CHAPTER 7

Gene Regulation by Glucosinolate Hydrolysis Products from Broccoli

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CONTENTS

INTRODUCTION

Experimental and epidemiological studies suggest a correlation between increased fruit and vegetable consumption and lowering of cancer incidence. In many of these studies, the cruciferous vegetables are singled out as having a more significant beneficial effect than fruits and vegetables in general.

This suggests that cruciferous vegetables may provide some benefit not shared by all fruits and vegetables. Cruciferous vegetables, such as broccoli, brussels sprouts, and cabbage, are a relatively unique dietary source of glucosinolates, a series of plant secondary metabolites derived from modified amino acids, and including thioglucose and N-sulfate moieties, Figure 7.1. Glucosinolates have not been directly associated with anticarcinogenic activity. However, when the plant is chopped or crushed, the plant enzyme myrosinase comes into contact with the glucosinolate and

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Progoitrin	2-hydroxy 3,4-butenyl glucosinolate
Glucobrassicin	indol 3-yl methyl glucosinolate
Glucoraphanin	4-methyl sulfinyl butyl glucosinolate

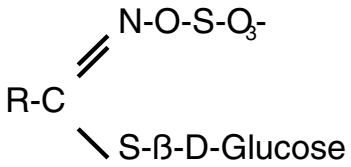

Figure 7.1 Major glucosinolates in broccoli.

hydrolyses it to release glucose. The resulting unstable thiono intermediate then rearranges, most frequently to form an isothiocyanate or a nitrile. It is the isothiocyante products of glucosinolate hydrolysis that have been associated most strongly with anticarcinogenesis, and it is their ability to trigger the upregulation of a number of host-defense genes involved in destroying chemical carcinogens that is the focus of this chapter.

ANTICARCINOGENICITY OF BROCCOLI

A recent study shows three or more servings a week of cruciferous vegetables, such as broccoli, brussels sprouts, or cabbage, can reduce risk for prostate cancer by 40%, compared with individuals only eating 1 or fewer servings per week.[1] Many more epidemiological studies suggest a role for cruciferous vegetables in prevention of cancers.[2–4] In particular, broccoli consumption has been associated with decreased incidence of cancers of the lung, colon, and prostate.[5]

In considering potential impact on the public health, we note that the Economic Research Service of the USDA reports that over the last 30 years, broccoli consumption has risen more than 5-fold, to 7.7 lb/capita, while brussels sprouts consumption remains unchanged over the same period, at 0.3 lb per capita.[6] This increased popularity of broccoli provides the means to translate basic science into improvement in the public health. Surveying a number of different varieties of cruciferous vegetables, one can identify four predominant glucosinolates; sinigrin, glucobrassicin, progoitrin and glucoraphanin.[7] The profile of the four glucosinolates is relatively similar for brussels sprouts, cabbage, cauliflower and kale, all particularly high in sinigrin, Figure 7.2. The glucosinolate sinigrin, upon hydrolysis, releases allyl isothiocyanate, which is responsible for the spicy bite in mustard or the raw core of white cabbage. Many people in the U.S. prefer the less spicy flavor of broccoli, which has little or no sinigrin. However, the total glucosinolate level is similar among broccoli and the other cruciferous vegetables, since the lack of sinigrin is balanced by a larger amount of glucoraphanin, the hydrolysis products of which are not as pungent as those of sinigrin.

There are no reported feeding studies showing protection by broccoli against chemically induced cancers in animal models. However, there is an abundant liter-

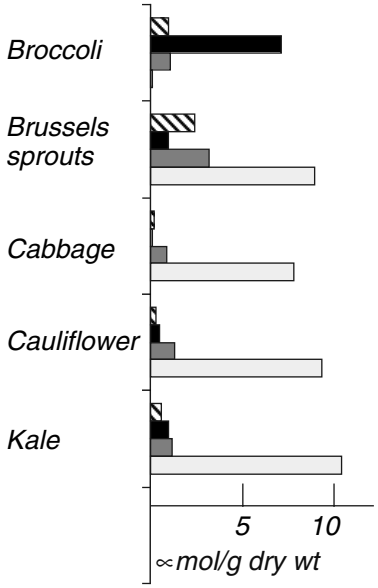

Figure 7.2 Distribution pattern of major glucosinolates in various vegetables belonging to *Brassica oleracea.* Fresh samples of the edible part of the vegetable were freeze-dried and analyzed for glucosinolate content by previously published methods.[7] Data are mean μmols glucosinolate/g dry weight vegetablel 50 varieties of broccoli, four varieties of Brussels sprouts, 6 varieties of cabbage, three varieties of cauliflower, and two varieties of kale.
Hatched bars = progoitrin; black-filled bars = glucoraphanin; grey-filled bars = glucobrassicin; open bars = sinigrin.

ature showing that diets containing other cruciferous vegetables, such as cabbage and brussels sprouts, can protect animals against many chemically induced cancers.[5] Bioactive hydrolysis products of glucosinolates were first recognized as the active components by their ability to increase the levels of detoxification enzymes in the livers of test animals, whether given in purified form, or as freeze-dried vegetables added to the diet.[8] Whereas broccoli feeding studies showing prevention of cancer have not been reported, many published studies show that purified sulforaphane, the isothiocyanate from glucoraphanin, can prevent chemically-induced cancer.[9] Data show that dietary broccoli, like its components and like other crucifers, is able to upregulate detoxification enzymes in rodents,[10] suggesting that broccoli is worthy of evaluation as a cancer preventative food. Furthermore, because it is a relatively popular food, broccoli is in a position to have a significant impact on the health of the public. There is a need for animal feeding studies to determine the efficacy of broccoli against chemical-induced cancers.

Glucobrassicin, progoitrin, and glucoraphanin are the three major glucosinolates found in broccoli, although the exact amount of each glucosinolate varies substantially across varieties and with growing and processing conditions.[11] Upon hydrolysis, these glucosinolates are converted to their biologically active hydrolysis products. Hydrolysis requires myrosinase (EC 3.2.3.1), a thioglucosidase that is absent

from mammalian tissue, but is present in cruciferous plant tissue, although compartmentally separated from the glucosinolate substrates. When the plant is chopped or crushed, the enzyme and substrate come together and hydrolysis occurs. Although little detail is yet known, it appears that the microflora of the gut are also able to support glucoinolate hydrolysis, leading to the understanding that even if cooked broccoli no longer contains an active myrosinase, that glucosinolate hydrolysis might occur in the colon.[12] Each of the three major glucosinolates in broccoli has bioactive hydrolysis products, Table 7.1.[13] Sulforaphane, an hydrolysis product from glucoraphanin, is present in the highest concentration, Table 7.1.

Phase I and Phase II Detoxification EnzymesDuring the late 1950s, when detoxification enzymes were first identified, they were divided into two groups, phase I and phase II, to differentiate between those that chemically alter the substrate through oxidation, reduction, or hydrolysis, and those that conjugate the substrate to an endogenous molecule, such as glucuronide.[14] Since that time, many more xenobiotic metabolizing enzymes have been identified and the categorization of phase II enzymes has broadened to include quinone reductase, epoxide hydrolase, γ-glutamyl-cysteine synthetase, and others that act to protect the cell from toxic or carcinogenic insult. In fact, the upregulation of quinone reductase has become a common biomarker for potential anticarcinogenic activity of natural products. In a cell culture system, quinone reductase can be rapidly measured and responds to many compounds that induce phase II enzymes.[15] In addition, induction of quinone reductase in cell culture appears to be a reliable predictor of induction in rodent organs. Furthermore, the induction of quinone reductase coordinates with the induction of many other detoxification enzymes, including glutathione-S-transferase, UDP-glucoronosyl transferase, and γ-glutamyl-cysteine synthetase.[16] Sulforaphane has been shown to be a potent upregulator of quinone reductase in cell culture, and this activity has led to an interest in developing diets high in sulforaphane-containing foods as cancer preventative diets.[17–19]

MONOFUNCTIONAL AND BIFUNCTIONAL INDUCTION

The upregulation of detoxification enzymes by glucosinolate hydrolysis products has been classified based on whether a compound activates enzymes from one or both of the phase I and phase II groups of detoxification enzymes. Monofunctional inducers upregulate a number of phase II enzymes; bifunctional inducers cause upregulation of phase I as well as phase II enzymes.[20] Sulforaphane is considered

Table 7.1 Concentration ranges of the major glucosinolates in 50 varieties of broccoli and their bioactive hydrolysis products.[7]

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	Concentration in Broccoli (μmol/g dry wt)	Bioactive Hydrolysis Product	Reference
Glucobrassicin	0.1-2.8	Indole-3-carbinol	24
Progoitrin	0.1-7.9	Crambene	38
Glucoraphanin	0.8-21.7	Sulforaphane	13

a highly potent monofunctional inducer, upregulating a battery of enzymes, including quinone reductase, γ-glutamyl-cysteine synthetase, aldo-keto-reductase and a number of glutathione-S-transferase isoenzymes.[21] Bifunctional inducers upregulate a few phase I cytochrome P450 monooxygenases, as well as a battery of phase II enzymes, overlapping, but not identical to those upregulated by monofunctional inducers. Metabolism of some bifunctional inducers by cytochrome P450 results in products that are monofunctional inducers and thereby upregulating the full battery of phase II enzymes.[20]

THE XENOBIOTIC RESPONSE ELEMENT AND INDOLE-3-CARBINOL

The genes for CYP1A 1/2, CYP1B1 and several phase II enzymes contain a 5' regulatory sequence termed the xenobiotic response element (XRE), also termed the aryl hydrocarbon response element or dioxin response element, Table 7.2.[22] The core XRE sequence is GCGTC, although flanking sequences that vary from gene to gene are also important for activation of transcription. This regulatory region is activated through binding of a ligand-receptor complex. When a ligand enters the cell, it binds to a cytosolic receptor, the aryl hydrocarbon receptor (AhR). The AhR is a member of the per-arnt-sim family of basic-helix-loop-helix transcription factors. Under basal conditions, the AhR is found in the cytoplasm associated with heat shock protein 90. Ligand binding releases AhR from the heat shock protein, and AhR translocates to the nucleus. In the nucleus, ligand-bound AhR forms a heterodimeric complex with a transcription factor termed the aryl hydrocarbon nuclear translocator (ARNT). This complex subsequently binds to the XRE, increasing transcription of the target gene. The transcription factor ARNT is known to dimerize with at least one other transcription factor, hypoxia inducible factor 1 β, and the resulting dimer binds to the hypoxia-response element found on a separate but overlapping battery of host-defense genes to those regulated by the XRE.[23]

Glucobrassicin is a glucosinolate derived from tryptophan, and when it undergoes hydrolysis, the major product is indole-3-carbinol. Diets containing indole-3-

Table 7.2 A partial list of genes containing an antioxidant response element (ARE) or a xenobiotic response element (XRE). More complete lists are reviewed in 60, 61.

ARE	XRE
NADPH:Quinone reductase	NADPH:Quinone reductase
Glutathione-S-transferase-Ya	Glutathione-S-transferase-Ya
Glutathione-S-transferase-A2	Glucuronosyl Transferase
Glutathione-S-transferase Pi	Cytochrome P450 1A1/2
γ-Glutamylcysteine synthetase (H & L)	Cytochrome P450 1B1
Glucuronosyl Transferase	Cytochrome P450 C
Heme oxygenase-1	Superoxide dismutase
Metallothionein	Xanthine oxidase/xanthine dehydrogenase,
Apolipoprotein-A1	
Ferritin L	
Thioredoxin	
Thioredoxin reductase	

carbinol cause induction of detoxification enzymes via the XRE, producing bifunctional induction. Although only a weak ligand for the AhR itself, when subjected to the acidic environment of the stomach, indole-3-carbinol forms multiple acid condensation products.[24] These I3C acid condensates are capable of more potently ligating AhR and activating transcription through the XRE.[22] Two products, diindolyl methane and indole-3-carbazole, have been isolated and studied individually, and have been found to be agonists of the XRE on CYP1A genes.[25,26]

A controversy exists in the literature as to whether dietary bifunctional inducers can be considered healthy, based on the idea that P4501A, which is upregulated by bifunctional inducers via the XRE, is able to activate many polycyclic procarcinogens. However, it appears that the bifunctional inducers like the indoles and some other dietary bioactive components may inhibit XRE-dependent pathways in carcinogenesis, possibly by acting competitively as antagonists to alternative XRE agonists,[25,26] possibly by disrupting cross talk between the XRE and the estrogen response element. This latter idea is supported by the successful clinical trials on indole-3-carbinol in fighting breast cancer, which have led to the development of other potentially therapeutic XRE antagonists, also called selective AhR modulators.[27]

THE ANTIOXIDANT RESPONSE ELEMENT, SULFORAPHANE AND CRAMBENE

Monofunctional inducers, which upregulate the phase II detoxification enzymes, have been found to be electrophiles, and transcriptional activation by these inducers has been traced to a cis-acting transcriptional enhancer termed the antioxidant response element (ARE) in rats and humans, and the electrophile response element (EpRE) in mice.[28,29] The molecular mechanism of ARE-dependent upregulation of phase II enzymes is not as completely understood as the mechanism of XRE-dependent bifunctional upregulation of enzymes. The ARE core sequence 5'-TGACnnnGC-3' has been identified in the 5' flanking sequence of several genes, including some rat, mouse, and human glutathione-S-transferase isoenzymes and quinone reductase; Table 7.2. Most recently the ARE has been identified in the regulatory region of thioredoxin and thioredoxin reductase genes.[30,31] These genes play an important role in regulating the sulfhydryl redox status of the cell.

When glucoraphanin is hydrolyzed by chopping broccoli in water, two products are formed, sulforaphane and sulforaphane nitrile.[32] In recent years, Talalay and colleagues have reported numerous studies on sulforaphane bioactivity, and have shown that it is potent at upregulating the detoxification enzyme quinone reductase in mouse hepatoma cell cultures, and have suggested that anticarcinogenesis is related to this upregulation of detoxification enzymes.[13,17] They have shown that this activity is ARE-dependent.[33] Induction of detoxification enzymes is not the sole activity of bioactive hydrolysis products from broccoli. For example, in addition to upregulating phase II detoxification enzymes, sulforaphane is reported to inhibit mRNA synthesis and enzyme levels of several cytochromes P450, including CYP 1A1, a major cytochrome P450 involved in the bioactivation of procarcinogens.[21]

There is also considerable evidence showing that sulforaphane interacts with the regulation of the cell cycle to promote apoptosis.[34]

There are several compounds in broccoli unrelated to glucosinolates, such as dithiothiones and polyphenolics, that are capable of mediating ARE-dependent regulation of the phase II battery of detoxification genes (Table 7.2). Furthermore, whereas sulforaphane is present in substantial quantity in broccoli, it is a minor component in other crucifers. Other glucosinolate hydrolysis products, such as benzyl isothiocyanate, phenethylisothiocyanate, and allyl isothiocyanate, the predominant glucosinolate hydrolysis products of gardencress, watercress, and mustard, respectively, are not found in high amounts in broccoli. These compounds, like sulforaphane, upregulate quinone reductase and the battery of ARE/Nrf2 regulated genes.[35]

Another major glucosinolate of broccoli and other crucifers is progoitrin. Progoitrin hydrolysis products have been far less studied as potential anticarcinogens. This may be due to the toxicity seen when progoitrin-containing plant products were used as the sole protein source in animal feed.[36] *Crambe abysinnica* seed meal, when used as the sole source of protein in animal feeds, produces pancreatic atrophy due to the high level of nitrile in the diet.[36] The amount necessary to cause these effects is far greater than that found in any normal American diet. Chopping fresh broccoli produces the nitrile, termed cyanohydroxybutene, or crambene. Interestingly, low dietary levels of crambene administered orally to rats, had a similar effect to that of sulforaphane, causing an increase in hepatic quinone reductase and glutathione-S-transferase, without increasing P4501A activity.[37] Even the effective dose range for a single dose, 0.5–1.5 mmol/kg BW, was very similar to the effective oral dose range for sulforaphane.[32,37,38] It is interesting to note that crambene, unlike the other bioactive hydrolysis products, is a nitrile. Sulforaphane nitrile is essentially without activity.[32] One possible reason for this discrepancy is that crambene may rearrange to form an α, β-unsaturated ketone, an electrophile.

Like sulforaphane, crambene triggers a response at the ARE, but not the XRE in *in vitro* reporter gene studies (Nho, personal communication). However, in contrast to the animal studies, in cell culture the dose required to cause a doubling of ARE reporter gene activity in human hepatoma cells is many times greater than the dose of sulforaphane required; 100 μM crambene compared with 0.6 μM sulforaphane. This low potency is consistent with the effect of crambene on quinone reductase enzyme induction in cell culture.[39] One possible reason for this is that conversion of the nitrile to a metabolite, such as the α, β- unsaturated ketone, is required for bioactivity, and that this may occur at a very slow rate in the hepatocyte. Alternatively, there may be a substantial difference in cellular accumulation between sulforaphane and crambene, since sulforaphane has been found to accumulate intracellularly in cell culture, reaching millimolar ranges following micromolar exposure.[40]

ACTIVATION OF TRANSCRIPTION FACTORS ASSOCIATED WITH THE ARE

Studies using knockout mice implicate the basic leucine zipper NF-E2 related factor (Nrf2) as part of the transcriptional complex directly involved in mediating ARE-

dependent transcriptional regulation of mouse glutathione-S-transferase and quinone reductase.[41–43] A recent publication utilized Nrf2 knockout mice and oligonucleotide array technology to identify genes regulated by Nrf2 for basal and sulforaphane-induced expression. The authors identified several previously unreported sulforaphane inducible genes under the control of Nrf2, including those involved in the inflammatory response, xenobiotic detoxification, and NADPH generation.[44] Nrf2 forms heterodimers with small Maf proteins and binds to the cognate ARE sequence. The exact Maf binding partner for Nrf2 may vary with stimulus and/or tissue, but MafG has been implicated as an activator and MafK as a repressor of transcriptional activation by Nrf2.[45] It has been suggested that in the cytoplasm Nrf2 may be largely bound to a protein, Keap1, which is anchored to the actin cytoskeleton.[46] Inducing agents like sulforaphane may oxidize critical sulfydryl groups of Keap1, disrupting the Keap 1-Nrf2 complex and permitting Nrf2 to migrate to the nucleus, where it can interact with the 5'-upstream regulatory ARE of phase II genes and increase their transcription.[46]

Other post-translational mechanisms may also regulate the activity of Nrf2. A role for protein kinase C and phosphatidyl inosital 3-kinase in this sequence of events may be to phosphorylate Nrf2 in response to inducer treatment and this phosphorylation may drive release of the Nrf2 from Keap1, causing migration of Nrf2 to the nucleus and subsequent activation of ARE driven gene trascription.[47–50] Alternatively, ERK2 and p38, members of the mitogen-activated protein kinase family, have been implicated as positive and negative regulators, respectively, in ARE-mediated induction of phase II detoxification enzymes.[51] Additionally, current studies suggest phosphorylation of Nrf2 by a protein kinase associated with the MAPK/ERK signaling cascade may lead to an increase in Nrf2 stability and transactivational activity.[52–54] While the exact mechanism remains undetermined, the possibility exists that all or several of these mechanisms function in the release of Nrf2 from Keap1.

Recently, it has been found that, in addition to its detoxification function and its function as a biomarker for upregulation of other phase II enzymes, upregulation of quinone reductase by monofunctional inducers may play a role in the stabilization of p53, the protein product of a tumor suppressor gene, which induces growth arrest and apoptosis.[55] Sulforaphane has also been shown to mediate growth arrest and induce cell cycle arrest and apoptosis in many cancer cell lines, including those of human prostate, colon, and T-cell leukemia origin.[34,56,57] The exact mechanisms, and whether all the bioactivities of sulforaphane involve the ARE, are not yet understood.

QUINONE REDUCTASE IS REGULATED BY BOTH AN XRE AND AN ARE

The promoter regions of glutathione-S-transferase Ya and quinone reductase have been shown to possess both an ARE and an XRE.[58,59] A survey of additional genes, as they are recognized to be regulated by the ARE/Nrf2 system[60] or the XRE/AhR system,[61] will no doubt identify more genes that are regulated by both pathways. Interestingly, when rats are exposed through their diets to a mixture of the monofunctional inducer crambene and the bifunctional inducer precursor indole-3-carbinol, substantial synergism in phase II enzyme induction was seen.[39] Individual

doses of crambene (50 mg/kg BW) and indole-3-carbinol (56 mg/kg BW) daily for 5 days each caused an approximate doubling of quinone reductase activity in rat liver. In contrast, quinone reductase levels in the livers of rats that were given both I3C and crambene were 4–5 fold greater than those of untreated rats.[39] These data support the possibility that a mixture of glucosinolate hydrolysis products, as found naturally in broccoli, may be more potent than the individual components.

SUMMARY

Epidemiolgical and animal studies suggest that a diet rich in crucifers protects against a number of cancers. These vegetables contain glucosinolates, which are hydrolyzed by a plant enzyme to form bioactive products able to upregulate detoxification enzymes. Whether or not this upregulation is causative in cancer prevention, the upregulation of detoxification enzymes serves as an excellent biomarker of exposure and effective dose of crucifers. Sulforaphane and crambene, two glucosinolate hydrolysis products from broccoli, are monofunctional indcers, upregulating phase II enzymes through an antioxidant response element (ARE)-dependent pathway. Brassica vegetables, including broccoli, all contain glucobrassicin, a glucosinolate that, upon hydrolysis, releases indole-3-carbinol. Passing through the acid stomach, indole-3-carbinol forms acid condensation products, ligands for the Ah receptor, causing bifunction induction: upregulation of both phase I and phase II detoxification enzymes through a xenobioitc response element (XRE)-dependent pathway. When rats were fed a mixture of crambene and indole-3-carbinol, both of which are present in broccoli, upregulation of the phase II detoxification enzyme quinone reductase in liver was not just additive, but synergistic. Thus the metabolites of glucosinolates in broccoli are capable of serving as both mono- and bifunctional inducers, and may have greater effects together than as isolated components. These data support the finding of epidemiological studies, that a diet that includes broccoli may slow or prevent cancer. Further research into the underlying genetic regulation of detoxification enzymes will aid in determining how to optimize the health benefits of broccoli and other cruciferous vegetables.

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Is this a book? If so, shouldn't the title be in initial capitals and what is the city for Chapman & Hall?

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