

# Inhibition of Benzo(a)pyrene-induced Mouse Forestomach Neoplasia by Dietary Soy Sauce<sup>1</sup>

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## ABSTRACT

We show that Japanese-style fermented soy sauce (shoyu) contains anticarcinogenic activity. Female ICR mice were fed a semipurified diet containing soy sauce (0–30%). Two weeks later a regimen consisting of 4 doses of benzo(a)pyrene (1 dose/week p.o. for 4 weeks) was begun to initiate forestomach neoplasia. Twenty-three weeks after the first initiation the animals were sacrificed, and forestomach neoplasms were counted and histologically confirmed. Soy sauce produced a significant dose-dependent reduction in forestomach neoplasms, which appeared to be maximal when soy sauce constituted 20% of the diet. Exposure to nitrite (0–500 ppm through drinking water) neither enhanced nor diminished the anticarcinogenic effect of the dietary soy sauce. Soy sauce was found to contain antioxidant activity which may be related to the observed anticarcinogenic effect. Contrary to expectations, mouse forestomach ornithine decarboxylase activity was induced by soy sauce. This appeared to be due at least in part to the relatively high sodium chloride content of soy sauce.

## INTRODUCTION

Wakabayashi *et al.* (1) reported that Japanese-style fermented soy sauce (shoyu) contains precursors of bacterial mutagens. They postulated that these substances may be related to the relatively high incidence of stomach cancer in Japan, which is about 8-fold higher than that observed in the United States (2). The model advanced by these investigators (1) held that nitrite in the gastrointestinal tract (from consumption of vegetables containing nitrate) might react *in vivo* with the mutagen precursors in soy sauce, resulting in the generation of one or more carcinogenic nitroso compounds.

Given this interesting hypothesis we undertook an investigation of the effects of soy sauce and nitrite on BP<sup>3</sup>-induced mouse forestomach neoplasia (3). Contrary to expectations we found that soy sauce (in the diet) plus nitrite (in the drinking water) significantly reduced forestomach neoplasm formation. Soy sauce given without nitrite produced a smaller, nonsignificant inhibitory effect. The cross-over design of the study permitted tentative separation of effects on initiation from effects on promotion and indicated that inhibition of neoplasia by soy sauce plus nitrite probably occurred during the tumor promotion stage.

One possible explanation for these observations is that nitrite (a reducing agent without antioxidant activity) may have generated an anticarcinogenic product, possibly an antioxidant, from an otherwise inert component of soy sauce. Japanese soy sauce is the result of a complex microbial fermentation of

soybeans and wheat; during storage oxidative reactions occur (4). Our previous study (3) was conducted with soy sauce that had been stored for some time and which likely contained many oxidized products that could have been reduced by nitrite.

In the present study care was taken to use newly manufactured soy sauce containing a minimum of oxidized products. The soy sauce by itself (without nitrite) exhibited a pronounced anticarcinogenic effect on BP-induced mouse forestomach neoplasia. Moreover, the anticarcinogenic effect was neither enhanced nor diminished by nitrite. The soy sauce was found to contain antioxidant activity. Surprisingly, despite its anticarcinogenic effect soy sauce induced ornithine decarboxylase activity in mouse forestomach.

## MATERIALS AND METHODS

**Materials.** BP (98% pure), NaNO<sub>2</sub>, TPA, potassium oxalate, bathophenanthroline, and dimethyl sulfoxide were purchased from Sigma Chemical Company (St. Louis, MO). *N*-Amyl acetate was purchased from Aldrich Chemical Co. (Milwaukee, WI). DL-[1-<sup>14</sup>C]Ornithine hydrochloride (specific activity, 50–60 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Ferric nitrate was from Mallinckrodt, Inc. (Paris, KY). At the beginning of each experiment freshly prepared soy sauce (shoyu) was supplied by the Kikkoman Company (Walworth, WI). The soy sauce contained no added antioxidants or other ingredients.

**Treatment of Mice.** Two independent experiments were conducted. For each, 300 five-week-old female ICR mice were purchased from Sprague-Dawley (Madison, WI). The animals were housed in polycarbonate cages (5 mice/cage) in a temperature- and humidity-controlled facility and permitted free access to water and food (TD86348; Teklad Test Diets, Madison, WI) for 2 weeks. The animals were then randomly assigned to 1 of 2 groups (150 animals each). In experiment 1, the groups were fed diet supplemented with either 20% soy sauce or water. Subgroups (25 animals from each group of 150) were given drinking water containing nitrite (0–500 ppm). In experiment 2, the groups were given either plain drinking water or water containing 500 ppm nitrite. Subgroups of 25 animals were fed diet containing soy sauce (0–30%). These regimens were maintained for the duration of the experiments. Body weight and intake were recorded once and twice weekly, respectively. Diet was prepared weekly and stored at 4°C. Nitrite-containing water was prepared just prior to dispensing.

**Induction of Forestomach Neoplasia.** Forestomach neoplasia was induced according to a previously described protocol (3). Two weeks after assignment to one of the experimental groups described above, animals were given 2 mg BP in 0.2 ml corn oil (p.o.). This dose was repeated once a week for 4 weeks. The mice were sacrificed at 211 days of age by CO<sub>2</sub> suffocation. The stomachs were removed and fixed in an expanded state by injection of 4% formalin. Twenty-four h later they were split longitudinally, and forestomach tumors (1 mm or larger) were counted under a dissecting microscope. Subsequently the tumors were confirmed histologically.

**ODC Assay.** Mice were intubated with 0.2 ml soy sauce, 0.2 ml 17% (w/v) NaCl in water, or 0.2 ml TPA (6 µg total, prepared by diluting 0.1% TPA in dimethyl sulfoxide with water). At intervals thereafter the animals were sacrificed by CO<sub>2</sub> suffocation. The forestomachs were rapidly excised, individually placed in 1.5 ml ice-cold buffer (50 mM Tris-HCl buffer, pH. 7.5, containing 0.1 mM pyridoxal phosphate, and 0.1 mM EDTA), and homogenized using a Polytron PT-10 homoge-

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<sup>3</sup> The abbreviations used are: BP, benzo(a)pyrene; ODC, ornithine decarboxylase (EC 4.1.1.17); TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

nizer for 30 s at 0–4°C. Samples were centrifuged at 30,000 × *g* for 30 min, and the supernatant fractions were assayed by measuring release of CO<sub>2</sub> from [1-<sup>14</sup>C]ornithine (5). Protein was quantified by the method of Lowry *et al.* (6).

**Determination of Antioxidant Activity.** Antioxidant activity was determined by the method described by Tsen (7). Assays contained 0.5 ml acetate buffer (pH 4.5), 0.05 ml ferric nitrate (10 mM), 0.2 ml bathophenanthroline (10 mM), and 0.8 ml amyl acetate extract of a given sample. The absorbance of the top pink layer was measured in a Beckman DU-7 spectrophotometer at 534 nm.

**Statistical Methods.** Data are expressed as means ± SEM and analyzed for statistical significance using Scheffe's test.

## RESULTS

In experiment 1, soy sauce produced a substantial anticarcinogenic effect. Control mice (139 surviving to completion) exhibited  $9.1 \pm 0.5$  neoplasms/animal, with an incidence of 98%. By contrast mice fed soy sauce (138 surviving to completion) exhibited  $2.5 \pm 0.2$  neoplasms/animal, with an incidence of 72%.

Fig. 1 shows data from experiment 1, plotted according to nitrite intake which was varied between 0 and 500 ppm. The data show that nitrite neither enhanced nor inhibited the anticarcinogenic effect of the soy sauce. Moreover, neither body weight nor caloric intake was affected by nitrite ingestion (data not shown). By contrast both these parameters were significantly different ( $P < 0.05$ ) for control *versus* soy sauce-fed animals: body weight was  $36.3 \pm 0.5$  g (controls) *versus*  $31.2 \pm 0.3$  g (soy sauce-fed); total calorie intake/mouse was  $2074 \pm 9$  kcal (controls) *versus*  $1967 \pm 9$  kcal (soy sauce-fed).

Experiment 2 was then conducted to determine the dose-response relationship between the level of soy sauce in the diet and forestomach neoplasm development. Both neoplasms per animal and the incidence of neoplasia were reduced by increasing the intake of soy sauce (Fig. 2). The maximal level of inhibition occurred at about 20% soy sauce in the diet. In confirmation of the results from experiment 1, concomitant exposure to 500 ppm nitrite neither enhanced nor diminished the anticarcinogenic effect of the soy sauce (Fig. 2). Also in confirmation of the results of experiment 1, the data of Fig. 3 show that increasing the amount of soy sauce in the diet resulted in a slowly progressive reduction in both caloric intake and body weight.

Given that many antioxidants are also anticarcinogens (8), we assayed soy sauce for antioxidant activity (Table 1). Substantial antioxidant activity was found. Extraction with ethyl acetate resulted in about 150-fold purification of antioxidant activity, which is 3 times greater than that displayed by a 1% solution of ascorbic acid (Table 1, Footnote c).

We also determined the effect of soy sauce on forestomach ODC activity (Fig. 4). Contrary to expectations, soy sauce induced ODC activity as did TPA. A solution containing the same amount of sodium chloride as that present in soy sauce also induced ODC activity, although not to the same extent as soy sauce.

## DISCUSSION

There is considerable interest in identifying anticarcinogens from food sources, with an eye toward using such substances in a rational manner to reduce cancer risk in humans. The first step is to identify foods or food extracts that exhibit anticarcinogenic activity in animal models. The active materials should

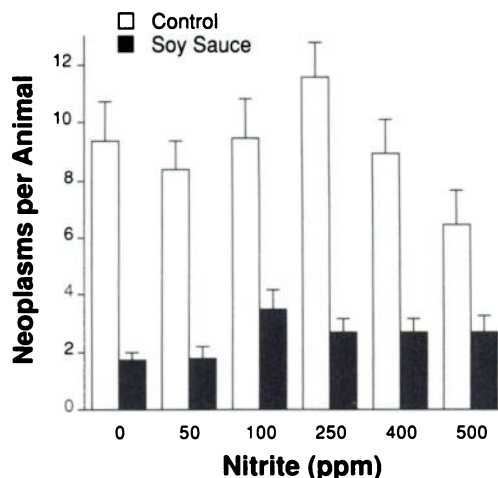


Fig. 1. Lack of effect of NaNO<sub>2</sub> in drinking water on the number of neoplasms per animal (columns, mean; bars, SEM) in mice fed a diet containing 20% soy sauce or a diet without soy sauce.

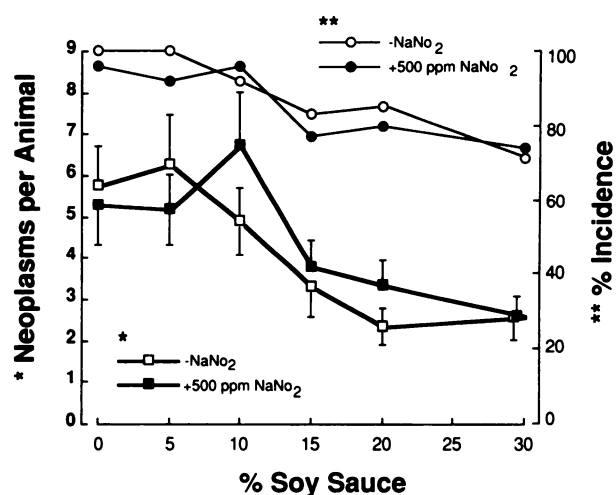


Fig. 2. Effect of dietary soy sauce on the number (points, mean; bars, SEM) and incidence of neoplasms per animal. ●, ■, 500 ppm nitrite in water; ○, □, plain water without nitrite.

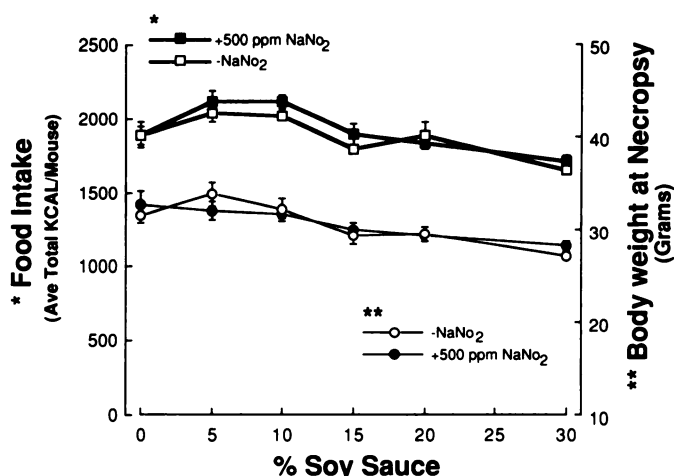


Fig. 3. Food intakes and body weights of animals from Fig. 2. Points, mean; bars, SEM.

Table 1 Antioxidant activity of soy sauce and its partial purification by ethyl acetate extraction

Sample	neq/ml sample <sup>a</sup>	Wt (mg/ml)	Specific activity (neq/mg) <sup>b,c</sup>
Soy sauce	66.8	389.5	0.17
Ethyl acetate-soluble fraction	46.9	1.7	27.6
Ethyl acetate-insoluble fraction	31.6	309.4	0.10

<sup>a</sup> neq of antioxidant activity.

<sup>b</sup> neq of antioxidant activity/mg.

<sup>c</sup> A 1% solution of ascorbic acid exhibited a specific activity of 9.79 neq of antioxidant activity/mg; a solution of amino acids mimicking the amino acid concentration of soy sauce exhibited a specific activity of 0.028 neq of antioxidant activity/mg.

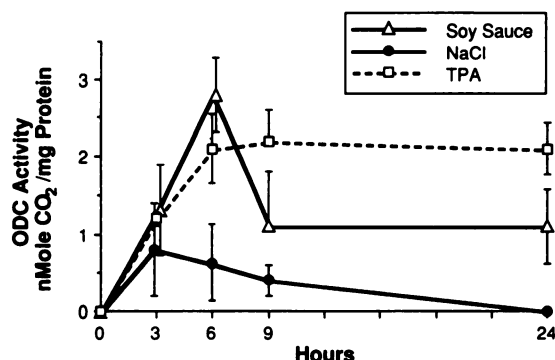


Fig. 4. Induction of forestomach ODC activity by soy sauce, NaCl, or TPA. Points, mean of 3 determinations; bars, SEM.

then be purified and identified. Finally, exhaustive mechanistic studies are required to determine if the anticarcinogen under study might be useful in preventing human cancer, particularly among persons at unusually high risk for one or more types of neoplasm.

The studies described in this report establish that soy sauce exhibits anticarcinogenic activity. Our previous observation (3) that nitrite was required to enhance the anticarcinogenic activity of soy sauce may have been dependent on the use of aged material which had to some extent oxidized, since the newly manufactured soy sauce used in this study was effective by itself. In the present study, nitrite neither enhanced nor diminished the effect.

We found that soy sauce contains substantial antioxidant activity (Table 1). Given that many antioxidants inhibit carcinogenesis (8), it is possible that one or more of the antioxidants in soy sauce are mechanistically linked at least in part to the anticarcinogenic effects of this food product. Current work to purify and identify these antioxidants is focused on the ethyl acetate fraction, where antioxidant activity was increased about 150-fold. Very recent findings, to be published elsewhere, indicate that both the ethyl acetate-soluble and -insoluble fractions (Table 1) contain anticarcinogenic activity. Hence there may be several anticarcinogenic components in soy sauce which act at different steps in the process of carcinogenesis in this model system, beginning with BP metabolism and continuing through to the inhibition of tumor promotion.

Feeding soy sauce resulted in a small but significant reduction in calorie intake and body weight gain (see "Results" and Fig.

3). To our knowledge there are no published data relating calorie intake to forestomach neoplasia in mice; hence the extent to which such effects contributed to the inhibition of neoplasia remains to be elucidated. However, our very recent findings with fractionated soy sauce (cited above) indicate that anticarcinogenic activity is associated with material that has no effect on body weight or food intake.

Many studies (9) have demonstrated that protease inhibitors from soybeans are effective in cancer prevention in rodents. However, during the fermentation and pasteurization steps involved in producing soy sauce, protease inhibitors are reduced and probably account for little if any of the anticarcinogenic activity observed in our experiments.

The enhancing effect of soy sauce on forestomach ODC activity came as a surprise. We had projected that the stimulatory effect of sodium chloride, a known tumor promoter for the glandular stomach in rats (10, 11), might be countered by the anticarcinogens in soy sauce, but this is clearly not the case (Fig. 4). Previously (3) we provided tentative evidence that soy sauce inhibits the tumor promotion stage in this model. Given this observation coupled with the strong possibility that soy sauce contains multiple anticarcinogens (discussed above) and the proposed relationship between the induction of ODC activity and tumor promotion (5, 12), it follows that at least some of the inhibitory action of soy sauce may be exerted at a step past the induction of ODC. Further mechanistic studies with purified compounds will be necessary to establish or disprove this conjecture.

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