Potent Induction of Phase 2 Enzymes in Human Prostate Cells by Sulforaphane[1]

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Abstract

Two population-based, case-control studies have documented reduced risk of prostate cancer in men who consume cruciferous vegetables. Cruciferae contain high levels of the isothiocyanate sulforaphane. Sulforaphane is known to bolster the defenses of cells against carcinogens through up-regulation of enzymes of carcinogen defense (phase 2 enzymes). Prostate cancer is characterized by an early and near universal loss of expression of the phase 2 enzyme glutathione *S*-transferase (GST)-π. We tested whether sulforaphane may act in prostatic cells by increasing phase 2 enzyme expression. The human prostate cancer cell lines LNCaP, MDA PCa 2a, MDA PCa 2b, PC-3, and TSU-Pr1 were treated with 0.1–15 μM sulforaphane *in vitro*. LNCaP was also treated with an aqueous extract of broccoli sprouts. Quinone reductase enzymatic activity, a surrogate of global phase 2 enzyme activity, was assayed by the menadione-coupled reduction of tetrazolium dye. Expression of NQO-1, GST-α, γ-glutamylcysteine synthetase-heavy and -light chains, and microsomal GST was assessed by Northern blot analysis. Sulforaphane and broccoli sprout extract potently induce quinone reductase activity in cultured prostate cells, and this induction appears to be mediated by increased transcription of the *NQO-1* gene. Sulforaphane also induces expression of γ-glutamylcysteine synthetase light subunit but not the heavy subunit, and this induction is associated with moderate increases in intracellular glutathione levels. Microsomal and α-class glutathione transferases were also induced transcriptionally. Sulforaphane induces phase 2 enzyme expression and activity significantly in human prostatic cells. This induction is accompanied by, but not because of, increased intracellular glutathione synthesis. Our findings may help explain the observed inverse correlation between consumption of cruciferae and prostate cancer risk.

Introduction

In the United States, prostate cancer is the most prevalent noncutaneous malignancy and the second leading cause of male cancer death (1). Prostate cancer has a long latency and estimates are that 10 to 12 years are required before prostate cancer becomes clinically manifest (2). Sakr *et al.* (3) have identified prostatic intraepithelial neoplasia, a prostate cancer precursor lesion, in 10% of men by 30 years of age and small foci of frank carcinoma in more than 10% of men before age 40. Prostate cancer is usually diagnosed clinically in the sixth and seventh decades of life, allowing a large window of opportunity for interventions to prevent or slow the progression of the disease.

The most common molecular genetic change in prostate cancer involves silencing of expression of GSTP1[3], a critical enzyme of carcinogen defense, through methylation of deoxycytidine residues in "CG islands" in the 5′ regulatory region of the *GSTP1* gene (4, 5). This change appears to occur early in prostate carcinogenesis, because it is found in virtually all of the cases of high-grade prostatic intraepithelial neoplasia and is a near universal finding in clinical prostate cancers regardless of grade or stage (6). The glutathione transferases protect cells against carcinogenic oxidative stress by conjugation of electrophiles to reduced glutathione. Up-regulation of phase 2 enzymes, including the glutathione transferases, can protect cells against carcinogens and has been documented to prevent carcinogen-induced tumors in a variety of animal models (7, 8).

Early loss of GSTP1 may predispose prostatic cells to the damaging effects of endogenous or exogenous carcinogens and may contribute to carcinogenesis. Two recent epidemiological studies (9, 10) suggest that such a preventive intervention may be possible. Both studies have found an association between decreased prostate cancer risk and high consumption of cruciferous vegetables. Cruciferae are known to contain high levels of the isothiocyanate sulforaphane, the most potent monofunctional phase 2 enzyme-inducing agent thus far identified (11).

Phase 2-inducing agents have been reported to increase phase 2 enzyme activity through increased transcription at phase 2 enzyme gene loci (12). A putative ARE in the regulatory regions of these genes is thought to be responsible for enhanced expression of many of these genes (13–19); *e.g.*, sulforaphane will increase expression of a reporter gene downstream of promoter constructs containing the ARE consensus sequence and a minimal promoter. Levels of reporter gene induction parallel endogenous QR induction in the same cell line (20).

Our hypothesis is that induction of phase 2 enzymes by sulforaphane may help explain the association between high consumption of cruciferae and decreased prostate cancer risk.

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[3] The abbreviations used are: GST, glutathione-*S*-transferase; ARE, antioxidant response element; GSH, reduced glutathione; QR, quinone reductase; γ-GCS, γ-glutamylcysteine synthetase; γ-GCS-L, γ-GCS-light chain; NAC, *N*-acetyl cysteine.

Because prostate cancer lacks expression of GSTP1, induction of other phase 2 enzymes by sulforaphane may offer a mechanistically based prostate cancer-preventive strategy. Because little is known about phase 2 enzyme expression, regulation, or activity in prostatic epithelial cells, we evaluated the effect of sulforaphane on the androgen-sensitive prostate cancer cell line LNCaP, three androgen-insensitive cell lines (PC-3, TSU-Pr1, and DU-145), and a normal prostate epithelial cell strain.

Materials and Methods

Cell Culture. LNCaP were obtained from the American Type Culture Collection and grown in RPMI 1640 with L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). PC3 and Tsu-Pr1 were a gift from William G. Nelson (Johns Hopkins University, Baltimore, MD) and grown in the same medium. MDA PCa 2a and MDA PCa 2b were kindly provided by Nora Navonne (M. D. Anderson Cancer Center, Houston, Texas) and were grown in HPC1 (BRFF) supplemented with 20% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (21).

Reagents. L-sulforaphane was purchased from LKT Laboratories (St. Paul, MN). All of the remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Broccoli sprouts were grown from seed on sterile agar and aqueous extracts prepared as described (22). Two separate batches of organic broccoli sprouts purchased from a local supermarket exhibited nearly identical inducer potency to those raised in the laboratory and were therefore used for subsequent experiments.

Northern Blot Analysis. Cells were harvested at approximately 70% confluency, and mRNA was isolated using Oligotex Direct mRNA isolation kit (Qiagen). For each lane, 6 μg of polyadenylate+ mRNA were electrophoresed through a 1% agarose gel and transferred to Brightstar-Plus nylon membrane (Ambion) using the Stratagene Posiblot pressure blotter and pressure control station (Stratagene). The RNA was cross-linked to the membrane by exposure to 125 mJoules of UV light in GS Gene Linker (Bio-Rad). cDNA probes were labeled with either [^{32}P]dCTP using the Nick Translation System (Promega) or psoralen-biotin using the BrightStar Psoralen-Biotin Kit (Ambion). Hybridizations were performed at 50°C in a buffer containing 6 × saline-sodium phosphate-EDTA, 5 × Denhardt's Reagent, 6% SDS, 25 μg/ml salmon testes DNA, and 50% formamide. Washes were performed at 55°C with 1 × SSC and 0.1% SSC. Northern blots hybridized with [^{32}P]dCTP-labeled probes were exposed to a Molecular Dynamics Phosphorimager screen and scanned ImageQuant software. Northern blots hybridized with psoralen-biotin-labeled probes were processed using the BrightStar Detection kit (Ambion) according to the recommended instructions. All of the images were analyzed using ImageQuant software.

Determination of Enzyme Activity in Cell Culture. LNCaP, MDA PCa 2a, and MDA PCa 2b were grown in 96-well plates at a density of 8×10^4 cells/ml. PC3 and Tsu-Pr1 were grown in 96-well plates at a density of 4×10^4 cells/ml. After 20 h of incubation, cells were treated with L-sulforaphane dissolved in DMSO (LKT Laboratories) at the indicated concentrations. Control wells were treated with the corresponding concentration of DMSO. QR activity was assessed by the menadione-coupled reduction of tetrazolium dye as modified from Prochaska *et al.* (23, 24). After 48 h of treatment with L-sulforaphane, media was gently aspirated and cells were lysed by incubation at 37°C with 50 μl of 0.08% digitonin and 2 mM EDTA (pH 7.8) with gentle agitation. While the cells were incubating, a stock solution was prepared by combining 16.7 mg of BSA, 7.5 mg of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, 0.6 mg of NADP, 1.25 ml of 0.5 M Tris-HCl (pH 7.4), 166.7 μl of 1.5% Tween 20, 166.7 μl of 150 mM glucose 6-phosphate, 16.7 μl of 7.5 mM FAD, 50 units of yeast glucose 6-phosphate dehydrogenase, and distilled water to a final volume of 25 ml for each plate to be assayed. Immediately before use, 25 μl of 50 mM menadione dissolved in acetonitrile was added to the stock solution. After 30-min incubation, 200 μl of the complete stock solution was added to each well. After 5 min, optical absorbance at 610 nm was determined in a LabSystems Multiscan Ascent microplate reader. GST activity was determined by reduction of CDNB in accord with methods described by Habig *et al.* (25).

Toxicity of L-sulforaphane was assessed in parallel plates treated identically to those used in assays for QR activity. Cytotoxicities were monitored by the LIVE/DEAD fluorescent assay (Molecular Probes) according to the suggested protocol.

QR activity, in arbitrary units, was calculated automatically from the mean activity for all of the three wells at each concentration. Activity was corrected for toxicity at each concentration as described by Prochaska *et al.* (23, 24). Inducer potency is expressed as the ratio of corrected QR activity for treated cells to corrected QR activity for the vehicle controls.

Determination of GSH Levels in Cell Culture. LNCaP were grown in 96-well plates at a density of 8×10^4 cells/ml. After 20 h of incubation, cells were treated with L-sulforaphane at the indicated concentrations. After an additional 48 h, the medium was removed, and the relative GSH levels were determined as described by Gerhauser *et al.* (26). GSH levels were determined in triplicate for each dose of sulforaphane and were corrected for toxicity as above. Reported values represent the average of two separate experiments.

Results

Sulforaphane Induces QR Activity in Cultured Prostate Cells. QR (NADPH menadione:oxidoreductase; EC 1.6.99.2) protects cells from quinones and their precursors by obligate two-electron reduction of quinones to hydroquinones, thereby preventing generation of highly reactive semiquinones (that arise from single electron transfer). QR is stably expressed *in vitro* and is induced coordinately with other phase 2 enzymes (27). QR has been used as a surrogate marker of global phase 2 enzyme activity *in vitro* and *in vivo*. To test whether sulforaphane has the ability to induce QR enzyme activity in prostate cells, we treated four prostate cancer cell lines and one primary prostate cell strain grown from histologically normal prostatic tissue harvested at surgery (courtesy of Donna Peehl). Cells were treated with sulforaphane or DMSO vehicle as control, and QR enzymatic activity was measured using the technique of Prochaska and Santamaria (23). Over a range of concentrations, sulforaphane induced QR activity in all of the prostate cell lines tested (Table 1). Sulforaphane was particularly potent at inducing QR enzymatic activity in the normal prostate cell strain with maximal induction (2.46-fold) at 1–3 μM and 1.35-fold induction occurring at 0.1 μM sulforaphane. Potent induction was also seen at micromolar doses in LNCaP, MDA PCa 2a, and MDA PCa 2b. All of these cell lines resemble human prostatic epithelia in that they express prostate-specific antigen and androgen receptor and possess relatively slow growth kinetics (21, 28). TSU-PR1, on the other hand, lacks these features of prostatic cells and shows somewhat diminished responsiveness to sulforaphane.

Broccoli sprouts have been reported to contain high levels

Table 1 Dose-dependent induction of quinone reductase activity in response to sulforaphane

	μM sulforaphane							
	15	10	8	5	3	1	0.5	0.1
LNCaP	1.98	2.29	1.93	2.11	1.39	1.28	1.11	1.00
MDA Pca 2a	1.67	1.60	2.10	1.99	1.89	1.31	0.94	1.04
MDA Pca 2b	1.52	2.47	2.10	1.92	1.90	1.70	1.25	1.04
TSU-Pr1	1.86	1.28	1.39	1.14	1.21	1.01	0.95	0.92
Normal strain	1.81	1.86	1.80	1.95	2.46	2.08	1.57	1.35

Table 2 Dose-dependent induction of quinone reductase activity in response to broccoli sprout extract

	Percentage of broccoli sprout extract						
	1.250	0.625	0.313	0.156	0.078	0.039	0.020
LNCaP	1.138	1.974	2.134	1.462	1.176	1.044	0.957

of sulforaphane and decrease the rate, incidence, and multiplicity of mammary tumors in dimethylbenz[*a*]anthracene-treated rats (22). To determine whether broccoli sprout extracts also have the ability to induce QR in human prostate cells, LNCaP cells were treated with water extracts of broccoli and assayed for QR enzyme activity. Table 2 illustrates the dose-dependent increase in QR in LNCaP cells with inducer potencies similar to those observed in cells treated with pure sulforaphane.

QR mRNA Levels Are Increased by Sulforaphane. Induction of phase 2 enzymes *in vitro* and *in vivo* is mediated by increased transcription at phase 2 enzyme gene loci. This transcriptional induction is thought principally attributable to the binding of specific proteins to an ARE in the 5′-regulatory regions of these genes (13–19). To evaluate whether increased QR enzymatic activity is attributable to increased transcription of the *NQO-1* gene in human prostatic cells, we treated five prostate cancer cell lines with 10 μM sulforaphane or with DMSO control for 8 h and then performed Northern blot analysis using the *NQO-1* cDNA as a probe. Hybridizations revealed marked induction of the 1.9- and 2.7-kb transcripts of the *NQO-1* gene. Transcriptional induction closely mirrored enzymatic activity in each of the cell lines. Densitometric measurements revealed that LNCaP, MDA PCa 2a, MDA PCa 2b, PC3, and TSU-Pr1 had a 2.6-, 2.2-, 1.9-, 1.8-, and 1.6-fold increase in *NQO-1* mRNA levels, respectively, as compared with control 8 h after treatment (Fig. 1*A*).

To determine the temporal induction profile of *NQO-1* by sulforaphane, we treated LNCaP cells with 10 μM sulforaphane over a 72-h time course and performed Northern blot analysis. *NQO-1* mRNA levels were measured by densitometry, and fold induction was calculated for each time point relative to DMSO-treated control cells. After treatment with 10 μM sulforaphane, *NQO-1* mRNA levels at 1, 4, 8, 46, and 72 h were induced 0.7-, 1.9-, 4.5-, 3.9-, and 4.6-fold, respectively (Fig. 2*A*). Thus, sulforaphane produces an early and sustained *NQO-1* transcriptional response. QR enzymatic activity was also induced and sustained over an identical time course (data not shown).

Sulforaphane Induces Glutathione Synthetic Pathways. The γ-GCS enzyme catalyzes the rate-limiting step in glutathione synthesis and is composed of two subunits, heavy and light chain. The 5′ regulatory regions of the heavy (29) and light (30) subunits of γ-GCS both contain an ARE, and their expression is induced coordinately by β-naphthoflavone, a well-characterized bifunctional (phase 1 and 2) enzyme-inducing agent. Northern blot analysis using the γ-GCS-L cDNA revealed potent transcriptional induction of this subunit similar to that observed with NQO-1. Sustained induction of γ-GCS-L mRNA levels of 0.5-, 6.5-, 7.8-, 3.6-, and 4.3-fold relative to DMSO controls were observed for the respective time points of 1, 4, 8, 46, and 72 h (Fig. 3*A*). Somewhat surprisingly, sulforaphane did not induce expression of γ-GCS-heavy chain in the LNCaP cell line at 8 h, although abundant message was expressed (Fig. 3*B*).

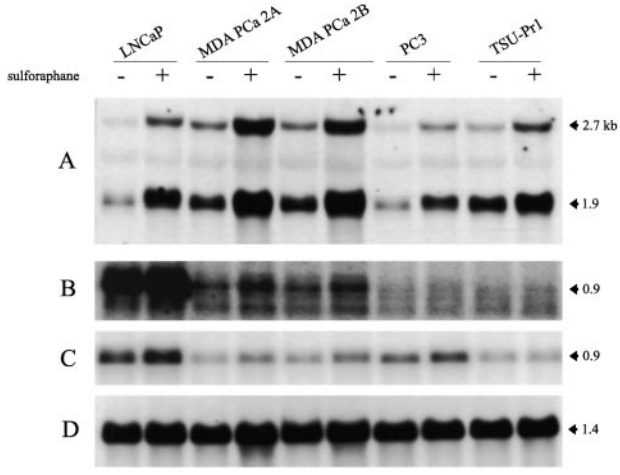

Fig. 1. Transcriptional response of phase 2 enzymes to sulforaphane in various prostate cancer cell lines. The cell lines LNCaP, MDA PCa 2A, MDA PCa 2B, PC3, and TSU-Pr1 were treated for 8 h with 10 μM sulforaphane or with the DMSO control. Northern blot analyses were performed using (*A*) *NQO-1*, (*B*) *GSTA1*, (*C*) microsomal GST, and (*D*) GAPDH cDNA probes.

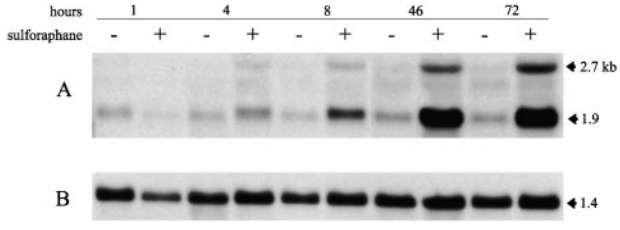

Fig. 2. Northern blot analysis of the transcriptional response to sulforaphane. LNCaP cells were incubated for 1 to 72 h with 10 μM sulforaphane or the DMSO control before the RNA was harvested. The membrane was probed with labeled (*A*) *NQO-1* and (*B*) GAPDH cDNAs.

Sulforaphane Elevates Glutathione Levels. Sulforaphane has been shown to decrease intracellular glutathione levels in murine hepatoma cells by direct conjugation to reduced glutathione (31). Because sulforaphane elevated γ-GCS-L but not γ-GCS-heavy chain mRNA levels in human prostate cells, we were curious whether it could increase glutathione levels in LNCaP cells. After treatment of LNCaP cells with 10 μM sulforaphane for 48 h, levels of reduced glutathione were measured and normalized to cell number. Between 5 and 10 μM, the amount of reduced glutathione/cell increased an average of 17% after treatment, and this increase appeared to be dose-dependent (Table 3).

Because intracellular glutathione levels increased in conjunction with phase 2 enzyme induction after treatment of the LNCaP cell line with sulforaphane, we wondered whether rais-

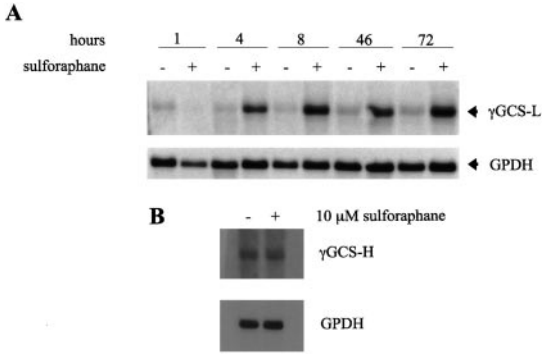

Fig. 3. Differential effects of sulforaphane on γ-GCS subunits. Northern blot analyses of (*A*) sulforaphane-induced γ-GCS light chain expression over time and (*B*) unchanged γ-GCS heavy chain expression treated with 9 h of 10 μM sulforaphane treatment in LNCaP cells.

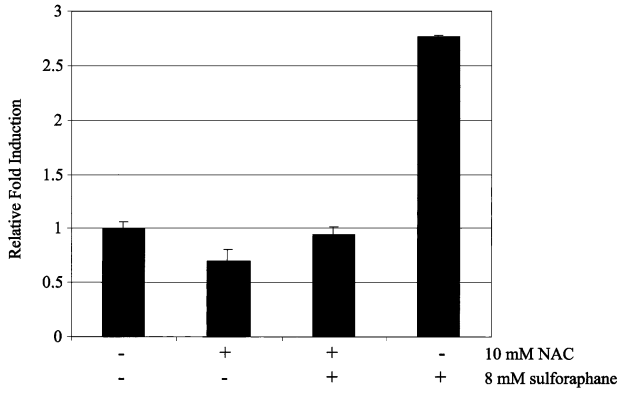

Fig. 4. QR response to sulforaphane is abolished in a reduced environment. LNCaP cells were pretreated for 2 h with 10 μM NAC followed by 8 μM sulforaphane or DMSO control before assaying QR activity. *Bars*, SD of triplicate data points.

Table 3 Dose-dependent induction of cellular glutathione by sulforaphane

	μM sulforaphane							
	15	10	8	5	3	1	0.5	0.1
LNCaP	1.103	1.179	1.152	1.172	1.122	1.051	1.034	1.009

ing intracellular glutathione levels with NAC could potentiate the effects of sulforaphane. LNCaP cells were pretreated with 10 mM NAC for 2 h, followed by either vehicle control or 8 μM sulforaphane for 48 h, and QR enzymatic activity was assayed. QR enzymatic activity was compared with that obtained from cells treated with 8 μM sulforaphane or vehicle control alone (Fig. 4). NAC alone did not induce QR activity, whereas sulforaphane alone did reproducibly. Intriguingly, pretreatment of LNCaP cells with 10 mM NAC abolished the induction of QR enzymatic activity.

Sulforaphane Induces Modest Increases of Expression of Glutathione Transferases. Unlike several species, the 5′-regulatory regions of most human phase 2 enzyme genes lack an ARE consensus sequence. Both human α-class and microsomal GSTs appear to lack this regulatory element (32). We investigated whether absence of this element abrogated the transcriptional response of these genes to sulforaphane. Northern blot analysis showed modest induction of expression of GST-α in LNCaP, MDA PCa 2a, and MDA PCa 2b (1.7-, 1.7-, and 1.4-fold, respectively; Fig. 1*B*), yet the 0.9-kb *GSTA1* band was unchanged in PC3 and TSU-Pr1. Microsomal GST was induced similarly in LNCaP, MDA PCa 2a, MDA PCa 2b, and PC3 (1.7-, 1.8-, 1.3-, and 1.4-fold, respectively; Fig. 1*C*), and again TSU-Pr1 was essentially unaffected. Global glutathione transferase activity was evaluated in all of the cell lines by reduction of CDNB. Unfortunately, like many cells *in vitro*, the prostate cell lines exhibited no measurable GST activity (data not shown; Ref. 33).

Discussion

Sulforaphane is a potent phase 2 enzyme-inducing agent in human prostate cells *in vitro*. Sulforaphane produced robust and sustained transcriptional induction of *NQO-1* gene expression that was accompanied by similar increases in QR enzymatic activity. Other members of the class of phase 2 enzymes were also induced transcriptionally. Intracellular levels of reduced glutathione increased after sulforaphane treatment, likely attributable to increased expression of the γ-GCS-L gene, an enzyme that catalyzes the rate-limiting step in glutathione synthesis. Together, the changes induced by sulforaphane buttress cellular defenses against carcinogens by increasing the reductive capacity of the cell.

All of the prostatic cells tested *in vitro* were affected similarly by sulforaphane, including a normal prostate cell strain, three hormone-responsive immortalized cell lines, and an androgen-insensitive cell line. Each of the cell lines demonstrated the same pattern of phase 2 enzyme response and glutathione induction, although there were quantitative differences. Regulation of the response to sulforaphane in these cell lines, therefore, appears to remain intact, although somewhat complicated. The light and heavy subunits of γ-GCS were not induced coordinately, despite the presence of a stereotypical ARE in the 5′-regulatory regions of both genes. Other investigators (29, 30) have reported coordinate induction of these subunits in response to phase 2-inducing agents. Furthermore, sulforaphane was capable of inducing expression of phase 2 enzymes known to lack AREs, namely GST-α and microsomal GST. Thus, regulation of these enzymes, at least in prostatic cells, is likely to involve more than binding of a protein complex to the ARE enhancer element. Detailed study of the regulatory regions of these genes will be necessary to understand the complex regulatory pathways that modulate the cellular response to sulforaphane.

Alteration of intracellular redox status may be one means by which sulforaphane acts to increase phase 2 enzyme expression. Pretreatment of prostatic cells with NAC, which is known to increase intracellular levels of reduced glutathione, completely ablated the effects of sulforaphane. Although it is possible that NAC acts directly on sulforaphane, we suspect that NAC reduces intracellular proteins mediating the phase 2 enzyme response. Understanding the role of intracellular redox in the regulation of phase 2 enzyme response has implications in the design of future clinical trials in cancer prevention; *e.g.*, one proposed intervention strategy for prostate cancer combines NAC (to increase intracellular reduced glutathione, a GST substrate) with a phase 2 enzyme-inducing agent such as sulforaphane (34). Our results suggest that such an approach may ablate the response to sulforaphane, at least in prostatic cells. Indeed, because sulforaphane increases intracellular glutathione pools by itself, such combined therapy may be unnecessary.

Our findings may help explain the recent observation (9, 10) that consumption of cruciferae, naturally rich sources of sulforaphane, may lower the risk of later development of prostate cancer. Because the loss of one phase 2 enzyme, namely π-class glutathione transferase, is an early and near universal finding in human prostate cancer, sulforaphane may help compensate for this loss by increasing global phase 2 enzyme activity. At first glance, it seems somewhat surprising that loss of expression of a single GST could increase risk of prostate cancer. Glutathione transferases comprise a family of enzymes with broad and overlapping substrate specificity; thus, loss of any single member should be compensated by the activity of the remaining GSTs (32). However, several epidemiological studies (35–38) have suggested that loss of individual GSTs (*e.g.*, GSTM1-null phenotype) can confer increased susceptibility to cancer at several organ sites. Low activity GSTP1 alleles have been associated with increased prostate cancer risk (39, 40). Indeed, mice engineered to lack π-class GST expression are more susceptible to carcinogen-induced tumors (41). Thus, loss of expression of a single GST appears to increase cancer risk, either from global decreases in GST activity or from loss of protection against a carcinogen inactivated solely by the lost enzyme.

Could the capacity of sulforaphane to induce phase 2 enzymes compensate for or prevent loss of GSTP1 expression? An intriguing study by Lin *et al.* (42) suggests that induction of phase 2 enzymes may be particularly pertinent in the setting of GST enzymatic deficiency. Patients with a previous history of colonic polyps were stratified for their subsequent risk of developing colorectal polyps based on levels of consumption of cruciferous vegetables. Compared with subjects that never consumed broccoli, those in the highest quartile of broccoli consumption had an odds ratio of 0.47 (95% confidence interval, 0.30–0.73), and this protective effect was only observed in subjects with the *GSTM1* null genotype. No protection was conferred in subjects with wild-type *GSTM-1* alleles. A similar interaction between *GSTM1* genotype and broccoli consumption has been observed in lung cancer (43). Because GSTP1 is lost in all of the human prostate cancers, induction of global phase 2 enzyme activity and increasing intracellular reduced glutathione may be have great relevance in preventing this disease.

In summary, sulforaphane is a potent inducer of phase 2 enzymes in human prostatic cells. Induction of phase 2 enzymes is one possible explanation for the association between high consumption of cruciferae and decreased prostate cancer risk. On the basis of these findings, intervention trials may be warranted, and broccoli sprouts, a rich natural source of sulforaphane, may be appropriate for use in such a trial. Additional work will be necessary to elucidate the mechanisms of phase 2 enzyme induction in human prostate cells.

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