Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2394–2398, March 1992
Medical Sciences

Rapid detection of inducers of enzymes that protect against carcinogens

(cancer/chemoprotection/enzyme induction/quinone reductase/vegetables)

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Contributed by Paul Talalay, December 19, 1991

ABSTRACT **Dietary composition is a major determinant of cancer risk in humans and experimental animals. Major and minor components of the diet may enhance or suppress the development of malignancy. Many dietary constituents also modify the metabolism of carcinogens by induction of enzymes involved in xenobiotic metabolism, and this is one well-established mechanism for modulating the risk of cancer. We have developed a simple system for rapid detection and measurement of the induction of enzymes that detoxify carcinogens (phase II enzymes), based on the direct assay of the activity of quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] in murine hepatoma cells grown in microtiter plate wells. Survey of extracts of a variety of commonly consumed, organically grown vegetables for quinone reductase inducer activity identified crucifers (and particularly those of the genus *Brassica*) as singularly rich sources. It is therefore of interest that high consumption of these types of vegetables has been correlated with decreased cancer risk in humans. The assay system also measures toxicity, which was unrelated to inducer potency among the vegetable extracts examined. By use of mutant hepatoma cells (defective in regulation of certain cytochrome P-450 enzymes) selective (monofunctional) inducers of protective phase II enzymes can be distinguished from (bifunctional) inducers that also elevate cytochromes P-450 (phase I enzymes) and thereby pose the risk of carcinogen activation. The assay system therefore permits not only rapid detection of inducers of anticarcinogenic enzymes in the human diet but also elucidation of effects of storage and processing on inducer activities.**

Extrinsic factors, including personal life-styles, play a major role in the development of most human malignancies (1–3). Cigarette smoking and consumption of alcohol, exposure to synthetic and naturally occurring carcinogens, radiation, drugs, infectious agents, and reproductive and behavioral practices are now widely recognized as important contributors to the etiology of cancer. But perhaps most surprising is the inference that normal human diets play causative roles in more than one-third (and possibly even two-thirds) of human neoplasia (1–3). Our food contains not only numerous mutagens and carcinogens but also a variety of chemicals that block carcinogenesis in animal models (4–11). Furthermore, carcinogens can even protect against their own toxic and neoplastic effects or those of other carcinogens—i.e., carcinogens may act as anticarcinogens (12–14). Clearly, dietary modifications modulate cancer risk in various ways: for instance, through changes in caloric intake, by altering the consumption of nutritive and nonnutritive major components, and by providing exposure to numerous minor chemicals that may be genotoxic or protective (4–7, 9–11, 15–19). Rational recommendations for modifying human diets to reduce the risk of cancer require identification of dietary carcinogens and chemoprotectors, even though interactions among such factors in the etiology of cancer are complex (20). Whereas extensive efforts have been made to identify dietary carcinogens and mutagens (4–6), chemoprotective components have received far less attention. This paper describes a method for detecting and identifying anticarcinogenic components in human diets.

Since a major mechanism regulating neoplasia is the balance between phase I enzymes, which activate carcinogens, and phase II enzymes‡ (25, 26), which detoxify them, we have developed a cell culture system for simple and rapid detection of dietary components that enhance phase II detoxication enzymes. With this procedure we surveyed extracts of a variety of vegetables for their ability to induce such protective enzymes. In the accompanying paper (27) we describe use of this method to isolate and identify a major inducer of protective enzymes from broccoli.

We chose vegetables as sources of inducers of detoxication enzymes for the following reasons. First, numerous epidemiological studies suggest that high consumption of yellow and green vegetables, especially those of the family Cruciferae (mustards) and the genus *Brassica* (cauliflower, cress, brussels sprouts, cabbage, broccoli), reduces the risk of developing cancer of various organs (28–34). Moreover, administration of vegetables or of some of their chemical components to rodents also protects against chemical carcinogenesis (9–11, 35). Second, well-documented evidence established that feeding of certain vegetables (e.g., brussels sprouts and cabbage) induces both phase I and phase II enzymes in animal tissues (36–44) and stimulates the metabolism of drugs in humans (36, 45, 46). The elevations of enzymes that metabolize xenobiotics may be highly relevant to the protective effects of vegetables, since relatively modest dietary changes not only affected the metabolism of drugs (44) but also modified the ability of carcinogens to cause tumors in rodents (15–19, 47–49).

Several lines of evidence provide compelling support for the proposition that induction of enzymes of xenobiotic

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Abbreviation: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2].

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‡Enzymes of xenobiotic metabolism belong to two families: (*i*) phase I enzymes (cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (21); and (*ii*) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and serve primarily a detoxication role (22). Quinone reductase (QR) is considered a phase II enzyme because it has protective functions (23), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that control glutathione transferase (24).

metabolism, and particularly phase II enzymes, results in protection against the toxic and neoplastic effects of carcinogens (25, 26): (*i*) Many seemingly unrelated compounds (including phenolic antioxidants, coumarins, cinnamates, 1,2-dithiole-3-thiones, isothiocyanates, lactones, thiocarbamates) can protect rodents against the effects of carcinogens under conditions that invariably evoke the induction of phase II enzymes in many tissues. Indeed, novel anticarcinogens have been isolated and identified solely on the basis of their ability to induce phase II enzymes (50, 51). (*ii*) Such anticarcinogens alter the metabolism of carcinogens and decrease the formation of mutagenic metabolites (52–54). (*iii*) Chemoprotection requires protein synthesis and is most effective if it precedes carcinogen challenge (12–14). (*iv*) Inducers of anticarcinogenic enzymes protect against a wide variety of structurally dissimilar carcinogens, suggesting the involvement of mechanisms that are not structurally fastidious, such as xenobiotic metabolism. (*v*) The enzymes that are elevated, e.g., glutathione transferases, quinone reductase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2], UDP-glucuronosyltransferases, protect against the toxicities of electrophiles such as ultimate carcinogens. (*vi*) Cells in which glutathione transferases are elevated (by development of resistance to alkylating chemotherapeutic agents or by transfection with cloned enzymes) show decreased susceptibility to the toxicity of carcinogenic electrophiles and reduced formation of DNA adducts (55–57).

Resolution of the issue whether the anticarcinogenic effects of vegetables are mediated through the induction of enzymes of xenobiotic metabolism requires the systematic bioassay of these plants for inducer activity. Since measurement of enzyme induction in animals is laborious and expensive, we developed a simple screening procedure in which the specific activity of QR, a phase II enzyme,‡ is measured in Hepa 1c1c7 murine hepatoma cells (58, 59). The feasibility of measuring inducer activity directly in cells grown in 96-well microtiter plates has simplified and accelerated the procedure (60), and the use of heat- and charcoal-treated serum in-

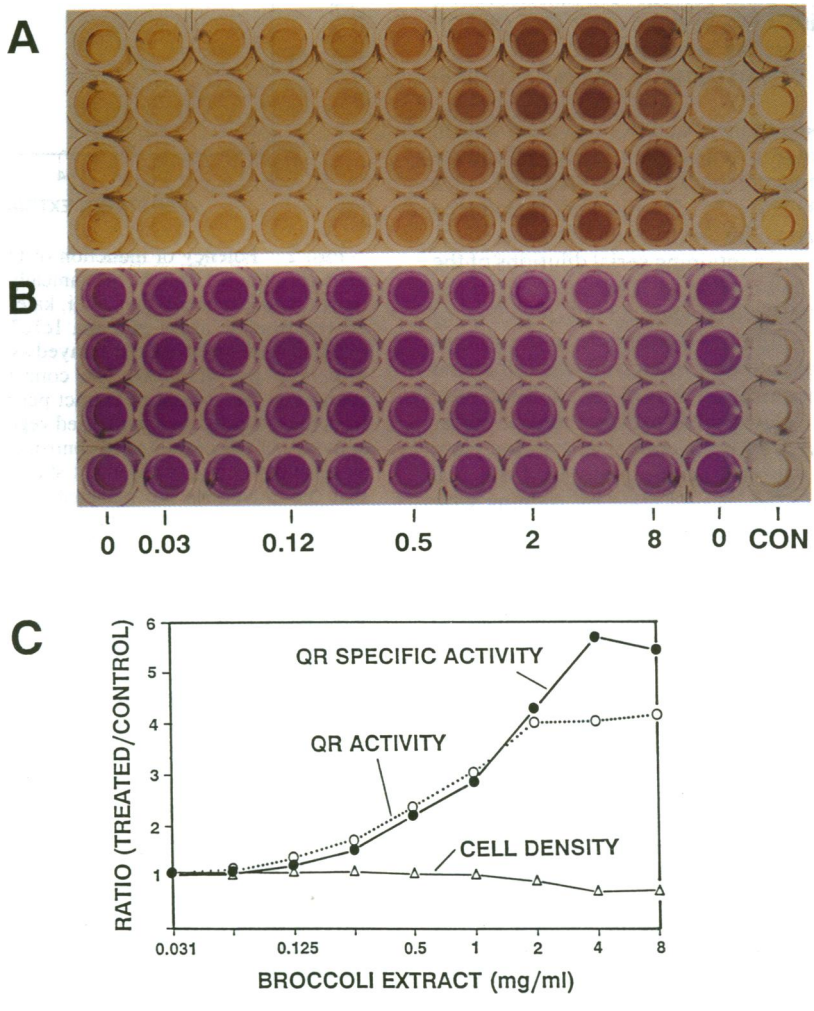

FIG. 1. Induction of QR in murine hepatoma cells by extracts of broccoli. (*A* and *B*) Photographs of sections of 96-well microtiter plates showing the induction of QR (*A*) and the cell density (*B*). (*C*) Graph showing the analysis of absorbances obtained from the plates. The assays were carried out on Hepa 1c1c7 murine hepatoma cells grown in microtiter plate wells and induced with serial 2-fold dilutions of acetonitrile extracts of lyophilized broccoli (Effie May). Details of the procedures are given in *Materials and Methods*. (*A*) The QR activities were measured in cell lysates by reduction of a tetrazolium dye. Note that the color (blue-brown) increases in intensity with the concentration of extract, indicating QR induction. (*B*) A parallel plate treated with the same dilutions of broccoli extract. The cells were stained with crystal violet. Note that there is a slight decrease in cell density at the highest concentrations (4–8 mg/ml), indicating mild cytotoxicity. (*C*) Graphical analysis of optical density information obtained from the above plates scanned at 610 nm (QR assay) and 490 nm (crystal violet assay) related to control wells that received the equivalent volume of acetonitrile only (0.2%). The total and specific activities of QR and the cell densities, expressed as ratios (treated/control), are shown on the ordinate. The concentrations of broccoli extract, shown below the designated microtiter plate wells and on the abscissa of the graph, are expressed as the amount of extract obtained from a given dry weight of broccoli (mg) added to each ml of culture medium (0–8 mg/ml). The QR activity and crystal violet density are related to cells that did not receive inducer. The columns of wells designated 0 mg/ml received no broccoli extract. CON designates the wells that contained no cells and served as the optical controls.

creased its sensitivity (61). These cells respond to nearly all compounds that induce phase II enzymes (e.g., QR and glutathione transferases) in rodents, and conversely induction of QR in these cells is a reliable predictor of inducer activity in various rodent organs *in vivo* (27, 61–63).

MATERIALS AND METHODS

Sources of Vegetables and Preparation of Extracts. All vegetables were grown under organic conditions without pesticides or artificial fertilizers that might contain enzyme inducers. They were stored at −20°C after arrival in our laboratory, although the intervening storage history of some vegetables is not known. Vegetables were homogenized with 2 vol of cold water in a Waring Blendor for 2 min at 4°C. The resultant soups were lyophilized to give dry powders, which were stored at −20°C. Portions (400 mg) of these powders were extracted for 6–24 hr with 14 ml of acetonitrile by shaking in glass vessels at 4°C. The extracts were filtered through 0.45 μm porosity organic solvent-resistant filters and evaporated to dryness either in a vacuum centrifuge (Speed-Vac; Savant) or on a rotating evaporator (<40°C). The residues were dissolved or suspended in 100 μl of acetonitrile.

Assay of Inducer Potency. Inducer activity was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (60, 61). Duplicate plates were prepared. In a typical experiment 10,000 Hepa 1c1c7 cells were introduced into each well initially, grown for 24 hr, and then induced for 48 hr by exposure to medium containing serial dilutions of the extracts (or compounds) to be assayed. Usually 20 μl of the acetonitrile solutions to be assayed were added to 10.0 ml of medium and 2-fold serial dilutions were made in the microtiter plates so that the final volume in each well was 150 μl and the organic solvent concentration was 0.2%. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) was measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and the cell density was determined in the second plate by staining with crystal violet. Quantitative information on specific activity of QR, the inducer potency, and the cytotoxicity of the extract or compound tested is obtained by computer analysis of the absorbances (see Fig. 1). One unit of inducer activity is defined as the amount that when added to a single microtiter well doubled the QR specific activity.

RESULTS AND DISCUSSION

Fig. 1 illustrates the measurement of inducer potency of extracts of organically grown broccoli (Effie May variety). The specific activities of QR were raised nearly 6-fold at the highest extract concentrations tested, at which less than 20% cytotoxicity was observed. The inductions obtained with broccoli (Fig. 1) and with other vegetable extracts (Fig. 2) were proportional to the quantity of extract added over a reasonably wide range. The toxicities of these extracts were modest and were unrelated to their inducer potencies (Fig. 2).

Extracts of a series of organically grown vegetables cultivated under a variety of conditions showed large differences in inducer potencies (Table 1). Because the dry weight content of the vegetables varied considerably, from 3.6% for a sample of bok choi to 26.5% for red leaf lettuce (mean 10% for 24 vegetables) (Table 1), we express the inducer activity of an extract in terms of the dry weight of vegetable yielding, upon extraction under standardized conditions, a given amount of inducer activity. This provides a measure of inducer potency, expressed as units per g of dry vegetable weight (see *Materials and Methods* for definition of unit). Although many vegetable extracts induced QR, certain families were consistently more potent inducers. For example,

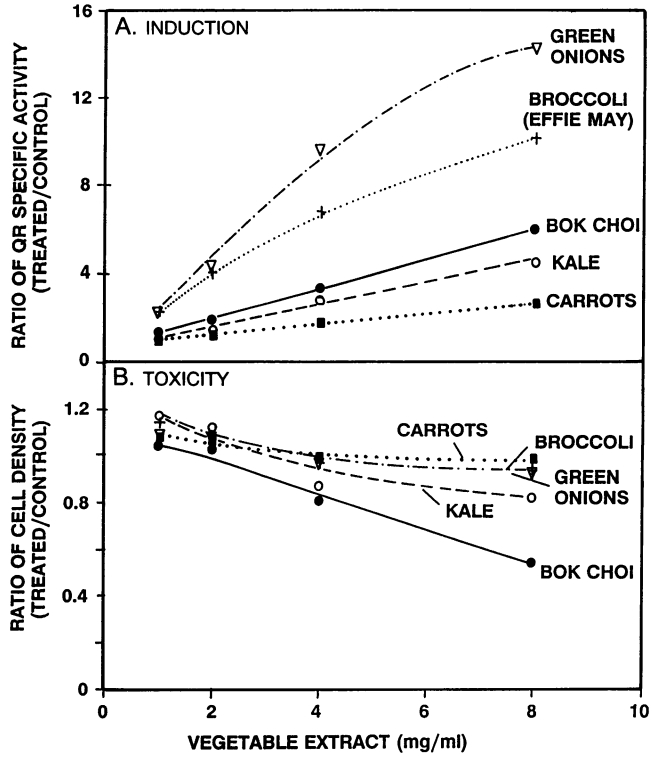

FIG. 2. Potency of induction of QR and toxicity of acetonitrile extracts obtained from five organically grown lyophilized vegetables (green onion, broccoli, bok choi, kale, and carrots) measured by the microtiter plate assay in Hepa 1c1c7 murine hepatoma cells. The extracts were prepared and assayed as described in Fig. 1 legend and *Materials and Methods*. The concentrations of extracts are expressed as the amount of extract per ml of culture medium derived from the indicated weight of dried vegetable. (*A*) Ratio of the specific activities of QR of treated to control cells. (*B*) Relative cell densities as determined by crystal violet staining measured at 490 nm. Note that the inductions are reasonably proportional to the amount of extract at lower induction ratios and that the inducer potencies and toxicities (which do not exceed 20% except in the case of bok choi) are not correlated.

whereas extracts of several Cruciferae had potent inducer activity, extracts of Solanaceae (peppers, potatoes, tomatoes) had low inducer activity. Of the 24 vegetables examined only 6 showed detectable toxicity; the others were nontoxic at the highest concentrations tested. Thus cytotoxicity of 20% was observed for red leaf lettuce at 8.0 mg/ml, for beets, cauliflower, and bok choi at 4.0 mg/ml, and for leeks and ginger at 2.0 mg/ml.

Cytotoxicity measurements are important because phase II enzyme inducers may be toxic and/or carcinogenic. Moreover, by use of mutant Hepa cells defective in aryl hydrocarbon receptor or cytochrome P-450 function (27, 63, 64), our assay system can distinguish *monofunctional* inducers (which elevate phase II enzymes selectively), from *bifunctional* inducers (which elevate both phase I and phase II enzymes) (63). Such information is crucial for identification of chemoprotective enzyme inducers for potential use in humans. Ideally such inducers should be monofunctional, because elevated activities of phase I enzymes may lead to carcinogen activation.

Since some crucifers (broccoli, brussels sprouts, cauliflower, cabbage) are consumed in substantial quantities in Western diets and are believed to protect against cancer, we examined the relation of inducer potency to variety, strain, location of growth, time of sowing, and time of harvest (Table 2). Although systematic examination of these factors under field conditions would require extensive studies over several

Table 1. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of representative samples of various vegetable families and species

Vegetable		Dry weight, %	Potency of QR induction, units*/g
Family	Species/variety		
Chenopodiaceae	Beets	17.3	<833
	Spinach	6.8	1,280
Compositae	Red leaf lettuce	26.5	3,030
Cruciferae	Cauliflower	9.8	2,220
	Bok choi	3.6	3,170
	Broccoli (Effie May)	10.2	16,700
	Broccoli (Winchester)	8.0	2,380
	Green cabbage	9.4	1,550
	Kale	9.2	2,220
	Radish	3.7	1,040
Cucurbitaceae	Zucchini	5.5	<833
Leguminosae	Green beans	6.7	2,150
	Sugar snap peas	14.5	<833
Liliaceae	Asparagus	5.6	1,110
	Green onions	5.1	22,200
	Leeks	8.3	2,780
Rosaceae	Apple	13.1	Inactive
Solanaceae	Green peppers	6.4	Inactive
	Red potatoes	15.7	Inactive
	Sweet potatoes	20.2	Inactive
	Tomatoes	6.2	<833
Umbelliferae	Carrots	10.8	1,230
	Celery	4.5	1,630
Zingerberaceae	Ginger	13.1	4,440

*One unit of inducer activity is defined as the amount of inducer required to double the QR specific activity of Hepa 1c1c7 cells growing in a microtiter well containing 150 μl of medium. An entry of <833 units/g indicates that at the highest concentration tested (extract from 1.2 mg of dry vegetable/150 μl medium) the QR specific activity was significantly elevated but not doubled. Inactive indicates less than 20% elevation of QR specific activity at highest concentration tested: extract from 1.2 mg of dry weight per 150 μl of medium.

Table 2. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of various cruciferous vegetables of the species *Brassica oleracea*

Vegetable (*B. oleracea* subspecies and variety)	Dry weight, %	Potency of QR induction, units/g
Broccoli (*B. oleracea italica*)		
Florets		
Emperor	9.6	6,670
Emperor (late sowing)	9.9	22,200
Green Valiant	10.4	16,700
Green Valiant (late sowing)	8.8	16,700
SAGA (older sample)	6.8	4,170
SAGA (younger sample)	8.0	11,100
SAGA (late sowing)	10.1	33,300
Violet Queen	9.6	3,700
Leaves only		
Emperor	7.0	5,560
SAGA (older sample)	9.4	3,030
SAGA (younger sample)	10.0	16,700
Violet Queen	10.6	7,410
Brussels sprouts (*B. oleracea gemnifera*)		
Jade Cross E	10.8	11,100
Oliver	15.6	6,060
Teal	14.0	11,100
Green cabbage (*B. oleracea capitata*)		
Nagoda 50	4.6	2,560
Perfect Ball	7.6	5,560
Primax	5.6	2,080
Red cabbage (*B. oleracea capitata*)		
Lasso Red	11.2	13,300
Ruby Perfection	7.0	4,760
Cauliflower (*B. oleracea botrytis*)		
Florets		
Andes	8.8	5,560
Montano	8.0	3,700
Leaves only		
Montano	7.4	3,330
Snow Crown	6.6	2,780
Kale (*B. oleracea acephala*)		
Konserva	10.4	3,170
Winterbor	8.4	4,760
Winterbor (late sowing)	15.4	16,700
Kohlrabi (*B. oleracea gongyloides*)		
Capri	5.2	1,330
Kolpak (late sowing)	6.0	1,590

The assays were performed as described in the text and legend to Table 1.

years of cultivation, it was important to determine whether such variables significantly affected the inducer activity. Except for a sample of kohlrabi, Cruciferae belonging to the species *Brassica oleracea* consistently and potently induced QR (Table 2), with broccoli and brussels sprouts generally the most potent inducers. The inductive capacity of most crucifers appears to be independent of geographic location of growth and time of harvest, although late sowing may have enhanced modestly the potency of the induction. On the basis of these results a particular variety of broccoli (SAGA) was selected for isolation and identification of monofunctional inducer activity as described in the accompanying paper (27).

In summary, epidemiological studies point to the inverse relationship between vegetable consumption and the risk of epithelial cancer, and they suggest a practical approach to achieving protection by emphasizing that the typical Western diet is low in fruits and vegetables (20). A striking but perhaps not surprising conclusion is that the microtiter plate assay for induction of QR identifies the same vegetables (crucifers) that display protective properties *in vivo* (9–11, 28–35). It is critical to our understanding of the relationship of diet to cancer, however, that we assess dietary constituents not only for their abilities to induce anticarcinogenic enzymes but also for their toxic and carcinogenic properties. The simple and rapid assay can also determine the toxicity of extracts and, by use of appropriate mutant cells, distinguish monofunctional inducers from less desirable bifunctional ones. Moreover, the assay of phase II enzymes makes possible further detailed analysis of the effects of treatment of vegetables (e.g., growth, storage, and cooking conditions) that might enhance or depress such induction, and also more definitive examination of the relationship of induction to chemoprotection.

We thank Stephen Collier (Surry, ME) for growing a number of vegetables under organic conditions (without use of pesticides and insecticides), Organic Farms (Beltsville, MD) for supplying a wide selection of organic produce, Dr. William Tew (Chesapeake Biological Laboratories, Baltimore) for lyophilizing numerous samples of vegetables, and Gale Doremus for preparing the manuscript and illustrations. These studies were supported by grants from the National Cancer Institute (PO1 CA 44530) and from the American Institute for Cancer Research. H.J.P. was supported by National Cancer Institute Training Grant CA 09243.

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