The Antioxidant Responsive Element (ARE) May Explain the Protective Effects of Cruciferous Vegetables on Cancer

Research supports the hypothesis that one’s diet has a great impact on his or her risk of cancer. Many studies have found that increased fruit and vegetable intake decreases the risk of cancer. Cruciferous vegetables such as broccoli and cauliflower seem to be especially protective against cancer. Most studies show that phytochemicals in crucifers up-regulate many detoxification enzyme systems in the animal that consumes them. Recent reports of the molecular events involved in the activation of a gene promoter called the antioxidant responsive element have begun to provide clues as to how a single substance may induce a battery of many genes.

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Epidemiologic and investigational studies support the hypothesis that increased fruit and vegetable intake reduces the risk of cancer. Vegetables of the genus Cruciferae are especially protective; epidemiologic studies have demonstrated inverse associations between cruciferous vegetable intake and the incidence of lung, pancreas, bladder, prostate, thyroid, skin, stomach, and colon cancer. Prospective dietary assessment of men with prostate cancer found that increasing crucifer intake from one to three or more servings per week decreased apparent risk by 41%. A cohort study concluded that intake of crucifers, but not other fruits or vegetables, is inversely associated with the risk of bladder cancer. A review of seven cohort and 87 case-control studies reported that two-thirds of all case-control studies found significant inverse associations between total crucifer intake and cancer risk. Inverse associations between intake and cancer risk were especially strong for the cruciferous vegetables cabbage, broccoli, cauliflower, and Brussels sprouts. Cohort studies reported inverse associations between intakes of cabbage, cauliflower, or broccoli and risk for lung cancer, between total crucifer intake and risk for stomach cancer, and between broccoli intake and risk for all cancers.

The chemoprotective mechanism of crucifers is not completely understood, but many animal and human studies have reported that factors in these foods modulate Phase-I and Phase-II detoxification enzyme levels and activities. Most investigators agree that the primary chemoprotective component of crucifers is the glucosinolate family of secondary plant compounds. There are more than 120 different known glucosinolates. They all have three common structural features: a β-D-thioglucose group, a sulfonated oxime group, and a variable sidechain derived from an aliphatic, aromatic, or heterocyclic modified amino acid (Figure 1). They are non-volatile, hydrophilic compounds that occur as unstable anions because of the highly acidic sulfate group. Glucosinolates are generally sequestered as potassium salts in the plant vacuoles.

Intact glucosinolates do not have biologic activity. When they are consumed and masticated, however, an enzyme (myrosinase) released from plant cells hydrolyzes glucosinolates molecules to active moieties such as sulforaphane and phenyl isothiocyanate. When absorbed from the diet these compounds strongly induct enzymes such as quinone reductase, epoxide hydrolase, and dihydriodiol dehydrogenase. Investigators have long noted that a single dietary factor may induce a battery of Phase-II enzymes.

Research has begun to unravel the molecular events that allow this coordinated response. Reports in 1991 first described a transcriptional regulatory element for glutathione-S-transferase A1 and quinone oxidoreductase. The element, called the antioxidant response element (ARE), responded to hydroquinones, catechols, isothiocyanates, peroxides, arsenicals, heavy metal salts, and t-butyl hydroquinone (tBHQ). This element was reported to have the following consensus sequence:

$$5’\gamma\alpha\ TGA \gamma\alpha\ NNN\ GC \gamma\alpha3’$$

and it has been found in the promoter region of numerous Phase-I and Phase-II detoxification enzymes.

Most inducible detoxification genes that contain an ARE transduce the signal through nuclear factor erythroid 2 p45-related factor 2 (Nrf2). In the nucleus, Nrf2 binds in conjunction with several small Maf proteins to the ARE in the promoter region, thus triggering transcription (Figure 2). Multiple enzymes are trig-
Figure 1. A generalized structure of a glucosinolate molecule includes (a) a β-thioglucose group, (b) a sulfate group, and (c) an R group. The R group is a modified aliphatic, aromatic, or heteroaromatic amino acid.

generated simultaneously by this mechanism; most of the induced enzymes are related to Phase-I or Phase-II detoxification pathways. This pathway appears to be a primary mechanism by which chemopreventive compounds from crucifers, such as sulforaphane in broccoli, exert their effects.5

The precise series of events that result in activation of a gene transcription by the ARE is not completely elucidated, but a picture is emerging. In the cytoplasm, the Nrf2 protein is tethered to a protein called KEAP1.17 Activation of the ARE requires release of Nrf2 from KEAP1 and translocation of Nrf2 to the nucleus. Chemical signals that can cause this translocation are called bifunctional inducers (e.g., indoles, B-napthoflavone) if they are able to activate both the ARE and the xenobiotic response agent (XRE), or monofunctional inducers (e.g., sulforaphane, ethoxyquin) if they only activate the ARE.18 Apparently, these inducers act to modify active sulfhydryls, although the precise location of the active groups, as well as which proteins are modified, is not completely known. These signals may be transduced through other proteins including mitogen-activated protein (MAP) kinases,19,20 extracellular regulated kinase (ERK), Jun N-terminal kinase (JNK), and protein kinase C.14,21,22 Phosphorylation of Nrf2 is involved, although it is unknown whether this event is essential for release and translocation.23 Nrf2 production is under transcriptional control and apparently acts as its own promoter. Thus, initial release of Nrf2 from KEAP1 leads to a rapid increase in intracellular Nrf2 concentration, resulting in saturation of cytosol KEAP1 and translocation of free Nrf2 to the nucleus.

In the nucleus, Nrf2 binds to the ARE in association with one of the Maf family of proteins.16 The exact role of Maf is unknown, but Nrf2 does appear to dimerize with Maf. Recent reports suggest that Maf proteins are actually transcriptional repressors, and binding of Nrf2 to Maf removes repressor activity.25 Kinases may phosphorylate Maf proteins and induce transcriptional activity of ARE-dependent genes. Some of these genes may use different activation pathways, which accounts for the observation that ARE-dependent proteins are affected to different degrees by similar molecular signals.

Genes containing active ARE sequences in their promoters share several similarities. First, transcription is up-regulated by Phase-II enzyme inducers; second, the ARE appears to up-regulate basal expression of the enzyme.26 The ARE was first characterized in the NADPH quinone oxidoreductase gene.27,28 This enzyme catalyzes the reduction of quinones and prevents redox cycling and oxidative stress. Glutathione (GSH) is a primary antioxidant, and a number of GSH-associated genes contain AREs in their promoters. Among this group is the gene for the rate-limiting enzyme in GSH production, γ-glutamylcysteine synthetase. Another member of this group codes for glutathione transferases, enzymes that catalyze the conjugation of glutathione to hydrophobic toxins or xenobiotics, thus facilitating their excretion. Additional ARE-containing GSH transferase genes include GSTA1, GSTA2, GSTA3, GSTM1, GSTM2, GSTM3, and GSTM4.29

Several approaches have been used to determine downstream targets of the Nrf2 protein. Chanas et al.29 used conventional biochemical techniques such as enzyme activities and Western blotting in conjunction with

![Figure 2](image)
real-time PCR to study the effects of knocking out Nrf2on multiple Phase-II enzymes in mice. Knockout mice exhibited increased sensitivity to 1-chloro-2,4-di-nitrobenzene, acetaminophen, BHT, ethoxyquin, and coumarin. Also noted were decreases in the activity of multiple GSH-S-transferase enzymes including GSTA1, GSTA2, GSTM1, GSTM3, GSTM4, and glutamate cysteine ligase. Real-time PCR demonstrated that the loss in activity was associated with decreases in mRNA of up to 10% of wild-type expression. These knockout mice showed increased sensitivity to the genotoxic effects of diesel fumes and greater accumulation of hepatic aflatoxin B1-DNA adducts, and developed more forestomach tumors. However, there must be other factors involved in the Nrf2/ARE model of gene induction, as enzymes also decreased in constitutive activity and such a decrease was independent of decreases in induced expression.

Thummulappa et al. used oligonucleotide microarrays in Nrf2 knockout and wild-type mice to search for genes regulated by Nrf2. Using this approach, they found that Nrf2-regulated GST genes included, in addition to the ones listed above, GSTμ, GSTμ1, GST8.7, GST 9.3, GSTμ3, GST YC2, and GST Ya. In addition to genes in the glutathione conjugation pathway, Nrf2 regulated multiple genes that affect glucuronidation detoxification pathways. Glucuronidation requires UDP-glucuronic acid as a cofactor and Nrf2 regulates many genes in the UDP-glucose pathway, including transketolase, transaldolase, fructose bisphosphatase, phosphoglucomutase, and UDP-glucose dehydrogenase. Nrf2 also regulated gene expression of oxireductases (e.g., carbonyl reductase, aldo-keto reductase), hydrolytic enzymes (e.g., carboxylesterase, peptidases), and oxidative enzymes (e.g., monoamine oxidase, amino oxidase).

Mammals deficient in the essential trace nutrient selenium (Se) often develop conditions that resemble oxidative damage to tissue (e.g., nutritional muscular dystrophy, pancreatic atrophy, exudative diathesis). Selenium supplementation prevents these conditions; consequently Se is often called an antioxidant. There is some molecular evidence for this as two different classes of proteins that require Se for activity have antioxidative capability in vitro. Selenium is needed for activity of four different glutathione peroxidase (GPx) isoforms, and GPx reduces peroxide radicals with the concomitant oxidation of GSH. GSH reductase then reduces GSH allowing the cycle to continue. GSH reductase expression has been demonstrated to be Nrf2 dependent. Basal expression of GPx is dependent on Nrf2, but it is not inducible with sulforaphane. GPx utilizes NADPH as the ultimate source of reducing equivalents, again demonstrating the regulation of many NADPH-dependent proteins by Nrf2.

Thioredoxin reductase (TR) is a Se-dependent protein and the primary enzyme used for the intracellular reduction of thioredoxin. The intracellular concentration of thioredoxin is sufficiently high to make it a major determinant of intracellular oxidative status. Because thioredoxin is used as a cellular growth signal, modulation of thioredoxinred-to-thioredoxinox ratios may have important implications for cancer.

Selenoprotein production classically has been considered to be controlled at the translational level by the existence of 3' stem loop elements and elongation factors that control mRNA stability dependent on the availability of Se. Although the control of expression of any selenoprotein by an ARE has not been demonstrated until recently, there was evidence for such a possibility. Thioredoxin and thioredoxin reductase gene expression are both up-regulated in oxidative environments and sulforaphane was demonstrated to induce TR activity and protein expression. Li et al. reported that tBHQ upregulated TR mRNA expression, consistent with the existence of a putative ARE sequence in the promoter region.

If thioredoxin reductase is regulated by an ARE, it opens the question of whether TR has any beneficial anticancer effects. Selenium supplementation reduces cancer risk, but reduced thioredoxin may actually turn on many pro-growth factors and signals. The antioxidant function of TR and the anticancer function of Se therefore may not be parallel mechanisms. Selenium may reduce cancer by inducing apoptosis, where oxidative stress may induce DNA destruction. Several reports show that Se induces apoptosis through the caspase pathway, and a recent report suggests that Nrf2 may be a substrate for caspase. It is therefore intriguing to theorize that Se supplementation may actually destroy Nrf2, which is the signal to turn on TR and may paradoxically increase oxidative tension, thus inducing apoptosis.

An estimated 40% of all human cancers are related to diet. The relationship of diet to cancer is not necessarily because of the inclusion of carcinogens in our diet, but may be a consequence of the exclusion of anticarcinogens from our diet. Cruciferous vegetables seem to be especially protective against cancer, which could be mediated by the ARE-dependent induction of a battery of protective enzymes.

Cancer is a complex molecular event; control of cancer-causing events is equally complicated. Recent advances in understanding the role of the ARE in coo-
ordinating the response of detoxifying enzymes are crucial
to understanding how these various defenses allow a
coordinated response to oxidative stress that may initiate
cancers. Progress in this area of research will certainly be
facilitated by new technologies such as gene arrays and
matrix-assisted laser desorption time-of-flight mass spec-
trometry (MALDI-TOF) that will allow simultaneous
measurement of activation of thousands of genes and
their multiple gene products.

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