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Grapefruit and Oroblanco Enhance Hepatic Detoxification Enzymes in Rats: Possible Role in Protection against Chemical Carcinogenesis

MICHAL HAHN-OBERCYGER, ALIZA H. STARK, AND ZECHARIA MADAR*

Faculty of Agricultural, Food and Environmental Quality Sciences, Institute of Biochemistry, Food Science and Nutrition, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

Citrus fruits are considered to be functional foods that promote good health. This study was carried out to assess the effect of oroblanco and grapefruit consumption on hepatic detoxification enzymes. Male Sprague−Dawley rats were provided with either regular drinking water (control) or experimental treatments of oroblanco juice, grapefruit juice, or a sugar mix for 6 weeks. After 1 week of treatment, half the animals in each group were injected with the procarcinogen 1,2-dimethylhydrazine. Grapefruit juice significantly increased activity and expression of the hepatic phase I enzyme, cytochrome P450 CYP1A1, with a marked trend toward enhanced NAD(P)H:quinone reductase (QR) activity. Oroblanco juice significantly increased glutathione *S*-transferase phase II enzyme activity along with CYP1A1 expression and a notable trend toward increased activity of both CYP1A1 and QR. These results suggest that these citrus fruits are bifunctional inducers, modulating both phase I and phase II drug-metabolizing enzymes to enhance hepatic detoxification.

KEYWORDS: Grapefruit; oroblanco; phase I enzymes; phase II enzymes; detoxification; rats

INTRODUCTION

It is well-known that dietary factors play an important role in the prevention of human diseases including cancer (*1*, *2*). Thus, identifying foods that promote health, "functional foods", and providing scientific evidence to support health claims are at the forefront of modern agricultural and nutritional research.

Results from numerous epidemiological studies have shown a protective effect of high vegetable and fruit intake and cancer development (*3*, *4*). It has been hypothesized that certain fruits and vegetables act as chemopreventive agents by enhancing the liver detoxification system. Several studies in cruciferous vegetables and garlic support this theory (*5*, *6*), but little is known about the effects of citrus fruits on detoxification and no studies have been carried out to assess the impact of oroblanco. Commonly known as "sweetie", this fruit was first produced by a University of California breeding program in 1958. Oroblanco originated from a cross between a low acid content pomelo and a white seedless grapefruit. The fruit is considered to be a good source of dietary flavonoids, and its juice contains naringin (470 mg/L) and hesperidin (7.7 mg/L). Grapefruit juice also contains considerable amounts of flavonoids, 540 mg/L naringin and 16.0 g/L hesperidin.

One of the body's early lines of defense against toxins and carcinogens is the hepatic detoxification enzymes, also called biotransformation enzymes and drug-metabolizing enzymes. Phase I enzymes (the cytochrome P450 family) found in the liver are known to "activate" compounds by adding a single nitrogen or oxygen, whereas phase II enzymes usually detoxify the harmful substances by converting them to hydrophilic metabolites that can readily be excreted from the body (*7*−*9*). The phase II enzymes include NAD(P)H:quinone reductase (QR), glutathione *S*-transferase (GST), epoxide hydrolase, and UDP-glucuronosyltransferase and are known to be inducible in animals and humans (*9*, *10*). A strong inverse relationship exists between tissue levels of phase II enzymes and susceptibility to chemical carcinogenesis (*11*, *12*). As a group, these inducible enzymes facilitate the metabolic detoxification of xenobiotics in mammals and can achieve chemopreventive activity by modification of carcinogen metabolism through increased carcinogen excretion and decreased carcinogen−DNA interactions.

The procarcinogen, 1,2-dimethylhydrazine (DMH), is an organotropic colon carcinogen that undergoes hepatic metabolic activation to azoxymethane and methylazoxymethanol, which leads to the formation of methylcarbonium ions, believed to be DNA-reactive metabolites. The cytochrome P450-1A enzymes are part of the hepatic DMH-metabolizing pathway (*13*).

The flavonoids are a family of polyphenol compounds found in many food items derived from plants and are considered to have a wide range of biological activities (*14*, *15*). Studies show that isolated flavonoids have the ability to inhibit cell cycle and cell proliferation (*16*, *17*) and can induce phase I and phase II hepatic detoxification enzymes including cytochrome P450, GST, and QR (*18*, *19*). With the exception of cruciferous vegetables, few in vivo studies using whole foods have been

* Corresponding author (telephone 972-8-9489008; fax 972-8-9363208; e-mail madar@agri.huji.ac.ilP.

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carried out to evaluate the impact on hepatic detoxification processes. The present study investigated the possible modifying effect of two citrus fruits, grapefruit and oroblanco, on hepatic detoxification enzymes in rats following exposure to the procarcinogen DMH. Because certain phytochemicals are known to increase activities of detoxification enzymes, the effects on the activity of both phase I and phase II liver detoxification enzymes GST, QR, and CYP1A1 were determined.

MATERIALS AND METHODS

Animals. Fifty-six male Sprague−Dawley rats (Harlan, Jerusalem, Israel), weighing 140−160 g, were maintained under controlled environmental conditions (12:12 h light/dark cycle and temperature of 23 °C). Animals were housed in plastic cages and given free access to a standard chow diet (Harlan), and treatments were administered through the drinking water. Body weight was recorded weekly, and liquid consumption was recorded daily.

Treatment. Animals were divided into four groups ($n = 14$), and control animals received tap water. Experimental treatments included oroblanco juice or grapefruit juice and an additional control diet including a simple sugar mix in concentrations identical to those present in the oroblanco juice (15.88 g/L fructose, 16.01 g/L glucose, and 50.13 g/L sucrose). Fruit juices were produced locally (Ganir Ltd., Sde Gat, Israel) by mechanical extraction and concentrated 5−6-fold by short-time evaporation and pasteurization. Concentrates were stored frozen until use. The juice was reconstituted by adding distilled water at a ratio of 1:5, concentrate to water, and given to animals in sterilized bottles. Along with flavonoids the juices contained minerals (K, Na, Ca, Mg, Cl, P), vitamin C, and organic acids. Juice was provided daily to prevent fermentation, which occurred rapidly at room temperature.

Experimental Design. Following a week on the experimental regimen, half the animals in each treatment ($n = 7$) received two intramuscular injections of DMH, a procarcinogen that specifically induces colon cancer in rodents. The injections were administered (30 mg/kg of body weight) with a week interval between doses. The experiment lasted a total of 6 weeks, and at the termination of the experiment, animals were anesthetized with Nembutal (8 mg/100 g of body weight), and liver samples were collected and stored at −80 °C for further analysis. All rats were cared for under the guidelines set forth by the Animal Care Committee of the Hebrew University, Jerusalem, Israel.

Preparation of Liver Samples. Liver tissue (1 g) was homogenized in 4 mL of assay buffer (50 mM Tris-HCl, pH 7.4, and 250 mM sucrose). The homogenates were centrifuged at 13500*g* for 20 min (2−4 °C), and the supernatant fraction was collected and centrifuged at 105000*g* for 60 min. The supernatant was taken for the cytosolic assays. The remaining microsomal pellet was suspended in 10 mM Tris-HCl buffer, pH 7.6, containing 150 mM KCl and centrifuged at 105000*g* (4 °C) for 60 min. The resulting washed microsomal pellet was resuspended in 2 mL of 0.1 M sodium−potassium phosphate buffer, pH 7.6. Protein concentration was determined using the Bradford method (*20*). Bovine serum albumin served as a standard.

Total GST Activity. Liver cytosolic GST activity was determined using the method described by Habig et al. (*21*) with glutathione (GSH) and 2,4-dinitrochlorobenzene (CDNB) as substrates. This method determines enzyme activity of the numerous isoforms of GST with the exception of GST-τ. The reaction mixture (1 mL) contained 0.1 M potassium phosphate buffer (pH 6.6), 5 mM GSH, 1 mM CDNB, and a suitable amount of cytosol (5−50 μg of protein). The reaction was started by the addition of the cytosolic enzyme. The increase in absorbance at 340 nm over a 5 min period was measured spectrophotometrically (UV 1601, Shimadzu) at 25 °C. An extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to determine activity from the initial slope of the reaction. GST activity was expressed as nanomoles of CDNB-GSH conjugates formed by 1 mg of protein per minute.

QR Activity. The activity of cytosolic QR in liver tissue was determined using a modified method described by Ernster (*22*) using 2,6-dichloroindophenol as a substrate. Briefly, cystoloic protein (5−50 μg) was added to 1 mL of assay buffer (25 mM Tris-HCl, pH 7.5, 60 μg of bovine serum albumin, 5 μM FAD, 0.2 mM NADH, 80 μM 2,6-dichloroindophenol, and 0.01% Tween 20). The reaction was performed at 25 °C for 5 min. Absorbance at 600 nm was monitored, and an extinction coefficient of 21 mM^{-1} cm^{-1} was used to determine activity from the initial slope of the reaction. QR activity was expressed as nanomoles of 2,6-dichloroindophenol reduced per minute per milligram of protein.

CYP1A1 Activity. Liver microsomal CYP1A1 was assayed using the method described by Burke et al. (*23*) with ethoxyresorufin as substrate. The reaction mixture (2 mL) contained 0.1 M potassium phosphate buffer (pH 7.8), 50 μM ethoxyresorufin, 50 mM NADPH, and a suitable amount of microsomal suspension (5−20 mg of protein/mL). A baseline of fluorescence was recorded at an excitation wavelength of 510 nm and an emission wavelength of 586 nm with a fluorometer (Quantech). Ten microliters of NADPH was stirred into the mixture to start the reaction, and the progressive increase in fluorescence, as ethoxyresorufin was deethylated to resorufin, was recorded. The reaction was performed at 30 °C. The fluorometer was calibrated with 10 μL of resorufin (0.01 mM in ethanol). CYP1A1 activity was expressed as nanograms of resorufin formed by 1 mg of protein per minute.

Western Blot Analysis. Protein extracts (100 μg/lane) were incubated at 100 °C for 5 min under reducing conditions and then size-fractionated on SDS−polyacrylamide gel. After electrophoresis, proteins were transferred at a constant current of 250 mA for 120 min onto nitrocellulose membranes using blotting buffer containing 10% methanol. Following transfer, the membrane was incubated in blocking solution (TBS) containing 5% nonfat dry milk. The membrane was washed with TBS and stained with Ponceau red. After five washes with TBS, the membrane was then incubated with primary antibodies in a solution of TBS with 5% nonfat dry milk (mouse monoclonal anti-GST at a dilution of 1:1000) and goat anti-CYP1A1 at a dilution of 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature. After washing with TBS, the membrane was then incubated with secondary antibodies in TBS buffer with 5% nonfat dry milk [goat anti-mouse for GST, donkey anti-goat for CYP1A1 (Jackson Immuno Research Laboratories, Inc., West Grove, PA].

Immunodetection was performed using the ECL detection kit (Amersham Life Science, Amersham, Bucks, U.K.). The film was scanned, and the density of the bands was calculated using scanning densitometry and expressed as arbitrary units.

Statistical Analysis. Numerical data were expressed as mean ± SE. Differences among means were determined by two-way analysis of variance (ANOVA) unless interaction between variables was significant, and then a one-way ANOVA followed by the Tukey−Kramer test was used. Where deemed to be appropriate, data were log transformed to equalize variance. Differences were considered to be significant at probability levels of $P < 0.05$.

RESULTS

Growth rates of experimental animals were similar in all treatment groups (**Figure 1**) with no significant differences in final body weights (**Table 1**). Both factors tested in this study, supplementation of the diet with citrus juice or sugar water and exposure to DMH, had no negative affect on growth in the 6 week treatment period.

CYP1A1 Activity and Expression. Liver microsomal CYP1A1 activity (**Table 1**) was greatest in grapefruit (19.03 ± 1.07 ng of resorufin·min^{-1} mg^{-1} of protein) and oroblanco (16.93 ± 1.54 ng of resorufin·min^{-1} mg^{-1} of protein) groups compared with water (14.01 ± 1.14 ng of resorufin·min^{-1} mg^{-1} of protein) and simple sugar mix (13.64 ± 0.89 ng of resorufin·min^{-1} mg^{-1} of protein) groups. Significance was reached with grapefruit juice, but oroblanco levels had borderline significance ($P = 0.0599$, two-factorial design). No significant differences were observed in CYP1A1 activity between healthy rats and those treated with DMH for all experimental groups (**Table 1**). When statistical analysis was performed only on rats

Table 1. Body Weight and Hepatic Enzyme Activity Measured on Day 42 in Rats That Consumed Oroblanco, Grapefruit, Sucrose Mix, or Water with or without Exposure to DMH[a]

	oroblanco		grapefruit		sucrose mix		water	
	+DMH	−DMH	+DMH	−DMH	+DMH	−DMH	+DMH	−DMH
final body weight (g)	325 ± 5	323 ± 11	303 ± 4	314 ± 5	316 ± 4	331 ± 12	328 ± 11	336 ± 9
CYP1A1 activity (ng of resorufin·min^{-1} mg^{-1} of protein)	18.1 ± 2.3ab	15.7 ± 2.1ab	18.7 ± 1.8a	19.4 ± 1.3a	13.0 ± 1.6b	14.2 ± 0.9b	14.8 ± 2.1b	13.4 ± 1.0b
GST activity (nmol·min^{-1} mg^{-1} of protein)	24.9 ± 1. 3a	16.1 ± 1.0a*	21.3 ± 1.2ab	14.3 ± 0.8ab*	21.5 ± 1.5ab	14.1 ± 1.2ab*	20.3 ± 1.0b	13.8 ± 1.5b*
QR activity (nmol·min^{-1} mg^{-1} of protein)	84.2 ± 22.7	57.2 ± 14.5	78.9 ± 23.0	59.6 ± 12.6	45.6 ± 7.0	42.0 ± 9.1	58.6 ± 9.5	37.2 ± 10.1

[a] DMH, 1,2-dimethylhydrazine. Results are means ± SE of seven rats. Values within rows with different letters represent significant differences between nutritional treatments (water, sugar mix, or citrus juices). An asterisk (*) indicates significant difference from the +DMH group ($P < 0.0001$).

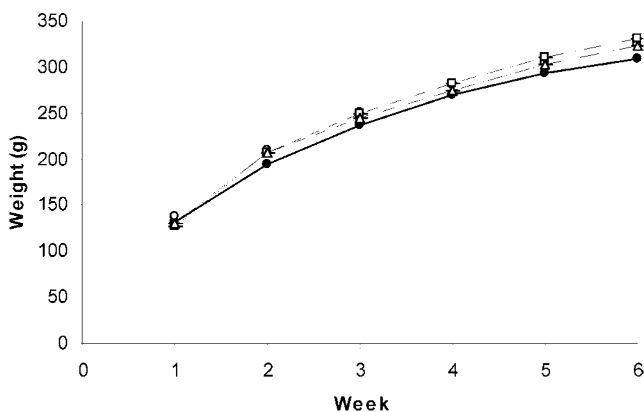

Figure 1. Effects of oroblanco (○), grapefruit (●), simple sugar mix (△), and water (□) on body weight over 6 weeks. Data in graph represent means of seven rats per treatment.

receiving the DMH treatment (single factorial), both grapefruit and oroblanco juices were significantly higher than the simple sugar mix and water controls. These results were confirmed by CYP1A1 expression, which was significantly increased ($P < 0.05$) in rats consuming oroblanco or grapefruit juice compared with the water and simple sugar mix groups. Results of protein expression also did not differ between healthy and DMH-treated rats (**Figure 1**).

GST Activity and Expression. Exposure to DMH significantly ($P < 0.0001$) increased hepatic GST activity in all groups of rats (**Table 1**). Oroblanco consumption significantly increased GST activity in cytosolic fractions in comparison to the water control group ($P = 0.031$). However, levels were not significantly greater than those of the simple sugar mix group or grapefruit group. The grapefruit group exhibited no increase in enzyme activity when compared to the water or simple sugar mix groups. Although some differences were observed in enzyme activity, the expression of GST was not significantly different among groups (**Figure 2**).

QR Activity. There was no significantly difference in QR activity among groups, although a distinct trend toward enhanced activity in animals treated with oroblanco and grapefruit was observed. In both healthy and DMH-treated rats this trend for increased QR activity was evident (**Table 1**).

DISCUSSION

Previous studies suggest that induction of hepatic detoxification enzymes is a relevant mechanism for cancer chemoprevention (*5*, *11*, *24*, *25*). In this study, oroblanco juice and grapefruit juice consumed by both healthy and DMH-treated rats increased CYP1A1 enzyme activity (**Table 1**). Enhanced enzyme activity was accompanied by a significant increase in CYP1A1 protein expression (**Figure 1**). Similar findings have been reported in whole animal models, where an increase in phase I enzyme activity was observed following dietary interventions with foods of plant origin (*6*, *13*, *18*). In contrast, Obermeier et al. (*26*) reported that a variety of isolated flavonoids inhibited the CYP1A enzyme in human liver microsomes. However, inhibition occurred at high concentrations, and moderate activation was actually observed at lower concentrations with some of the compounds. The variable effects of flavonoids on CYP1A activity may be due to the different models used in each study, which include cell culture models and other in vitro designs. Very few studies use whole animal models, and for the most part, isolated flavonoids are studied, not whole foods (citrus juice). It must be taken into account that other compounds present in the juice may be responsible for the enhanced enzyme activity and expression. Additionally, when whole foods are consumed, exposure levels are considerably lower in comparison to studies using isolated concentrates of biologically active compounds.

Interactions between grapefruit juice and other cytochrome P-450 isoforms have been documented. Grapefruit juice is a known inhibitor of intestinal CYP3A4, but the hepatic form of this phase I enzyme is not thought to be affected by juice consumption (*27*). In the present study, hepatic phase II enzyme activity was also enhanced by citrus consumption. Both oroblanco and grapefruit induced the activity of GST (**Table 1**). The observed increase in GST activity is consistent with previous animal studies, in which treatment of rats with dietary polyphenols resulted in significant induction of hepatic GST activity (*11*, *28*). This effect has also been observed in human studies in which addition of specific vegetables to the diet modified serum GST activity (*29*). However, results of Rijnkels et al. (*13*) differ from those reported here. Wistar rats fed a diet containing a vegetable−fruit mixture and treated with DMH exhibited decreased hepatic GST activity, suggesting that fruit and vegetable consumption might enhance carcinogenesis in this particular animal model.

The change in GST activity (total GST) was not accompanied by a concomitant increase of GST-π expression (**Figure 3**). This may be explained by the different mechanisms involved in the regulation of the various isozymes of GST. Furthermore, GST-π is only one of the many isoforms of the enzyme present in the liver, and it is possible that the levels of other isoforms were enhanced. Alternatively, enzyme activity may be enhanced without a concurrent increase in protein expression.

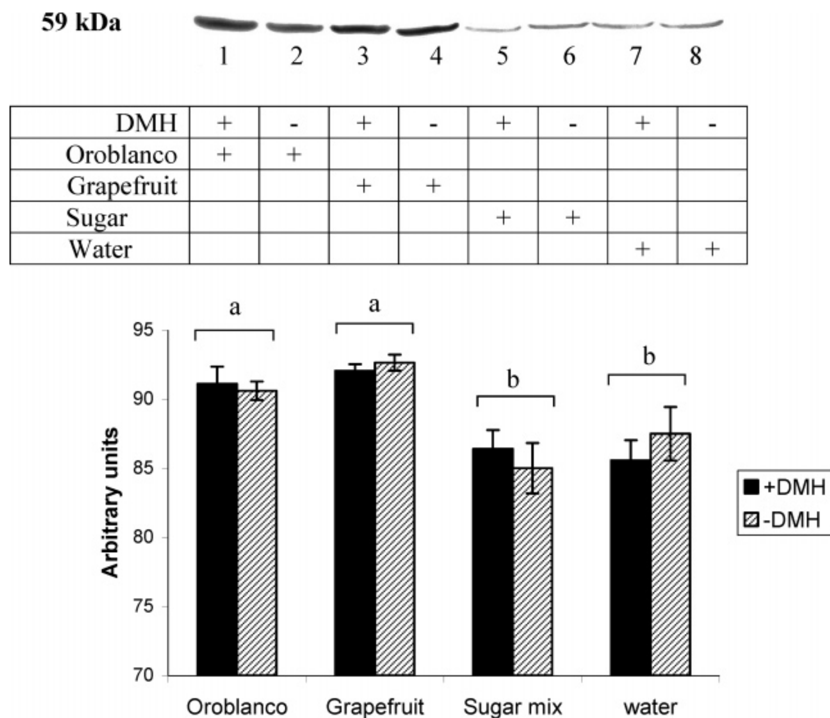

Figure 2. Liver CYP1A1 expression as detected by immunoblot analysis in DMH-treated and healthy rats that consumed oroblanco, grapefruit, simple sugar mix, and water. Data represent mean ± SE ($n = 7$). Different letters (a, b) represent significant differences among groups at $P < 0.05$.

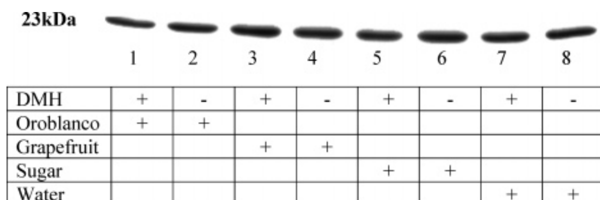

Figure 3. Expression of liver GST pi isoform (GST-π) as detected by immunoblot analysis in DMH-treated and healthy rats that consumed oroblanco, grapefruit, simple sugar mix, or water.

This study also investigated the effect of citrus juice on QR activity. A trend toward increased QR enzyme activity was observed in rat liver following treatment with oroblanco and grapefruit (**Table 1**), but differences did not reach significance. The trend was most notable in animals treated with DMH. Overall, the data indicate that citrus consumption has a positive impact on the phase II enzymes GST and QR, suggesting that they may enhance detoxification of carcinogens/toxins and their removal from the body. Various compounds, natural and synthetic, are capable of elevating phase II enzyme activity and are classified as monofunctional (phase II) inducers (*24*). Bifunctional inducers have the capacity to increase both phase I and phase II enzymes. The ability to induce both CYP1A1 and GST or QR enzymes indicates that citrus fruits operate as bifunctional inducers. These data are consistent with the finding of Talalay et al. (*9*) that the flavonoids, which occur abundantly in many common edible plants, are typical bifunctional inducers. DMH-treated rats had higher levels of GST and QR activity in comparison to healthy rats in all experimental groups. DMH is a potent carcinogen, and in order to exert its carcinogenic effect, DMH undergoes bioactivation via cytochrome P450 enzymes to produce the ultimate DMH carcinogen, methylazoxymethanol. DMH itself induces phase I and phase II enzyme activity, as well. However, in this study the activity of CYP1A1 was similar in DMH-treated and healthy rats. This observation does not support the findings of Delker et al. (*30*), who found a reduction in CYP1A1 activity in DMH-treated animals, or those of Rijnkels and Alink (*25*), who reported elevated CYP1A1 activity following DMH administration. Differences in results are most likely due to variability in the time between exposure to the carcinogen and enzyme measurement or the DMH dose. In the study carried out by Delker et al. (*30*) enzyme activity was measured 20 h following exposure to DMH, whereas Rijnkels (*13*) measured enzyme activity in samples taken a week after the final exposure to DMH following a 4 week regimen of injections. In the present study, animals received injections of DMH for a 2 week period, and samples were taken 3 weeks following the final injection.

In conclusion, our data indicate an elevation in the activities of both phase I and phase II enzymes in the liver of rats that consumed either grapefruit or oroblanco juice. Potentially, this allows for greater detoxification and removal of harmful substances from the body, an important protective mechanism useful in chemoprevention.

ABBREVIATIONS USED

DMH, 2-dimethylhydrazine; CYP1A1, cytochrome P450; QR, NAD(P)H:quinone reductase; GST, glutathione *S*-transferase; TBS, blocking solution.

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