Induction of the Phase 2 Response in Mouse and Human Skin by Sulforaphane-containing Broccoli Sprout Extracts

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Abstract

The isothiocyanate sulforaphane was isolated from broccoli extracts in a bioactivity-guided fractionation as the principal and very potent inducer of cytoprotective phase 2 enzymes and subsequently shown to inhibit tumor development in animal models that involve various carcinogens and target organs. Because broccoli and broccoli sprouts are widely consumed, extracts obtained from them are viewed as convenient vehicles for sulforaphane delivery to humans. In relation to our current interest in devising strategies for protection against UV light–induced skin cancer, it was necessary to examine the safety and efficacy of topical application of sulforaphane-containing broccoli sprout extracts as single and multiple doses in both mice and humans. Topical application of an extract delivering 100 nmol sulforaphane/cm² increased the protein levels of NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase A1, and heme oxygenase 1, three representative phase 2 enzymes, in mouse skin epidermis. Quantitative assessment of the activity of NQO1 24 h after dosing showed increases of 1.5- and 2.7-fold after application of single and multiple (thrice, every 24 h) doses, respectively. A dose-escalation safety study in healthy human subjects revealed no adverse reactions when doses as high as 340 nmol of sulforaphane in the form of broccoli sprout extracts were applied topically to the center of a 1-cm-diameter circle drawn on the volar forearm. A subsequent efficacy study showed that despite the interindividual differences in basal levels, the enzyme activity of NQO1 in homogenates of 3-mm full thickness skin punch biopsies increased in a dose-dependent manner, with maximum increases of 1.5- and 4.5-fold after application of 150 nmol doses, once or three times (at 24 h-intervals), respectively, thus providing direct evidence for induction of the phase 2 response in humans. (Cancer Epidemiol Biomarkers Prev 2007;16(4):847–51)

Introduction

It is now widely recognized that the up-regulation (induction) of the phase 2 response is a powerful and highly efficient strategy for protection against the continual damaging effects of reactive oxygen intermediates and electrophiles, both of which participate in the pathogenesis of cancer and many other chronic diseases (1-5). Phase 2 enzymes [e.g., glutathione S-transferases (GST), NAD(P)H-quinone acceptor oxidoreductase 1, and heme oxygenase 1] catalyze diverse reactions that are present in edible plants because they are already part of the human diet. Using a microtiter plate bioassay for quantifying potencies for induction of NAD(P)H-quinone oxidoreductase 1 (NQO1; ref. 7), a prototypic phase 2 enzyme, the isothiocyanate sulforaphane was isolated as the principal and very potent inducer from broccoli (8). Sulforaphane was subsequently shown to increase tissue levels of phase 2 enzymes in rodents and to inhibit incidence and multiplicity of dimethylbenz(a)anthracene-induced mammary tumors in rats (9). During the past 5 years, several research groups have shown that sulforaphane inhibits tumor development in at least eight other rodent models that involve various carcinogens and target organs (10).

An important requirement for translation of laboratory findings to humans is demonstration of safety. Our recent randomized, placebo-controlled, double-blinded clinical phase I study of safety, tolerance, and pharmacokinetics of repeated oral doses of broccoli sprout extracts containing either glucoraphanin or sulforaphane has revealed no evidence of any systematic, clinically significant, adverse events that could be attributed to ingestion of the sprout extracts (11). To our knowledge, the evidence for induction of the phase 2 response by dietary inducers in humans is limited to the demonstration of suppression by broccoli sprout extracts of formation of aflatoxin B1-DNA adducts in individuals at high risk to liver cancer (12) and the elevation of GSTs in plasma, urine, and rectal biopsies of volunteers fed cruciferous...
vegetables (13, 14). In relation to our current interest in devising strategies for protection against UV light–induced skin cancer, it was necessary first to examine the safety and efficacy (in terms of induction of the phase 2 response) of topical application of sulforaphane-containing broccoli sprout extracts in humans. This study directly shows the elevation of NQO1 in skin of human volunteers treated with sulforaphane by topical application of broccoli sprout extracts and the safety of this intervention.

Materials and Methods

Preparation and Standardization of Broccoli Sprout Extracts. Broccoli (Brassica oleracea italica, cv. DeCicco) sprout extracts were prepared and hydrolyzed with daikon sprout myrosinase as described (11, 15). The final preparations were dissolved in 80% acetone:20% water (v/v) and their isothiocyanate concentration (of which 90% was sulforaphane) was determined by the cyclocondensation reaction (16). NQO1 inducer bioassay (7) confirmed previously reported potency values (8).

Animals. Female SKH-1 hairless mice (Charles River, Wilmington, MA) were maintained in a 12-h light/12-h dark cycle at 35% humidity and given free access to water and pelleted AIN 76A diet without antioxidants (Harlan Teklad, Madison, WI). Experiments were in compliance with the NIH guidelines, approved by the Johns Hopkins University Animal Care and Use Committee. Two groups of 9-month-old mice were treated topically with either a single dose or three repeated doses at 24-h intervals of (a) 50 µL of broccoli sprout extract (in 80% acetone:20% water by volume) containing 0.5 µmol of sulforaphane, applied to the caudal area of the back, thus delivering 100 nmol sulforaphane/cm²; and (b) 50 µL of vehicle, applied to the rostral area of the back. The animals were euthanized 24 h after the last dose. Two identical rectangular segments (1.5 × 1 cm) of dorsal skin within each treated area were removed: one was frozen in liquid N2 and stored at −80°C until analyzed.

NQO1 Enzyme Activity. Frozen skin tissue was pulverized in liquid N2 (Biopulverizer, Biospec Products, Bartlesville, OK) and the resulting powder was homogenized in 0.25 mol/L sucrose-10 mmol/L Tris-HCl (pH 7.4). The clear supernatant fractions obtained after centrifugation at 14,000 × g for 30 min at 4°C were analyzed for protein concentration and enzyme activity levels (7).

Immunohistochemistry. Frozen tissue blocks were sectioned by a Microm International HM 505 E microtome cryostat operated at −25°C and the resulting cryosections (10-µm thickness) were mounted on microscope glass slides. Sections were immunostained using highly specific primary antibodies against NQO1 (1:200 dilution), GSTA1 (1:200 dilution), and HO-1 (1:1,000 dilution; Stressgen, Victoria, Canada), followed by FITC-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). A Leica MZ FL III microscope equipped with an excitation filter of 470/40 nm and a barrier filter of 525/50 nm was used to view the FITC fluorescence.

Statistical Analysis. All statistical comparisons were done using Stata 7.0. Comparisons of NQO1 activity (expressed as treated over control ratio) were evaluated by ANOVA and “nptrend” on all doses, by subject, and across multiple

Human Subjects. Protocols for studies that involve healthy human volunteers were approved by the Institutional Review Board at the Johns Hopkins School of Medicine. Subjects were recruited by advertising. The Drug Development Unit of the Division of Clinical Pharmacology carried out the screening of the human volunteers, and its staff obtained the skin punch biopsies. Seventeen subjects participated after giving written informed consent [7 males and 10 females, 11 Caucasians and 6 African Americans; mean age, 37.7 (range 25-51) years]. For the safety study, a circle (1 cm in diameter) was drawn on the skin of the volar forearm and the extract was applied to the center of the circle by using a positive displacement pipette. Two broccoli sprout extracts prepared in 80% acetone:20% water (v/v) that differed 10-fold in isothiocyanate concentration (2.7 and 26.2 mmol/L) were used. The single volumes of extract applied did not exceed 3.25 µL. To maintain localization, multiple applications were made and each was allowed to dry before applying the next. A maximum volume of 26 µL was applied at a single site. Each subject received a placebo-treated “spot” with the equivalent volume of the vehicle. Subjects were instructed not to wash the treated areas for at least 8 h after application and returned on 2 consecutive days for visual inspection. An investigator who was unaware of the treatment groups inspected and photographed the sites of application of extract or placebo. The next higher dose was applied only if there was no evidence of reaction to the previous one.

For the efficacy studies, subjects were instructed to maintain a low vegetable diet for 2 weeks, to refrain from consuming cruciferous vegetables and condiments, and to keep a food diary 2 days before and throughout the study. Broccoli sprout extracts or vehicle were applied topically to the center of 1-cm-diameter circles drawn on the posterior waist region of the back either as single doses or three repetitive doses at 24-h intervals. This anatomic site was chosen because normally it is not exposed and there is a small risk of scarring due to the biopsies. Full thickness skin punch biopsies (3-mm diameter, maximum number of six per volunteer) were taken from treated and control sites 24 h after the last treatment, after s.c. application of lidocaine. The area of the biopsy was sutured and dressed. Specimens were immediately frozen in liquid N2 and stored at −80°C until analyzed.

Figure 1. Induction of NQO1 in mouse skin by topical application of broccoli sprout extracts. The backs of SKH-1 hairless mice were treated topically with either a single dose (n = 4) or three doses (n = 3) at 24-h intervals of (a) 50 µL of broccoli sprout extract (in 80% acetone:20% water by volume) containing 0.5 µmol of sulforaphane, applied to the caudal area, thus delivering 100 nmol sulforaphane/cm²; and (b) 50 µL of vehicle, applied to the rostral area. The animals were euthanized 24 h after the last dose and their dorsal skin within the treated areas was harvested. NQO1-specific activity was measured in pulverized total skin homogenate supernatants obtained from vehicle-treated (white columns) and broccoli extract–treated (hashed columns) areas.
Results and Discussion

Topical Application of Broccoli Sprout Extracts as a Source of Sulforaphane Elevates NQO1, HO-1, and GSTA1 in Mouse Skin. We first evaluated the phase 2 response in the skin of SKH-1 hairless mice in vivo after topical application of standardized myrosinase-hydrolyzed broccoli sprout extract containing the equivalent of 0.5 nmol sulforaphane/cm² to the caudal area of the mouse back, whereas the rostral back area received vehicle. Mice were euthanized 24 h after the last dose of treatment; full thickness dorsal skin within each treated area was collected; and NQO1 activity was evaluated. In full agreement with our previous studies (15), there was a substantial 1.6-fold induction (P = 0.004) in homogenate supernatants of skin to which a single dose of the extract was applied (Fig. 1). The 3-day treatment resulted in even greater (2.7-fold; P = 0.04) induction. The higher magnitude of induction observed after multiple dosing could be due to a combination of higher cumulative dose delivered to the target cells and the kinetics of the response. Immunohistochemical analysis of fresh frozen tissue sections confirmed increase of NQO1 protein levels...
Specific activities of treated to control.

Application of the last dose.

Enzymes in skin in vivo may be of particular importance because the skin is constantly exposed to a variety of environmental xenobiotics and sunlight. Indeed, polymorphisms in genes encoding phase 2 proteins have been associated with increased skin cancer risk. In mice, deletion of the gene encoding for GSTP led to increased skin tumor incidence, providing direct evidence that a single phase 2 enzyme can profoundly affect carcinogen susceptibility in the skin (17). Similarly, NQO1-deficient mice are much more sensitive to skin carcinogenesis compared with their wild-type counterparts (18). In humans, an inverse association between NQO1-positive genotype and the number of cutaneous basal cell carcinomas has been observed (19), as well as higher sensitivity to UV-radiation-induced cutaneous damage in individuals homozygously deficient in GSTT1 and/or GSTM1 (20). Thus, both human polymorphisms of phase 2 enzymes with deletion of their genes in mice increase susceptibility to skin tumor development.

More than 15 years ago, Keyse and Tyrrell (21) identified HO-1 as the major protein that is induced in cultured human skin fibroblasts by UVA radiation, as well as by various thiol reagents, all of which (i.e., menadione, hydrogen peroxide, sodium arsenite, and iodoacetamide), just like sulforaphane, are now known to react with Keap1, the sensor for phase 2 inducers (6). We have previously shown the antioxidant response element–dependent inducibility of HO-1 by phase 2 inducers of many different chemical classes and proposed that HO-1 induction is part of the phase 2 cytoprotective response (22). Indeed, a dose-dependent protective effect by UVA radiation against UVB-induced erythema/edema and immunosuppression mediated by induction of HO-1 was recently observed in skin of hairless mice (23), consistent with the observation that UVA but not UVB radiation caused nuclear translocation and accumulation of Nr2f2 in cultured murine dermal fibroblasts (24). Perhaps the most practically important aspect of our present finding is that it shows the feasibility of enhancing cytoprotective phase 2 enzymes in skin by topical application of an agent that is immediately suitable for human use.

Induction of NQO1 by Broccoli Sprout Extracts in Skin of Healthy Human Volunteers. The development of means for topical application of potential chemoprotective agents offers several advantages over dietary route of administration: (a) direct delivery to the target organ (skin); (b) selective application to sun-exposed areas of the skin; (c) minimal risk of potential side effects due to systemic distribution. Encouraged by the effectiveness of induction of the phase 2 response in the mouse skin by topical application of sulforaphane-containing broccoli sprout extracts, we next undertook a dose escalation safety study in healthy human volunteers. Doses were applied topically on the volar forearm to the center of 1-cm-diameter circles and reactions were observed and photographed. Each subject received a maximum of two doses (each at a different site). Doses were as follows: 0.534 and 5.34 nmol (four subjects); 10.7 and 21.4 nmol (two subjects); 42.7 and 85.4 nmol (two subjects); 170 and 340 nmol (two subjects); and 681 nmol (two subjects). No significant adverse reactions were observed except in one subject at the highest dose (681 nmol) who developed transient 3-mm flat erythema 24 h after dosing.

We then conducted efficacy studies. The end point was determination of the enzyme activity of NQO1 in 3-mm skin punch biopsies of healthy human volunteers after topical application of a single dose of broccoli sprout extract to the skin at the posterior waist region. Each subject served as his/her own control and, in addition to the extract-treated regions, received two vehicle-treated spots. Both NQO1 activity and protein content could be reliably determined in the supernatants of total skin homogenates (average fresh weight of each biopsy, ~30 mg), although the NQO1-specific activity differed nearly 2-fold among individuals. No NQO1 activity was detected in one individual. This was not surprising in view of the known polymorphisms in the nqo1 gene in the human population that exhibit a clear gene-dose effect with respect to phenotype (25). Thus, homozygous individuals NQO1*1/*1 (wild type) have the highest levels of NQO1 protein. In stark contrast, homozygous individuals NQO1*2/*2 have no detectable protein levels because the mutant protein that carries a single amino acid substitution (proline to serine at position 187) is unstable and undergoes rapid ubiquitination and proteasomal degradation. Heterozygous individuals NQO1*1/*2 have intermediate levels of protein and enzyme activity.

Importantly, despite the interindividual differences in basal levels, the specific activity of NQO1 increased by ~1.5-fold in all three subjects who received a single dose of extract.
containing 170 nmol of sulforaphane, as well as in those who received the 340 nmol dose (Fig. 3A). No significant changes in NQO1 activity were seen in subjects who received single applications of extracts containing 5 or 40 nmol of sulforaphane. There was a significant effect of treatment on induction of NQO1 (P = 0.015) and trend analysis was also highly significant (P_trend < 0.01) when dose levels were compared across multiple subjects.

The effect of three repeated topical applications (at 24-h intervals) of broccoli sprout extracts was examined next. Each subject was treated with four escalating doses of extract flanked by two vehicle-treated spots. This treatment regimen led to even greater elevations of the specific activity of NQO1 in skin of healthy volunteers, thus providing direct evidence for induction of the phase 2 enzyme activity of NQO1 in skin of healthy volunteers, thus providing direct evidence for induction of the phase 2 response in humans. Devising strategies for protection against development of skin carcinogenesis is particularly important for the growing population of solid organ transplant recipients who, compared with the general population, are at a greatly increased risk of cutaneous squamous cell carcinoma (65-fold), malignant melanoma (3-fold), Kaposi’s sarcoma (84-fold), and lip squamous cell carcinoma (20-fold; ref. 26).

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References

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