it was significant that Mannervik and colleagues showed that these propenals are very efficient substrates for conjugation with GSH by several GSTs (Berhane *et al.*, 1994). These studies complement the earlier finding of Mannervik and his colleagues (Danielson *et al.*, 1987) that 4-hydroxyalkenals that can arise endogenously from free radical-initiated lipid peroxidation are likewise very good GST substrates. As GST P1-1 is especially efficient for these alkenal substrates, the Mannervik group has drawn the conclusion that "GST P1-1 serves an important function in the cellular response to oxidative stress", and that this reactivity complements the aforementioned GSH peroxidase activity of GSTs in protecting cells against the damaging effects of various oxidative processes (Berhane *et al.*, 1994).

Protection against catecholamine quinone toxicities. Very recently, it has been shown that the oxidized (*o*-quinone) metabolites of neurotransmitter catecholamines (e.g. dopamine, dopa, epinephrine and norepinephrine) which contribute to redox cycling toxicity are efficiently conjugated with GSH by GST (Baez *et al.*, 1997).

Antioxidant role of NAD(P)H:quinone reductase (DT-diaphorase; NQO1)

The role of QR in catalysing the obligatory two-electron reduction of quinones and thereby diverting these agents from one-electron oxidoreductions and the resulting oxidative stress is well recognized (Prochaska and Talalay, 1991). Prochaska *et al.* (1987) have shown that quinone reductase suppresses menadione-dependent chemiluminescence

arising from the formation of active oxygen species generated by oxidative cycling. The antioxidant actions of phenolic antioxidants such as BHA can therefore be ascribed not only to their direct chemical properties as hindered phenols but also to the induction of QR (Benson *et al.*, 1980). Although QR undoubtedly affords antioxidant protection through the detoxification of exogenous quinones, very recent evidence indicates strongly that QR probably plays additional important roles in supporting the function of two naturally-occurring quinones: coenzyme Q (ubiquinone) and α -tocopherolquinone. In experiments involving both natural and artificial lipid membranes, Beyer *et al.* (1996) demonstrated that QR maintains various types of coenzymes Q in the reduced form, which is required for the protection of these membranes against oxidation. These authors suggest that QR evolved to provide antioxidant protection for membranes. Furthermore, Siegel and colleagues (1997) demonstrated the efficient reduction of α -tocopherolquinone to its hydroquinone by human QR, and provided convincing evidence that this reduction plays a significant role in maintaining cellular antioxidant function.

It has also recently been demonstrated that treatment with Phase II enzyme inducers (e.g. dimethyl fumarate) that evoke a pleiotropic antioxidant response (elevation of both QR and GST) in non-neuronal cells, can reduce the toxicity of dopamine prooxidative metabolites in neural cells, leading to the suggestion that this may protect against certain neurodegenerative diseases (Duffy *et al.*, 1998; Murphy *et al.*, 1991). The very recent finding that QR (NQO1)-null mice exhibited increased toxicity to menadione compared to wild-type mice has not only solidified the evidence supporting a role for QR in protection against quinone toxicity, but has provided a model for the further study of the role of this enzyme in carcinogenesis and neurodegenerative diseases (Radjendirane *et al.*, 1998).

Antioxidant role of heme oxygenase

Sulforaphane is also a very potent inducer of the enzyme heme oxygenase-1 (HO-1) (Prester *et al.*, 1995) that catalyses the conversion of heme to bilirubin which in turn is reduced enzymatically to bilirubin. These widespread reactions are involved in the continuous breakdown of haemoglobin. Bilirubin is a very potent and versatile antioxidant, especially at low oxygen tensions such as prevail in tissues (Stocker *et al.*, 1987). Direct evidence has now been obtained that cells in which heme oxygenase-1 activity has been overexpressed by transfection of the cognate cDNA are indeed more resistant to oxygen toxicity (Lee *et al.*, 1996). It therefore seems likely that heme oxygenase-1 induction confers antioxidant protection.

Conclusions

There is ample evidence that sulforaphane is a very potent inducer of Phase II enzymes and also raises cellular glutathione levels. It is well known that Phase II enzymes play an important role in protecting cells against the toxicities of electrophiles. But we show here that some Phase II enzymes also protect cells against a wide variety of oxidative stresses, and the induction of these enzymes contributes to the mechanisms by means of which cells guard against the toxicities of reactive oxygen species and other forms of oxidative toxicity. Indirect antioxidants such as sulforaphane and other Phase II enzyme inducers are actually very efficient and rather versatile antioxidants for the following reasons: (a) unlike direct antioxidants, they are not consumed stoichiometrically while exerting antioxidant functions; (b) they have a longer duration of action and high cellular concentrations need not be maintained continuously since the induced enzymes have half-lives measured in days; (c) they support the functions of important naturally-occurring, direct-acting antioxidants such as the tocopherols and coenzymes Q; (d) they enhance the synthesis of glutathione, one of the most abundant intracellular direct antioxidants; and (d) they elevate enzymes that can cope with a wide variety of types of oxidants.

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