

Selenium and vitamin E inhibit radiogenic and chemically induced transformation *in vitro* via different mechanisms

(glutathione peroxidase/catalase/glutathione/free radicals/carcinogenesis)

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ABSTRACT Results from *in vivo* and *in vitro* studies showing that antioxidants may act as anticarcinogens support the role of active oxygen in carcinogenesis and provide impetus for exploring the functions of dietary antioxidants in cancer prevention by using *in vitro* models. We examined the single and combined effects of selenium, a component of glutathione peroxidase, and vitamin E, a known antioxidant, on cell transformation induced in C3H/10T-1/2 cells by x-rays, benzo[a]pyrene, or tryptophan pyrolysate and on the levels of cellular scavenging systems and peroxide destruction. Incubation of C3H/10T-1/2 cells with 2.5 μ M Na₂SeO₃ (selenium) or with 7 μ M α -tocopherol succinate (vitamin E) 24 hr prior to exposure to x-rays or the chemical carcinogens resulted in an inhibition of transformation by each of the antioxidants with an additive-inhibitory action when the two nutrients were combined. Cellular pretreatment with selenium resulted in increased levels of cellular glutathione peroxidase, catalase, and nonprotein thiols (glutathione) and in an enhanced destruction of peroxide. Cells pretreated with vitamin E did not show these biochemical effects, and the combined pretreatment with vitamin E and selenium did not augment the effect of selenium on these parameters. The results support our earlier studies showing that free radical-mediated events play a role in radiation and chemically induced transformation. They indicate that selenium and vitamin E act alone and in additive fashion as radioprotecting and chemopreventing agents. The results further suggest that selenium confers protection in part by inducing or activating cellular free-radical scavenging systems and by enhancing peroxide breakdown while vitamin E appears to confer its protection by an alternate complementary mechanism.

A growing body of data now provides compelling evidence that reactive oxygen species produced by radiation and some chemicals including tumor promoters play a role in the process of carcinogenesis *in vivo* and *in vitro* (1-11). Agents that catalytically scavenge intermediates of oxygen reduction (3, 12, 13, 15) and serve as antioxidants (4, 6, 7) have been shown to inhibit various steps in neoplastic transformation and defend the cells in a manner that may vary among species and tissues (3, 5, 14). For example, superoxide dismutase (3), catalase (5), vitamin C (11), vitamin A analogs (14), and the food additive bisulfite (15) have been shown to suppress cell transformation and, in some cases, inhibit the action of tumor promoters (3, 10, 14).

The identification of nutritional antioxidants, which act alone or in concert to inhibit carcinogenesis, is of public concern because of their potential role in cancer prevention (4, 6). Selenium and vitamin E represent two ubiquitous

dietary antioxidants whose role in carcinogenesis has been of interest for some time (16-23).

Epidemiological findings (16) and data from animals exposed to chemical carcinogens (17, 18) have supported the notion that dietary selenium is a cancer preventive agent. However, at a cellular level our data on its inhibition of radiation and chemically induced transformation have been preliminary (14, 23) and the mechanisms by which selenium confers its protection have remained obscure.

Vitamin E (α -tocopherol) has also been shown to suppress chemically induced carcinogenesis in some animal systems (21); however, its anticarcinogenic action at a cellular level and its effects on radiation-induced transformation are unknown.

The metabolic function of vitamin E and selenium are interrelated (22), though each of these nutrients control oxidative damage in a different manner. Selenium is an essential constituent of glutathione peroxidase. This enzyme destroys hydrogen peroxide and organic hydroperoxides by using reducing equivalents from glutathione. Vitamin E, an integral component of the cell membrane, is a free-radical scavenger and prevents the propagation of peroxidative processes. While selenium plays a role in the transport and storage of vitamin E, it is unknown whether these two nutrients act in concert to modify the neoplastic process.

In the present work we addressed the question of the interrelation between selenium and vitamin E by using *in vitro* cell transformation as a model for multistage carcinogenesis (24, 25). We tested whether treatment of C3H/10T-1/2 mouse cells with Na₂SeO₃ (selenium) and/or with tocopherol succinate (vitamin E) modifies cell transformation following exposure to x-rays, benzo[a]pyrene, or to tryptophan pyrolysate, a carcinogen derived from protein foods (26-28). We also determined by using parallel, pretreated cultures whether selenium and vitamin E, alone or in combination, alter the levels of cellular scavenging systems and enzymes that catalyze peroxide breakdown.

We report here that nontoxic levels of Na₂SeO₃ (selenium) significantly inhibit cellular transformation by x-rays, benzo[a]pyrene, and tryptophan pyrolysate. We also find that selenium increases cellular capacity to destroy peroxides. An enhancement of total cellular peroxidase and catalase activities were observed. A small increase in nonprotein thiols was found, but no enhanced levels of glutathione-S-transferase were observed. We also find that α -tocopherol succinate (vitamin E) inhibits transformation by x-rays, benzo[a]pyrene, and tryptophan pyrolysate, but does not enhance peroxide destruction or alter the levels of catalase and peroxidase. Though vitamin E and selenium are additives in inhibiting transformation, vitamin E does not augment the biochemical effects induced by selenium.

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MATERIALS AND METHODS

Cell Cultures and Transformation Assays. Mouse embryo C3H/10T-1/2 cells at passage 8 were cultured in basal Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO) as described (24, 25).

For transformation experiments cells were treated with 2.5 μM Na_2SeO_3 (selenium, Sigma) or 7 μM tocopherol succinate (vitamin E, Sigma) or both at plating time and exposed 24 hr later to x-rays (400 rad; 1 rad = 0.01 Gy) (25, 29) benzo[*a*]pyrene (1.2 $\mu\text{g}/\text{ml}$ for 48 hr), or tryptophan pyrolysate (Trp-P-2) (0.5 $\mu\text{g}/\text{ml}$ for 48 hr) (14, 26–28). Vitamin E and/or selenium were kept in the medium for 72 hr, after which subsequent medium changes were free of these agents.

Cell survival and cell transformation were assayed as reported (24, 25). Both type II and type III foci were scored as transformed. The relationship between the morphological transformation of the cells and their ability to grow in agar and form tumors in the animal has been described (24, 25).

Peroxide Formation and Reduction of Hydrogen Peroxide, Peroxidase, and Catalase. Peroxide formation and its reduction by intact C3H/10T-1/2 cells was measured in cultures parallel to those employed in the transformation studies. We used a model 25 oxidase meter fitted with an oxidase electrode, model 2510 (Yellow Springs Instrument). Hydrogen peroxide was monitored continuously as described (30, 31). To initiate the reaction, 50–60 nM peroxide was added to phosphate-buffered saline (20 mM, pH 7.3) containing 10 mM KCl and 5 mM glucose. Once a stable baseline was achieved, C3H/10T-1/2 cells, pretreated with 2.5 μM Na_2SeO_3 (selenium) for 24 hr, or untreated cells were added as 10^6 cells/ml in phosphate-buffered saline in a total volume of 3 ml. The decrease in peroxide concentration was monitored for 1–15 min after the addition of the cells.

Glutathione peroxidase activity was determined by adding 20 mM sodium azide to the cells. Sodium azide is an inhibitor of catalase activity (31). To determine catalase activity the glutathione reactive reagent *N*-ethylmaleimide was added at 30 mM to inactivate cellular glutathione, thereby indirectly inhibiting glutathione peroxidase.

Catalase activity was measured also with an oxygen electrode (32) by monitoring oxygen evolution ($2\text{H}_2\text{O}_2 \rightarrow \text{O}_2$

+ $2\text{H}_2\text{O}$) following the addition of peroxide to the cell suspension (32). Cellular nonprotein thiols were measured by using a modified Ellman's procedure (33).

Glutathione-*S*-transferase was assayed spectrophotometrically as described (31) by adding 0.1 mM chlorodinitrobenzene to cells suspended in 20 mM phosphate-buffered saline, pH 7.3, with 2 mM glutathione.

Vitamin E. Vitamin E, 7 μM , α -tocopherol succinate was added to parallel C3H/10T-1/2 cultures employed in the transformation studies and left in the medium for 24 hr. Another group of cells was pretreated for the 24-hr period with vitamin E and selenium to assay for a synergistic or additive effect of selenium with vitamin E. Control cells (no selenium and no vitamin E treatment) served for comparison. Peroxide breakdown, catalase, and glutathione peroxidase were assayed in the cells as described above.

RESULTS

Cell Transformation. Our studies show that pretreatment of C3H/10T-1/2 cells with selenium (Na_2SeO_3) for 24 hr prior to their exposure to X-rays, benzo[*a*]pyrene, or tryptophan pyrolysate markedly reduces the frequency of cell transformation (Table 1). Selenium at 2.5 μM was nontoxic and did not alter cell growth. The protective effect of selenium prevailed throughout the 6-week period of the experiment (24, 25), even though it was removed from the medium 72 hr after its addition.

Vitamin E inhibited cell transformation, induced by x-irradiation, benzo[*a*]pyrene, and tryptophan pyrolysate, and augmented the inhibitory action of selenium in additive fashion (Table 1). Vitamin E (7 μM) alone or with selenium did not alter cell survival or cell growth.

The interference of selenium and vitamin E in the process of radiation and chemically induced transformation appears to be one of protection. Ongoing time-course studies indicate that the inhibitory action of selenium and vitamin E is decreased when these nutrients are added at various times after carcinogen treatment (C.B., unpublished results).

Peroxide Breakdown, Glutathione Peroxidase, and Catalase. Following a 24-hr treatment by Na_2SeO_3 (selenium) the

Table 1. The effect of selenium and α -tocopherol succinate (vitamin E) on radiogenically and chemically induced transformation in C3H/10T-1/2 cells

Treatment	Survival fraction	Transformed foci/surviving cells, no./no.	Transformation frequency*
Control	1.00	0/33,931	0
Na_2SeO_3 †	0.97	0/49,875	0
Vitamin E	0.98	0/37,621	0
Na_2SeO_3 + vitamin E	0.98	0/41,781	0
400 Rad	0.75	65/66,168	9.8 ± 1.2
400 Rad + Na_2SeO_3	0.79	16/86,152	1.8 ± 0.8
400 Rad + vitamin E	0.81	14/79,765	1.7 ± 0.4
400 Rad + Na_2SeO_3 + vitamin E	0.79	10/110,361	0.9 ± 0.09
B[<i>a</i>]P‡	0.81	76/65,600	11.6 ± 1.3
B[<i>a</i>]P + Na_2SeO_3	0.82	14/87,501	1.6 ± 1.1
B[<i>a</i>]P + vitamin E	0.79	10/73,509	1.4 ± 0.9
B[<i>a</i>]P + Na_2SeO_3 + vitamin E	0.81	10/120,132	0.8 ± 0.06
Trp-P-2§	0.75	105/79,010	13.3 ± 1.1
Trp-P-2 + Na_2SeO_3	0.76	13/75,011	1.7 ± 0.7
Trp-P-2 + vitamin E	0.80	10/73,105	1.4 ± 0.3
Trp-P-2 + Na_2SeO_3 + vitamin E	0.81	10/108,200	0.9 ± 0.05

*The results represent pooled data from 3 experiments and are presented as mean \pm SE $\times 10^4$.

†Cells were treated with 2.5 μM Na_2SeO_3 or 7 μM α -tocopherol (vitamin E) or with both at plating time, 24 hr prior to carcinogen treatment and removal 72 hr later.

‡Benzo[*a*]pyrene (B[*a*]P) was added at 1.2 $\mu\text{g}/\text{ml}$ 24 hr after plating and maintained for 48 hr.

§Tryptophan pyrolysate (Trp-P-2) (26) was added at 0.5 $\mu\text{g}/\text{ml}$ and maintained in the culture for 48 hr.

C3H/10T-1/2 cells possessed a greater capacity to reduce peroxide compared to the untreated controls (Table 2). Cellular catalase levels were determined with the peroxide electrode by adding sodium azide (31). Azide has no effect on glutathione peroxidase, thus the differences in the rates of peroxidase reduction indicated the contribution of catalase to peroxide inactivation (Table 2). Direct measures of catalase, with an oxygen electrode (30), confirmed the findings illustrated in Table 2 (results not shown). The data indicate that the activities of both glutathione peroxidase and catalase increased with selenium treatment (Table 2).

In another series of experiments we treated cells with *N*-ethylmaleimide to deplete cells of glutathione thereby removing the substrate necessary for peroxide reduction by peroxidase (31) (Fig. 1). *N*-ethylmaleimide treatment (inhibition of glutathione peroxidase) combined with azide treatment (inhibition of catalase) completely inhibited cellular peroxide reduction. However, treatment of cells with *N*-ethylmaleimide in the absence of azide showed an enhanced peroxide breakdown in the selenium-treated cells compared to untreated controls.

Glutathione-S-Transferase. We determined glutathione-S-transferase activity in the C3H/10T-1/2 cells. Glutathione-S-transferase possesses a nonselenium-linked glutathione peroxidase activity. No increase in the activity of the enzyme was found following selenium treatment (values for the enzyme in untreated cells were 2.3 nmol per min per mg of protein and 2.4 nmol per min per mg of protein for selenium-treated cells).

Nonprotein Thiols. Nonprotein thiols including glutathione were determined in the C3H/10T-1/2 cells by using Ellman's method (33). A higher level of nonprotein thiol was observed in selenium-treated cells than in untreated cells (Table 3). The elevated nonprotein thiols in selenium-treated cells was due, mostly but not completely, to an increase in glutathione. This was seen by exposing the selenium-treated cells to L-buthionine sulfoximine (BSO) (34) for 24 hr thereby depleting cellular glutathione. As seen in Table 3 the nonprotein thiols were depleted to values less than 1% by L-buthionine sulfoximine in selenium-untreated cells and to 9.5% of control in the selenium-treated cells.

The results indicate that selenium treatment can elevate cellular glutathione, though it is unknown whether the increases in glutathione levels are confined to specific cellular compartments or are increased throughout the cell milieu.

We determined the effects of vitamin E (α -tocopherol succinate) alone and in conjunction with selenium on peroxide breakdown and on levels of catalase and glutathione peroxidase in the C3H/10T-1/2 cells. Vitamin E (7 μ M vit E) was added to selenium-treated and untreated cells and kept

Table 2. The effect of Na_2SeO_3 on glutathione peroxidase and catalase in C3H/10T-1/2 cells

Enzyme monitored	Control cells	Selenium-treated cells
Total peroxide reduction	9.8 \pm 0.2	26.7 \pm 1.3
Glutathione peroxidase	5.6 \pm 0.2	17.2 \pm 1.2
Catalase	4.2 \pm 0.1	9.0 \pm 0.6

Assays were carried out on 1×10^6 cells/ml in 50 mM phosphate-buffered saline, pH 7.4, with 5 mM glucose. In selenium-treated cells Na_2SeO_3 (2.5 μ M) was added to the cultures 24 hr prior to enzyme assay. Total cellular capacity to reduce peroxide was measured in the absence of azide. Glutathione peroxidase was measured by adding 20 mM sodium azide to inhibit catalase activity (31). The difference between the values obtained in the presence and absence of azide represents the amount of catalase activity. The data represent the average of three trials. Enzyme activity is expressed as nmol peroxide reduced per min per mg of protein \pm SEM.

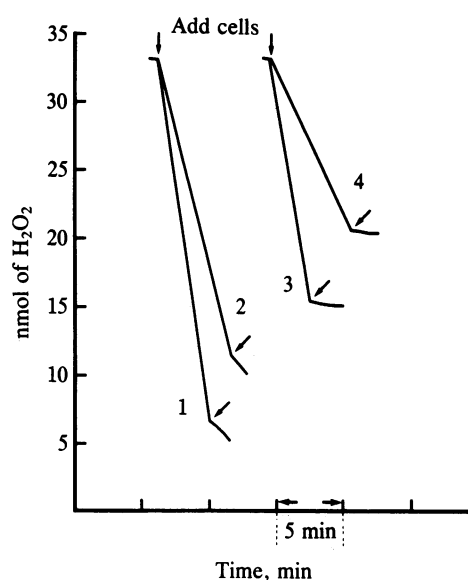


FIG. 1. Decomposition of H_2O_2 by C3H/10T-1/2 cells using an oxidase meter (30). Selenium-treated cells, traces 1 and 3, or untreated cells, traces 2 and 4, were added to the buffer mixture at 10^6 cells/ml at times indicated by upper arrows. Peroxide decomposition was recorded for 5 min. In the absence (traces 1 and 2) or presence of 20 mM sodium azide (traces 3 and 4), 0.2 mM *N*-ethylmaleimide (an inhibitor of glutathione) was then added at times indicated by lower arrows to inhibit peroxidase activity. The reaction medium contained 20 mM Hepes, 10 mM KCl, and 0.15 M NaCl, pH 7.2 at 37°C.

on for 24 hr. No enhancement of peroxidase breakdown or of catalase and peroxidase activities were observed in cells treated with vitamin E alone. In addition, no amplification of the selenium effect was observed in enhancing peroxide breakdown or in increasing peroxidase and catalase activities (data not shown).

DISCUSSION

Recent experimental findings and some current thoughts have underscored the important role of free radical reactions (12, 13) in the process of carcinogenesis (1–6, 8–10, 25). Much of the compelling evidence has emanated from the observations that antioxidants and enzymes that scavenge free radicals or that react with their products serve as powerful anticarcinogens and inhibit the neoplastic process at its various stages (2–11, 15, 16, 35, 36).

An initiating event in many free radical reactions may be the production of superoxides (O_2^-) (12, 13), which are formed naturally in cellular metabolic processes and are enhanced in living cells by the action of radiation and a variety of chemicals (3, 5, 10, 12, 13, 36–38).

Superoxides dismutate to form hydrogen peroxides that can react with reduced metals (Fenton reactions) to produce

Table 3. The effect of Na_2SeO_3 on nonprotein thiols (glutathione and other nonprotein thiols)

Treatment	Nonprotein thiol, nmol/mg of protein*
– Na_2SeO_3 – BSO	10 \pm 0.7
– Na_2SeO_3 + BSO	None detected
+ Na_2SeO_3	21 \pm 0.9
+ Na_2SeO_3 + BSO	2 \pm 0.5

BSO, L-buthionine sulfoximine (34).

*Mean \pm SEM.

the more toxic reactive hydroxyl radicals. Hydroxyl radicals react with biologically important molecules such as lipids and DNA to produce additional chain reactions with oxygen. The results are a variety of products such as aldehydes, which cross-link cellular macromolecules including DNA (37, 38) and may play a role in the carcinogenic process. While x-ray action directly leads to oxygen radicals that play a role in initiation and promotion (3–5), procarcinogens like benzo[*a*]pyrene or pyrolysates produce free radicals but also employ oxygen radicals for their activation via the P-450 system (13).

Cellular defenses include vitamin E, which prevents peroxidative processes by sequestering free radicals, as well as catalase and glutathione peroxidase with its coordinating mineral, selenium, which detoxify peroxides and protect the cells from subsequent deleterious effects.

Our present studies show that when selenium is added to C3H/10T-1/2 cells as Na₂SeO₃ at concentrations of 2.5 μM, it inhibits transformation induced by x-rays and by two chemical carcinogens, benzo[*a*]pyrene, an environmental pollutant, and tryptophan pyrolysate (Trp-P-2), a pyrolysis product from broiled protein foods (26–28). All three oncogenic agents are producers of free oxygen species. Since selenium inhibits radiation-induced as well as chemically induced transformation (Table 1), its mechanism of action is mediated at least in part via a route that is independent of metabolic modification of the carcinogens.

We find that selenium pretreatment of C3H/10T-1/2 cells results in an increased cellular breakdown of peroxide (Fig. 1) and in enhanced levels of specific peroxide detoxifying enzymes, i.e., catalase and the selenoenzyme glutathione peroxidase. Glutathione, a nonprotein thiol required for glutathione peroxidase activity and in itself a scavenger of free radicals (12, 13, 34) was also elevated. However, glutathione-*S*-transferase, a selenium-independent enzyme (39), was unaltered.

Pretreatment of the C3H/10T-1/2 cells with vitamin E at 7 μM alone or in conjunction with 2.5 μM selenium inhibited transformation by x-rays, benzo[*a*]pyrene, and tryptophan pyrolysate (Table 1) but had no effect on peroxide breakdown or on cellular glutathione peroxidase, catalase, or glutathione in the C3H/10T-1/2 cells. The inhibition of transformation by vitamin E and selenium suggests an additive effect. It is possible that the abundance of vitamin E in the cell precludes its effectiveness in altering enzyme levels and peroxide destruction and that the inhibitory action of vitamin E on transformation proceeds via a different mechanism than that of selenium.

Morphological transformation *in vitro* represents an early step in the conversion of the cells to malignancy (24, 25, 40–46).

The results of the above study indicate that active oxygen species (i.e., hydroperoxides) may play a role in radiogenic and chemically induced transformation and that the initiation of the neoplastic process by these agents can be prevented by nontoxic levels of selenium, a micronutrient in our diet and by vitamin E. The data suggest that the protective action of selenium as an anticarcinogen is mediated via the enhancement of cellular protective systems and by an increased cellular capacity to destroy peroxides.

The results further suggest that selenium and vitamin E can serve as cancer preventing agents that act in concert but operate via different mechanisms. The work underscores the concept that appropriate dietary measures in our lifestyle (2, 7, 14, 26, 28) can lower the rate of human affliction by cancer.

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