PHARMACODYNAMICS AND DRUG ACTION

Grapefruit juice and its flavonoids inhibit 11β-hydroxysteroid dehydrogenase

Introduction: The enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) oxidizes cortisol to inactive cortisone. Its congenital absence or inhibition by licorice increases cortisol levels at the mineralocorticoid receptor, causing mineralocorticoid effects. We tested the hypothesis that flavonoids found in grapefruit juice inhibit this enzyme in vitro and that grapefruit juice itself inhibits it in vivo.
Methods: Microsomes from guinea pig kidney cortex were incubated with cortisol and nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) and different flavonoids and the oxidation to cortisone measured with use of HPLC analysis. In addition, healthy human volunteers drank grapefruit juice, and the ratio of cortisone to cortisol in their urine was measured by HPLC and used as an index of endogenous enzyme activity.
Results: Both forms of 11β-OHSD requiring either NAD or NADP were inhibited in a concentration-dependent manner by the flavonoids in grapefruit juice. Normal men who drank grapefruit juice had a fall in their urinary cortisone/cortisol ratio, suggesting in vivo inhibition of the enzyme.
Conclusion: Dietary flavonoids can inhibit this enzyme and, at high doses, may cause an apparent mineralocorticoid effect. (CLIN PHARMACOL THER 1996;59:62-71.)

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The enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) oxidizes cortisol to inactive cortisone. This enzyme in the kidney regulates the amount of mineralocorticoid activity there, because cortisol binds as avidly to the mineralocorticoid receptor as aldosterone does. Deficiency of this enzyme in children, first described by Ulick et al.[1] in 1977, causes high cortisol levels in the kidney that result in hypertension and hypokalemia. Licorice-induced hypermineralocorticoidism is probably due to the inhibition of 11β-OHSD by glycyrrhizic acid, the active principle of licorice.[2-4] Much research has been done since 1977 on syndromes of apparent mineralocorticoid excess.[5,6]

Gossypol, a polyphenolic constituent of cotton seed, has been studied in China as a potential male oral contraceptive, but hypokalemia developed in some Chinese men while they were taking it.[7] We found that gossypol inhibited 11β-OHSD activity in guinea pig[8] and human renal cortical microsomes.[9] Because there are structural similarities between gossypol and some flavonoids, we tested some of these and some other compounds, such as diuretics, that cause hypokalemia[9-10] and discovered that some inhibit this enzyme. Narin-

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Supported by grant RR47 from the National Institutes of Health (Bethesda, Md.) and by grants from Hoffmann-La Roche Inc. (Nutley, N.J.), Sandoz Pharmaceuticals Inc. (East Hanover, N.J.), The Rockefeller Foundation (New York, N.Y.), and Han-Dok Remedia (Seoul, Korea).
Received for publication May 11, 1995; accepted Aug. 17, 1995.
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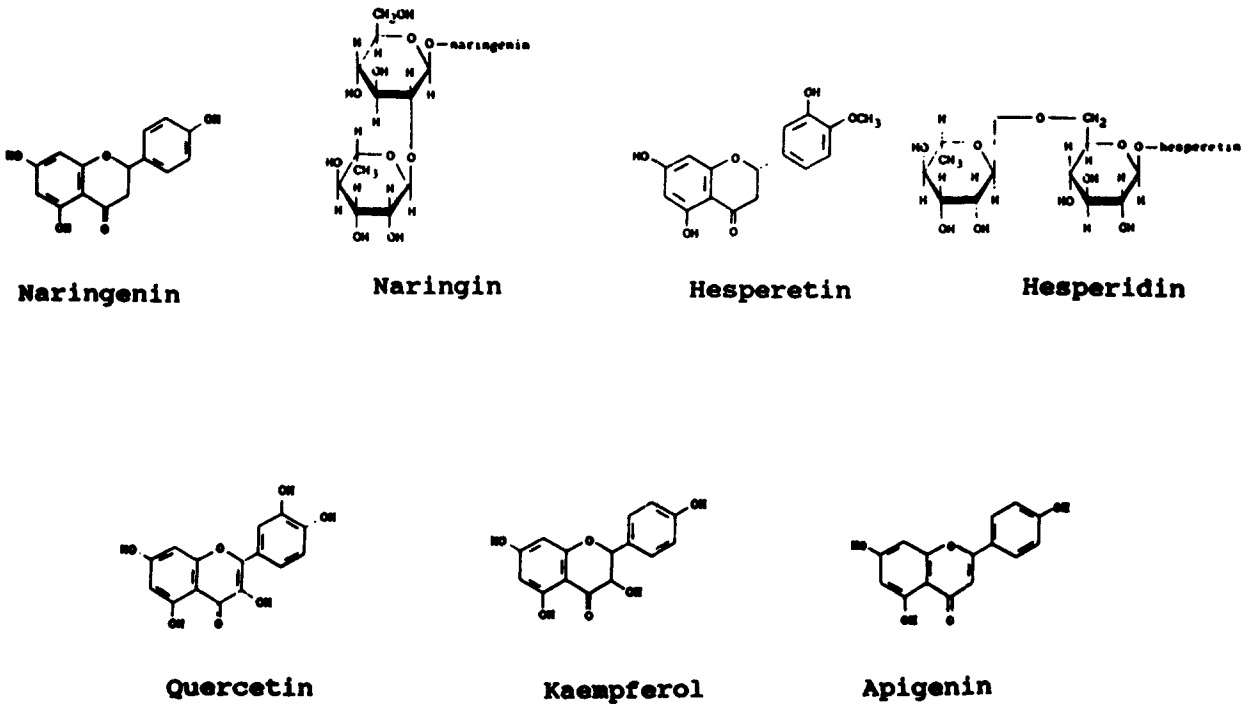


Structures of flavonoids.

genin, the aglycone of naringin, is a major flavonoid in grapefruit juice and inhibits this enzyme.[10] Recent work suggests that there are two isoforms of this enzyme, nicotinamide adenine dinucleotide (NAD)–dependent 11β-OHSD and nicotinamide adenine dinucleotide phosphate (NADP)–dependent 11β-OHSD with specific tissue distributions.[11-14] The effects of these flavonoids are worth study because about 25 mg of flavonoids has been recently estimated to be ingested daily in the diet,[15] whereas older studies cite as much as 1 gm per day.[16]

The objective of this study was to learn which other flavonoids in grapefruit juice inhibit 11β-OHSD in vitro and whether grapefruit juice inhibits the enzyme in vivo.

MATERIAL AND METHODS

In vitro study

Chemicals and solutions. All flavonoids (see Structures), cortisone, cortisol, corticosterone, NAD, NADP, 99.9% dimethyl sulfoxide (DMSO), and Sigma Diagnostic Total Protein Kit (cat. No. 690-A) were purchased from Sigma Chemical Co., St. Louis, Mo. All flavonoids were dissolved in DMSO. Cortisone, cortisol, and corticosterone were dissolved in methanol (J.T. Baker HPLC grade purchased from VWR Scientific, Piscataway, N.J.) (1.4 mmol/L) and kept at −4° C. NAD and NADP (5 mmol/L) were dissolved in Tris hydrochloric acid buffer (0.1 mol/L, pH 8.0).

Microsomal preparation and assay of 11β-OHSD activity. Guinea pig kidney cortex was obtained from long-haired male Hartley guinea pigs (Hilltop, Pa.). Tissue was homogenized by a Tekmar Tissuemizer (Cincinnati, Ohio). Microsomes were prepared, diluted to a concentration of 1.25 mg protein/ml as measured by the Sigma Diagnostics Total Protein Kit, and stored at −70° C. The enzyme activity in the microsomes was determined by measuring the rate of conversion of cortisol to cortisone in the presence of NAD or NADP as described previously.[8-10] Each flavonoid was studied with use of NAD and NADP as the cofactor. The conversion rates from cortisol to cortisone were determined, and the extent of inhibition was calculated. The drug concentrations that inhibited the enzyme by 50% (IC_{50}) were estimated from duplicate incubations at each concentration of at least three different concentrations of each flavonoid by use of the dose-response program of Chou and Chou (*Dose-effect Analysis with Microcomputers,* Elsevier-Biosoft, Cambridge, En-

gland, 1989). For each flavonoid studied, at least one concentration was above and one below the IC_{50}.

Analytical method for urinary cortisone and cortisol

We modified our HPLC method for measurement of these compounds from microsomal incubation mixtures.[9] The equipment consisted of a Waters Automated Gradient Controller with two Waters 6000A pumps (Waters Chromatography, Milford, Mass.). The injector was a Waters U6K and the detector was a Waters 486 Tunable Absorbance Detector set at a wavelength of 246 nm and 0.15 absorbance units full scale. The separation was performed with a Waters Nova-Pak C_{18} 3.9 × 150 mm stainless steel column (4 μm spherical particle size, pore size 60 Å, 7% carbon load, end-capped) or with a Waters μBondapak C_{18} 3.9 × 300 mm column (10 μm irregular particle size, pore size 125 Å, 10% carbon lead, end-capped). The peak areas were recorded on a SE120 plotter purchased through Waters Chromatography.

The mobile phase was methanol/water, initially at 70% water:30% methanol. Conditions were changed over the first 6 minutes to 56% water:44% methanol in a linear gradient that was then held isocratically for 14 minutes. The gradient was then reversed linearly to 70:30 over 3 minutes and the column equilibrated for 5 minutes before the next injection. The flow rate was 1 ml/min.

Procedure

To each 10 ml aliquot of every standard and sample (performed in duplicate) was added 40 μl of the 25 μg/ml corticosterone* as the internal standard. The samples were briefly vortexed to mix. One milliliter of 0.1 mol/L of sodium hydroxide was added to each test tube and again briefly vortexed to mix. Three milliliters of methylene chloride were added to each sample, capped with Teflon-lined screw tops, and rotated for 45 minutes on a mechanical rotator at approximately 20 rpm. The samples were centrifuged at 3000 rpm (1000*g*) for 15 minutes. The aqueous layer (top) was aspirated to waste. Again the samples were centrifuged for 10 minutes at 3000 rpm and the remainder of the aqueous phase was aspirated. A small spatula full of sodium chloride (~150 mg) was added to each sample, and any emulsion was broken up with a Pasteur pipet. The samples were then again centrifuged for 10 minutes. The organic layer was carefully transferred to clean test tubes and evaporated to dryness in a warm water bath (~45° C) under a stream of nitrogen. The residue was redissolved in 150 μl of HPLC grade methanol and injected into the HPLC.

The retention times were 16.5, 19.0, and 23.5 minutes for cortisone, cortisol, and corticosterone, respectively, on a Waters 10 micron, 300 × 3.9 mm stainless steel μBondapak C_{18} column. On a Waters 4 micron, 150 × 3.9 mm Nova-Pak, the retention times for cortisone, cortisol, and corticosterone were 12.8, 13.6, and 17.8 minutes. Levels measured in about 60 human urine samples ranged from 7.1 to 215.4 ng/ml for cortisone and 4.5 to 230.1 ng/ml for cortisol. The ratio of cortisone to cortisol was 0.2 to 5.7.

The absolute recovery was 70% for cortisol and 69% for cortisone. The interday coefficient of variation for cortisone was 6.5% for 25 ng/ml and 1.5% for the 100 ng/ml standard. For cortisol, the values were 6.3% for 25 ng/ml and 1.1% for 100 ng/ml. Cortisone dissolved in methanol was chromatographed and the peak was collected. The putative cortisone peak from extracted urine was also collected, and both fractions were scanned with a Varian Cary 219 spectrophotometer. The peaks had identical absorption spectra, with absorption maximums at 239 nm. (The *CRC Handbook of Chemistry and Physics* states that the absorption maximum of cortisone in alcohol is 237 nm).

All samples were assayed twice in duplicate. Standard curves for cortisone and cortisol were determined and plotted as in the in vitro study. Concentrations of these steroids in unknown samples were extrapolated from these standard curves.[10]

In vivo preliminary study

Six male volunteers aged from 35 to 65 years (two investigators and four other members of the Department of Pharmacology) who were living at home gave daily morning urine samples for 4 days. They then drank grapefruit juice, requested to be at a dose of a quart a day, for 7 days, and gave daily morning urine samples on the last 4 days of this period. After a 3-day washout period, the subjects again gave daily morning urine samples for 4 days.

*Corticosterone is excreted by humans at a rate that averages 6 μg/24 hours[17] or less than of 1% of 1.5 to 4.0 mg/24 hour production rate.[18] Thus the concentration from endogenous sources is less than 10% of that added, a negligible amount for this assay.

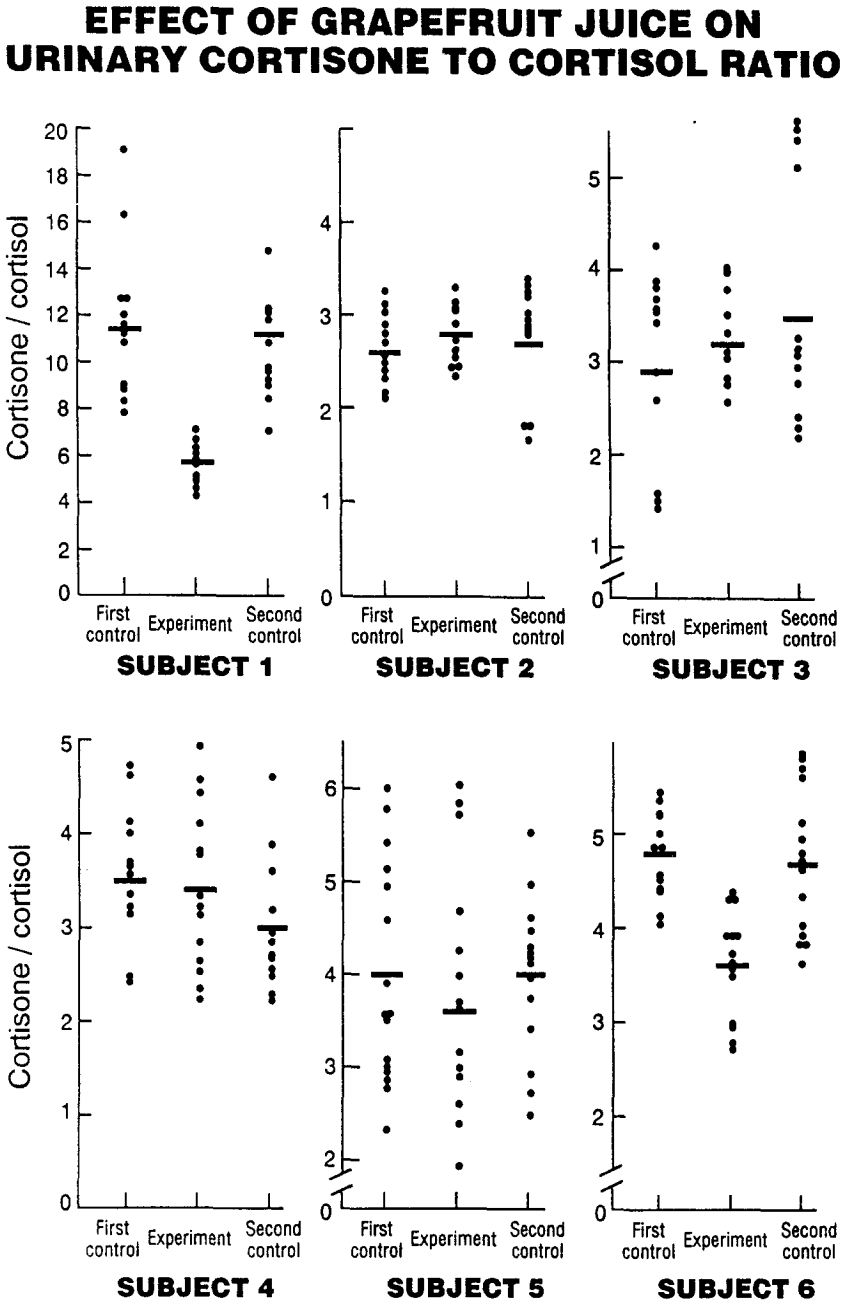


Fig. 1. Preliminary study results in six subjects living at home. Subjects 1 and 6 were two of the authors, who are known to have consumed the full amount of grapefruit juice.

The cortisone and cortisol concentrations were measured in each urine sample. The two investigators (subjects 1 and 6) had a decrease in the ratio of urinary cortisone to cortisol during the grapefruit juice period compared with the control periods before and after grapefruit juice (mean ± SD for subject 1 was 11.4 ± 3.1, 5.7 ± 0.9, and 10.2 ± 2.1; mean ± SD for subject 6 was 4.8 ± 0.4, 3.6 ± 0.6, and 4.7 ± 0.8). The other four subjects had no significant change. All data are shown in Fig. 1. Subjects 1 and 6 then volunteered for the dose-response study.

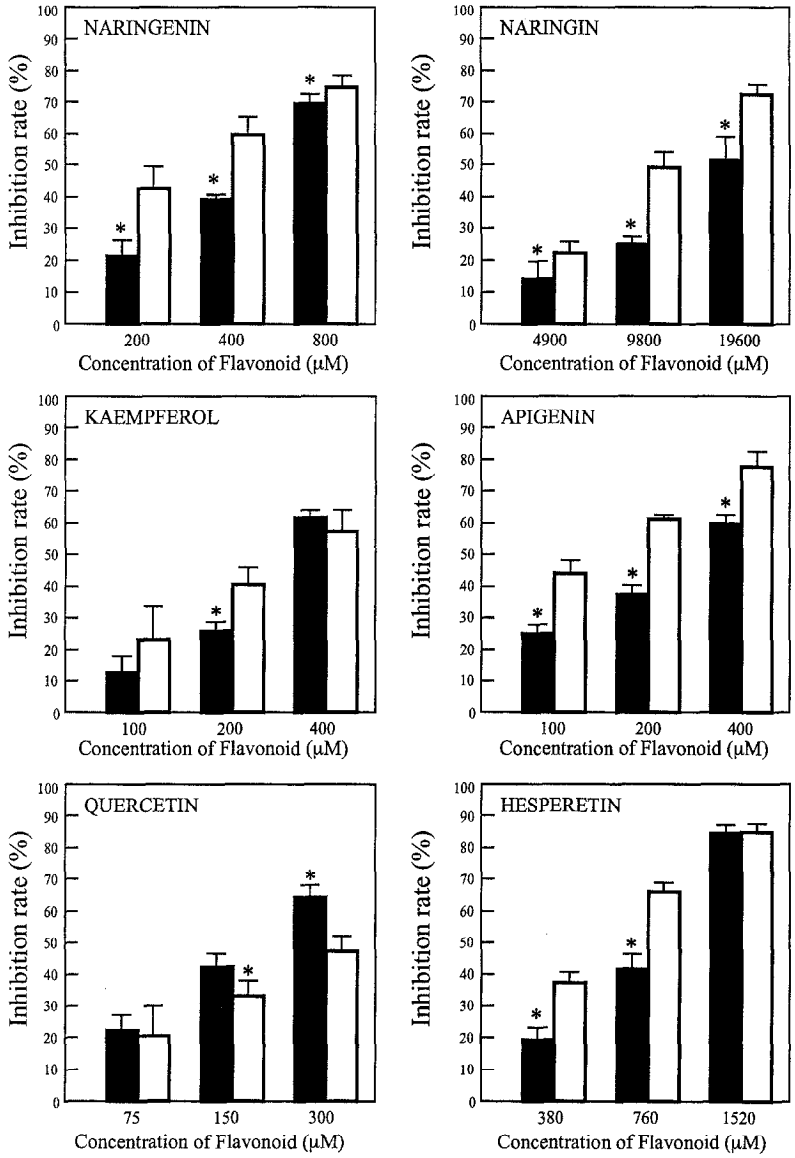


Fig. 2. Concentration-response relationships for the inhibition of 11β-hydroxysteroid dehydrogenase by different flavonoids with use of nicotinamide adenine dinucleotide (NAD; *solid bars*) or nicotinamide adenine dinucleotide phosphate (NADP; *open bars*) as a cofactor. $*p < 0.05$.

In vivo dose-response study

These two volunteers (subjects 1 and 6) gave urine samples for the last 4 days of four 7-day study periods. (1) First control period: Each subject collected a 10-hour (7 AM to 5 PM) urine sample daily for 4 days (one subject missed 1 day of sample collection). (2) Low-dose period: Each subject drank 950 to 1060 ml grapefruit juice a day for 7 days and gave 10-hour urine samples for the last 4 days of the 7-day period. (3) High-dose period: Each subject drank 1900 to 2100 ml (double volume of low-dose period) grapefruit juice for 7 days and gave daily 10-hour urine samples for the last 4 days. (4) Second control period: Each subject gave daily 10-hour urine samples for 4 days after 3 days of a washout period.

In vivo metabolic balance study

Protocol. Two different healthy male volunteers (aged 26 and 31 years), not previously screened for responsiveness to grapefruit juice, gave informed written consent and were admitted to the clinical research center for 3 weeks. An evaluation before the study

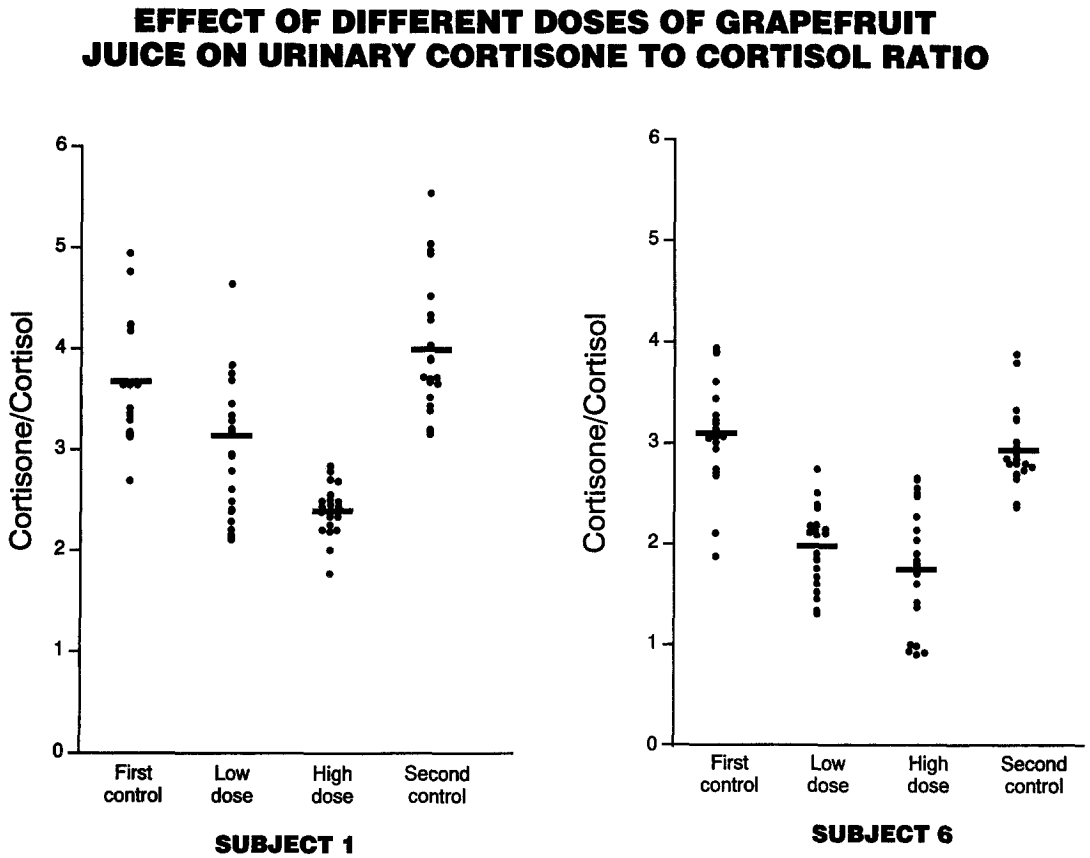


Fig. 3. Urinary cortisone/cortisol ratios in subjects in dose-response study. Each period include four daily urine collections. Each urine sample was assayed twice, each assay in duplicate. Each *point* represents a single assay (four points per daily urine).

showed normal physical findings, serum chemistry, hematology, and ECG for both subjects. They ate a diet that had a constant amount of sodium and potassium during the study (potatoes, bananas, and lemonade during control periods to balance the grapefruit juice during the experimental period). Their blood pressures and body weights were measured daily. Twenty-four-hour urine was collected for free cortisone, cortisol, Na^+, and K^+ for the last 4 days of three 7-day study periods. Blood samples were drawn for Na^+ and K^+ for the same periods. Plasma renin activity, aldosterone and cortisol, and urinary aldosterone excretion were measured at the end of each period. The first and third weeks were the control periods. The second week was the experimental period in which 1500 ml grapefruit juice (100% from concentrate, Ocean Spray Cranberries Inc., Lakeville, Mass.) was consumed daily.

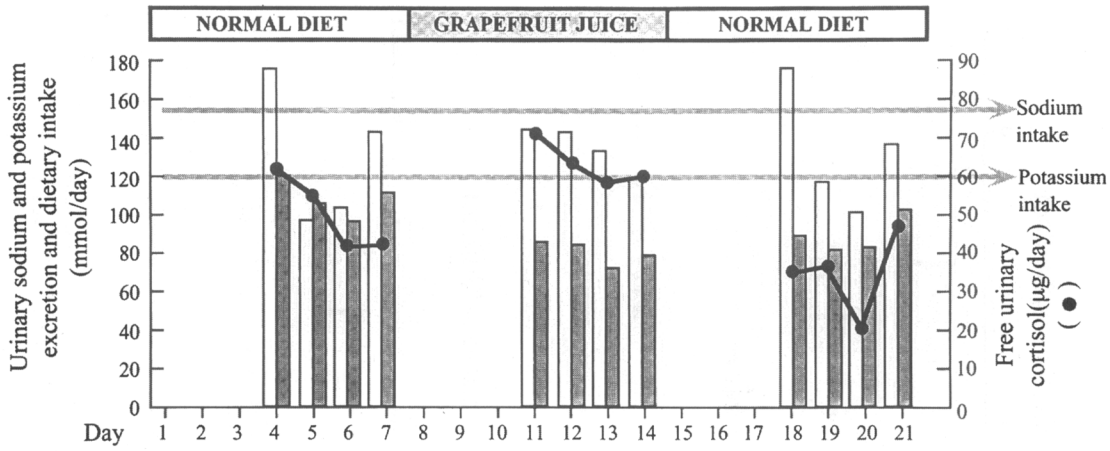
Statistics. The Bonferroni *t* test after a one-way ANOVA was used to assess statistically significant differences. Statistical significance was assumed when the corresponding *p* values were lower than $\alpha = 0.05$.

Approval. All human studies were approved by the Cornell Institutional Review Board.

Table I. Inhibition of 11β-OHSD in microsomes of guinea pig kidney by various flavonoids in the presence of NAD or NADP

	IC_{50} (μmol/L)	
Flavonoids	*NAD*	*NADP*
Quercetin*	192 ± 18	355 ± 82
Apigenin*	284 ± 25	125 ± 16
Kaempferol	322 ± 13	293 ± 62
Naringenin*	496 ± 77	264 ± 63
Hesperetin*	769 ± 69	509 ± 45
Naringin*	21,191 ± 4,949	10,550 ± 1,136
Hesperidin	>55,000	>50,000

Data are mean values ± SD.
11β-OHSD, 11β-Hydroxysteroid dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; IC_{50}, drug concentration that inhibited the enzyme by 50%.
*$p < 0.05$; NAD compared with NADP.



Urinary cortisone/cortisol	2.7 2.9 3.1 3.1	1.9 2.2 2.2 2.1	3.5 3.0 3.8 2.8	
Plasma cortisol (mcg/dl)	9.5	11.8	12.8	13.5
Plasma renin activity(ng/ml/hr)	0.33	1.4	1.4	1.6

Fig. 4. Values for subject A in metabolic balance study. *Open bars,* Daily urinary sodium excretion; *solid bars,* potassium excretion. The cortisone/cortisol ratios in the grapefruit juice period are significantly different from each normal diet period. The urinary free cortisol during grapefruit juice differs significantly from the first but not the second control period.

RESULTS

In vitro study

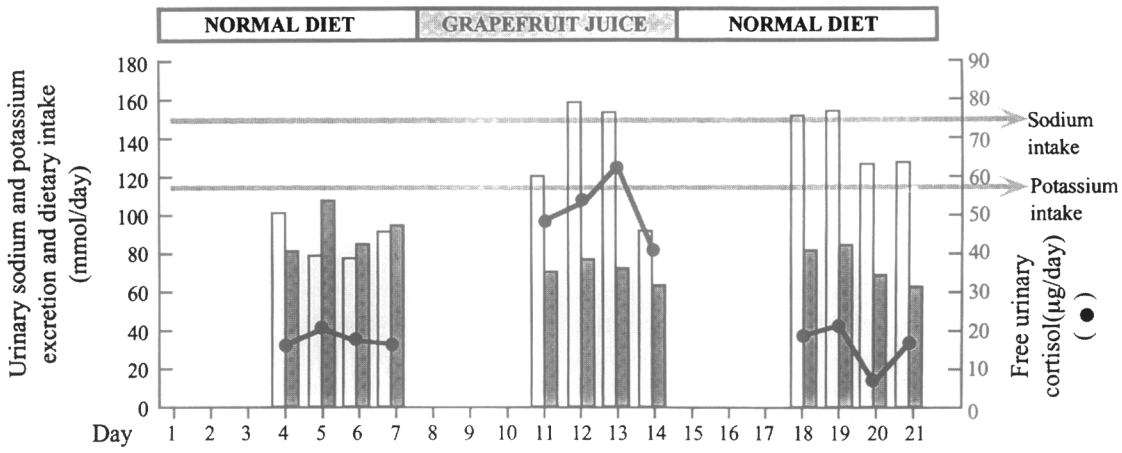
The renal cortex homogenate obtained from male guinea pigs readily converted cortisol to cortisone after 1 hour of incubation at 37° C with NAD or NADP as a cofactor. There was no difference in the conversion rate between NAD and NADP (mean ± SD, 75.1% ± 7.53% with NAD versus 71.0% ± 6.85% with NADP; $p > 0.05$). However, the Michaelis-Menten constant (K_m) values for NAD and NADP calculated from the double reciprocal plots were significantly different (36.4 ± 7.02 μmol/L with NAD versus 57.6 ± 13.1 μmol/L with NADP; $p < 0.05$).

Each flavonoid inhibited the enzyme in a concentration-dependent manner. The inhibition rates for most flavonoids with use of NAD differed from that with use of NADP (Fig. 2). The IC_{50} values of the flavonoids to inhibit the NAD- or NADP-utilizing form of 11β-OHSD are given in Table I. Quercetin was the most potent inhibitor with NAD; apigenin, kaempferol, and naringenin had similar potencies. Apigenin was found to be the most potent inhibitor with NADP, whereas the potency of naringenin, kaempferol, and quercetin were similar. Naringin and hesperidin were poor inhibitors, and their IC_{50} values were much less than that of their aglycons, naringenin and hesperetin. The IC_{50} values of each flavonoid with use of NAD as a cofactor differed from the IC_{50} values with NADP as the cofactor, except for kaempferol.

In vivo dose-response study

The two subjects who drank grapefruit juice showed a dose-dependent decrease in their urinary cortisone/cortisol ratios, indicating inhibition of 11β-OHSD by grapefruit juice (Fig. 3). Each 4-day period was statistically significantly different from the control periods, and the low- and high-dose periods differed in subject 1 statistically and in subject 6 numerically but not statistically.

SUBJECT B



Urinary cortisone/cortisol	3.7 3.8 3.6 2.3	1.8 1.5 1.6 1.8	3.4 3.8 4.3 3.7	
Plasma cortisol (mcg/dl)	7.1	10.9	9.6	10.0
Plasma renin activity(ng/ml/hr)	1.3	3.5	2.5	2.1

Fig. 5. Values for subject B in metabolic balance study. *Open bars,* Daily urinary sodium excretion; *solid bars,* potassium excretion. The cortisone/cortisol ratios and the urinary free cortisol during the grapefruit juice period are significantly different from both control periods.

In vivo metabolic balance study

The actual values for each subject are presented in Figs. 4 and 5. The mean ratios of the urinary cortisone to cortisol fell with grapefruit juice and recovered during the second control period (mean ± SD, 3.27 ± 0.48 during the first control period, 1.88 ± 0.28 during the grapefruit juice period, and 3.52 ± 0.46 during the second control period). Urinary free cortisol levels also were increased during the grapefruit juice period and returned to the control level after subjects ceased to drink grapefruit juice (mean ± SD, 34.3 ± 19.0 for the first control period, 58.2 ± 9.2 for the grapefruit juice period, and 26.3 ± 12.9 for the second control period; $p < 0.05$ for each control period compared with grapefruit juice period). There was a little change in the body weight during the study (67.3 ± 0.4, 67.8 ± 0.3, and 67.5 ± 0.1 kg for subject A and 73.5 ± 0.8, 74.4 ± 0.2, and 74.4 ± 0.1 kg for subject B, in the first control, grapefruit juice, and second control periods, respectively). The urinary sodium and potassium values were variable during the study. There were no significant changes in plasma potassium levels and blood pressure values during the study.

DISCUSSION

Grapefruit juice is known to inhibit the first-pass oxidation of felodipine and nitrendipine,[19,20] presumably because of compounds in the juice that inhibit cytochrome P450 3A. We did this study to learn if it also inhibited another in vivo oxidation, that of 11β-OHSD. We tested several flavonoids present in grapefruit juice for their ability to inhibit 11β-OHSD from guinea pig renal cortex microsomes. The two different isoforms of the enzyme, NAD-dependent and NADP-dependent 11β-OHSD, had different K_m values for cortisol, and the flavonoids had different IC_{50} values for the two forms. We confirmed

the finding of Walker et al.[11] of similar conversion rates for the two forms.

There are a number of flavonoids in grapefruit juice. Naringin is the most abundant flavonoid, present in concentrations of up to 1 mmol/L.[21] It is thought to be converted to the aglycone naringenin in the intestine after oral administration. Because the flavonoids in grapefruit juice inhibited 11β-OHSD in vitro, we evaluated the ability of grapefruit juice to inhibit the enzyme in vivo. Drinking grapefruit juice lowered the urinary cortisone/cortisol ratios in the two investigators and both inpatient subjects, indicating in vivo inhibition of the enzyme. At the doses consumed, it did not change renal electrolyte clearance. Natural licorice in very high doses causes mineralocorticoid effects by inhibition of this enzyme.[4,22,23] We think that grapefruit juice inhibited 11β-OHSD, but the effect was too mild to cause electrolyte changes in these subjects because their urinary free cortisol did not exceed the normal range. A possible alternative explanation is that ring A reduction of cortisol and not 11β-OHSD inhibition is the major cause of the syndrome of apparent mineralocorticoid excess.[23-26]

If the conventional view that 11β-OHSD inhibition is the cause of the syndrome, and if there are differences in different people's enzyme sensitivity to these inhibitors, as we found with different strains of guinea pigs for gossypol inhibition,[8] some people may increase their potassium clearance if they drink large amounts of grapefruit juice. Furthermore, flavonoids are sold in tablet form in health food stores and drug stores. If people take large quantities of flavonoids as dietary supplements, it is possible that the flavonoids may cause sufficient 11β-OHSD inhibition to produce the syndrome of apparent mineralocorticoid excess.

We thank Patricia Danton for her help.

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