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Modulatory Effects of Citrus Flavonoids Towards the Metabolism and Mutagenicity of Environmental Carcinogens

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by
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A Thesis in Partial Fulfillment
of the Requirements for the Degree
of Master in Science in Pharmacology

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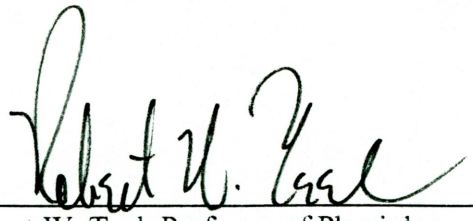

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ABSTRACT

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by

Wayne L. Bear

Master of Science, Graduate Program in Pharmacology
Loma Linda University, March 2002
Dr. Robert Teel, Chairperson

The environmental carcinogens classified as heterocyclic amines (HCA's) and the tobacco-specific nitrosamine NNK generally require internal enzymatic activation. This activation occurs via cytochrome P450 (CYP) enzymes and leads to metabolites that bind to human DNA which may in turn cause mutations which may then in turn lead to carcinogenesis. Certain nonnutritive plant compounds termed phytochemicals may protect against HCA and NNK-induced mutagenesis and carcinogenesis. We investigated the potential protective effects of five different phytochemicals of the flavonoid class which naturally occur in citrus. These flavonoids were diosmin, naringin, naringenin, rutin, and quercetin. Our results suggest that naringenin and quercetin were the strongest inhibitors of the metabolic activation of NNK, while diosmin, naringin, naringenin, and rutin each demonstrated protective effects toward HCA-induced mutagenesis.

CHAPTER ONE

INTRODUCTION

A. Phytochemicals and Chemoprevention

Epidemiological studies have clearly demonstrated that diets high in fruit and vegetable consumption provide some type of protection against many different cancers (1). Our specific area of research focused on the mechanisms of action at the molecular and cellular level, i.e., we are looking for the exact compounds in fruits and vegetables that possess such anticancer properties. These chemicals have been collectively and suitably termed "phytochemicals," and are defined as being nonnutritive substances in plants that possess health-protective benefits (1,2). Our investigations focused on the following five citrus phytochemicals: diosmin, naringin, naringenin, quercetin, and rutin.

Phytochemicals can usually be classified chemically as belonging to one of the following families: bioflavonoids, phenolic compounds, terpenoids, pigments, allyl sulfides, phytates, glucarates, lignans, isoflavones, saponins, indoles, isothiocyanates, tocopherols, phytoestrogens, plant sterols, phytases, phthalides, or polyacetylenes. Figure 1 categorizes the foods containing phytochemicals with the most chemopreventive activity as scientific and epidemiological studies have demonstrated to date. In particular, research has shown that the carcinogenic activating pathway of promutagens and procarcinogens such as heterocyclic amines (HCA's) by cytochrome P450 (CYP) isozymes 1A2 (primarily) and 1A1 can be inhibited by various phytochemicals (3-6).

Methoxyresorufin O-demethylase (MROD) activity is mediated by isozymes of cytochrome P450 present in liver microsomes, particulary the isozyme CYP1A2. By

Figure 1. Phytochemical food pyramid based on increasing chemoprotective and health-related importance. (Modified from Caragay, 1992; In reference 27, N. Back, et al., 1996.)

increasing
importance

GARLIC
CABBAGE
LICORICE
GINGER
SOYBEANS
UMBELLIFERAE
(CARROTS, CELERY, PARSNIPS)
ONIONS TEA TUMERIC
CITRUS
WHOLE WHEAT FLAX BROWN RICE
SOLANACEA (TOMATO, EGG PLANT, PEPPERS)
CRUCIFEROUS (BROCCOLI, CAULIFLOWER, BRUSSELS SPROUTS)
OATS MINTS OREGANO CUCUMBER
ROSEMARY SAGE POTATO THYME CHIVES
CANTALOPE BASIL TARRAGON BARLEY BERRIES

utilizing fluorescence spectrophotometry the amount of methoxyresorufin that is dealkylated by the 1A2 isoform can be determined. Enzyme inhibition studies performed in our lab have utilized a variety of phytochemicals including citrus flavonoids. Results suggest that phytochemicals inhibited the CYP enzyme-mediated activation of MROD. We have demonstrated dose-dependent inhibition by phytochemicals, such as the effect of naringenin on MROD activity described in chapter three. Dose-dependent chemoprevention was profiled for the citrus phytochemical auraptene towards large bowel tumorigenesis in rats (7).

Mutagenesis assays involving *Salmonella typhimurium* TA98 bacteria and five citrus phytochemicals are described in chapter three. It has been demonstrated that isothiocyanates and other phytochemicals inhibit the CYP enzyme-mediated activation of HCA's (3,5). Genistein, a soy bioflavonoid with a very similar structure to quercetin, has been shown to inhibit the growth of B16 melanoma cells as well as breast carcinoma cells (8,9). Evidence also supports the in vitro inhibition of aflatoxin B1 activation in human liver microsomes by flavonoids (10).

The correlation between the effects of citrus flavonoids on the dealkylation of alkoxyresorufins and inhibition of HCA-induced mutagenesis and NNK metabolism suggests that the citrus flavonoids exerted their action through the inhibition of cytochrome P450 activity.

B. Heterocyclic Amines

Pyrolysis of meat juices during high-temperature cooking leads to the formation of heterocyclic amines (HCA's). Charred, broiled, grilled, and/or fried proteinaceous foods

containing creatinine are recognized sources of HCA's (11). Twenty-one HCA's that originate from such cooking processes have been identified. Four of the HCA's were used in the mutagenesis assays described in chapter three: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ); 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx); 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1); and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). (See Figure 3, page 18, for the chemical structures.) Many of these HCA's typically exist in the ng/g level in various pyrolyzed meats, e.g., approximately 9 ng MeIQx per gram of pan-fried beef (11).

HCA's are classified as promutagens and in some cases as procarcinogens. They are activated in vitro as well as in vivo by CYP enzymes (12,13). The specific isozymes responsible for these reactions are CYP1A1 (P-448L) and primarily by CYP1A2 (P-448H) (14-16). N-hydroxylation by either of these isoforms may be followed by the action of phase II enzymes, either acetyltransferase or sulfotransferase (11,17,18). DNA adduction may then occur with the newly transformed electophyllic compounds, i.e., the activated HCA's (18). (See Figure 8, page 44, for details of the metabolic bioactivation of HCA's and DNA adduct formation.) Both MeIQx and IQ are highly mutagenic in *Salmonella typhimurium* TA98 (11). Certain HCA's have also induced tumors when fed to mice and rats (15,19,20). Given that HCA's are promutagens new chemopreventive agents can be identified by testing their ability to inhibit the activating enzymes, e.g., CYP1A2. An assay of enzyme activity that is selective for CYP1A2 is the demethylation of resorufin by methoxyresorufin O-demethylase (MROD) (1,3,21). Studies have shown a strong correlation between inhibition of CYP1A2 activity and inhibition of HCA-induced mutagenicity by flavonoids (22) and isothiocyanates (3).

C. The Tobacco-Specific Nitrosamine NNK

In addition to the dietary associated HCA's, those who smoke or breath second-hand smoke are exposed to the tobacco-specific nitrosamine NNK, 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone. NNK induces lung tumors in rats and is a likely agent in human lung carcinogenesis (23,24). DNA alkylating agents are formed in the body after NNK is metabolically activated by CYP isoforms 1A1, 1A2, 2B1, 2D6, and 2E1 (25,26). NNK metabolism may occur via three pathways: α-hydroxylation, NNK reduction, or N-oxidation. Metabolism of radioactively labeled [5-H^3] NNK by hamster liver microsomes has been measured by HPLC methods and documented (25). (See Figure 2, page 16, for details of NNK metabolism pathways.)

Since cancer is estimated to become the number one cause of death in this century there is a practical role as well as an urgent necessity for examining phytochemicals that may afford protection against environmental carcinogens like HCA's and NNK.

D. Citrus Flavonoids

Our studies have focused on citrus flavonoids and their potential anitmutagenic/anticarcinogenic properties. Citrus fruits are a ready source for various phytochemicals: glucarates, carotenoids, coumarins, mono-terpenes, tri-terpenes, and phenolic acids in addition to flavonoids (27). Flavonoids are abundant throughout the plant kingdom being found in green tea, cruciferous and solanaceous vegetables, cereal grains, soybeans as well as citrus (27). Flavonoids are generally categorized by chemical structure into some 15 classes. Four classes of flavonoids are known to occur in citrus:

flavanones, flavones, flavonols, and anthocyanins. The anthocyanins are only found in blood oranges giving the oranges their characteristic blood red flesh. The polyhydroxylated flavonoids rutin, along with its aglycone, quercetin, belong to the flavonol class. Some examples of flavones include the interesting and almost uniquely citrus polymethoxylated chemicals tangeretin and nobiletin in addition to diosmin and apigenin (28). Naringin, the most abundant flavonoid in grapefruit, its aglycone, naringenin, and the principal flavonoid found in oranges, hesperidin, fall into the flavanone class.

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CHAPTER TWO

EFFECTS OF CITRUS PHYTOCHEMICALS ON LIVER AND LUNG CYTOCHROME P450 ACTIVITY AND ON THE IN VITRO METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK

Wayne L. Bear and Robert W. Teel

Anticancer Research *20:* 3323-3330, 2000.

Abstract. NNK is a potent environmental carcinogen to which both smokers and nonsmokers are exposed. The response to NNK may be affected by factors including nutrition. We investigated the effects of five citrus phytochemicals on the in vitro metabolism of the tobacco-specific nitrosamine NNK and on the dealkylation of methoxyresorufin (MROD) and pentoxyresorufin (PROD) in liver and lung microsomes of the Syrian golden hamster. In the NNK metabolism experiments in vitro incubations contained 3 μCi [5-H^3] NNK, 0.5 mg microsomal protein and 0.5 μmole of the citrus phytochemical diosmin, naringin, naringenin, quercetin or rutin. In the dealkylation studies incubations contained 0.5 μM methoxyresorufin or pentoxyresorufin, 0.5 mg microsomal protein and 0.5 μmole of citrus phytochemical. The major NNK metabolism pathway in hamster liver microsomes was NNK-reduction while in lung microsomes it was α-hydroxylation. The α-hydroxylation pathway produces metabolic products that methylate and pyridyloxobutylate DNA. Naringenin, a metabolite of naringin, and quercetin were the most potent inhibitors of α-hydroxylation of NNK in both liver and lung microsomes. This inhibition correlated with a potent inhibition of MROD and PROD activity in liver but not in lung microsomes. The metabolic activation of NNK is associated with cytochrome P450 isoforms 1A1, 1A2, 2B1, 2D6 and 2E1. Our results suggest that naringenin and quercetin from citrus fruits inhibit the activity of cytochrome P450 (CYP) isoforms that activate NNK and may afford protection against NNK-induced carcinogenesis.

Introduction

Environmental factors have considerable influence on the etiology of cancer (1). When tobacco is processed or smoked tobacco-specific N-nitrosamines such as the potent carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are formed. In order for NNK to exert its carcinogenic effects in vivo it must be activated (2). CYP isoforms 1A1, 1A2, 2B1, 2D6, and 2E1 are reported to activate NNK (3,4). Mutagenic and carcinogenic properties of NNK metabolites relate to their ability to methylate and pyridyloxobutylate purine bases of DNA (3). The three major metabolic pathways labeled in Figure 2 as *a*, *b*, and *c* are carbonyl reduction, pyridine N-oxidation, and α-carbon hydroxylation, respectively.

The biblical verse Rev. 22:2, "And the leaves of the tree were for the healing of the nations" has emerging practical applications as numerous epidemiological and experimental studies have shown (5-7). The consumption of herbs, fruits, and vegetables has health benefits. The citrus flavonoids utilized in our assays possess multifunctional biochemical activities, from the estrogen receptor blocking and anti-protein kinase properties of quercetin (8,9) to the drug interacting and CYP enzyme binding abilities of naringenin (10). Figure 3 shows the structures of five citrus flavonoids used in our study. Regular consumption of certain phytochemicals or a combination of phytochemicals, as in a diet consisting of a mixture of fruits and vegetables, may afford protection against many of the environmental mutagens and carcinogens to which humans are exposed. More specifically, those who are exposed to cigarette or cigar smoke on a daily basis are at high risk for developing lung cancer. Therefore, it is pertinent to identify and isolate

Figure 2. Simplified pathways of NNK metabolism. Pathway *a* is carbonyl reduction; *b* is pyridine N-oxidation; and *c* is α-carbon hydroxylation. (Modified from Hecht, 1996, reference 3.)

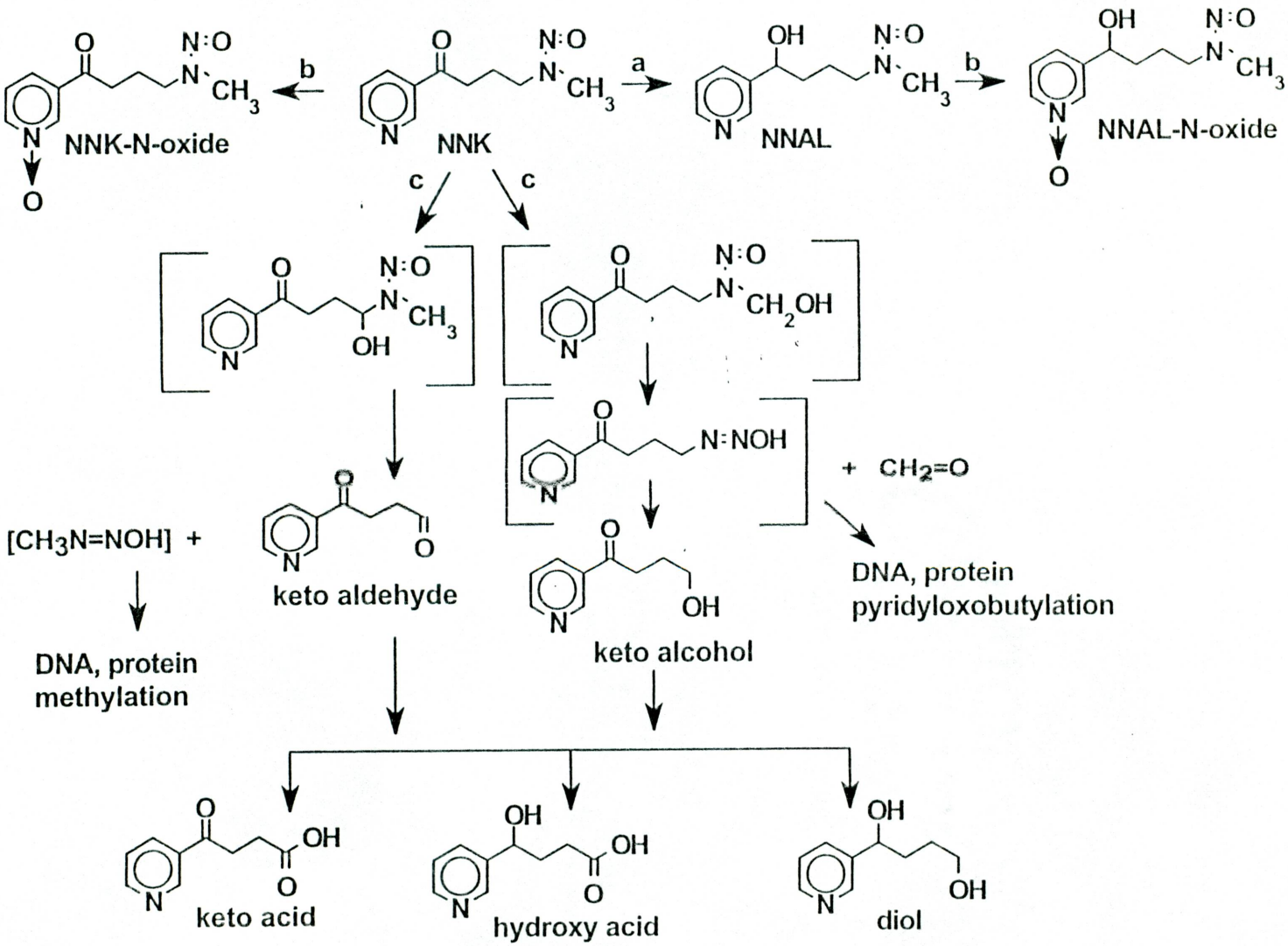
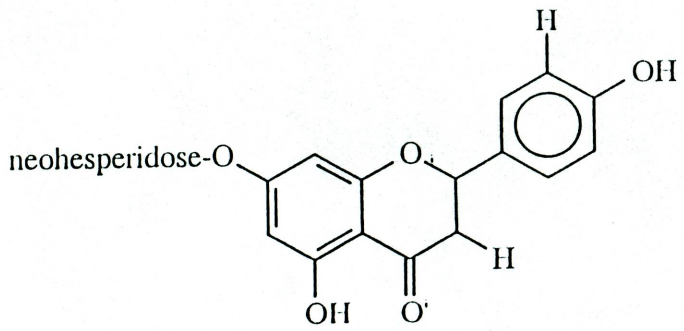
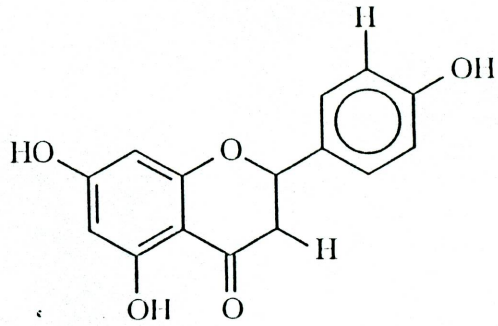

NNK-N-oxide
b
NNK
a
NNAL
b
NNAL-N-oxide
c
c
[CH3N=NOH] +
keto aldehyde
+ CH2=O
DNA, protein
pyridyloxobutylation
DNA, protein
methylation
keto alcohol
keto acid
hydroxy acid
diol

Figure 3. Chemical structures of five citrus flavonoids.



naringin



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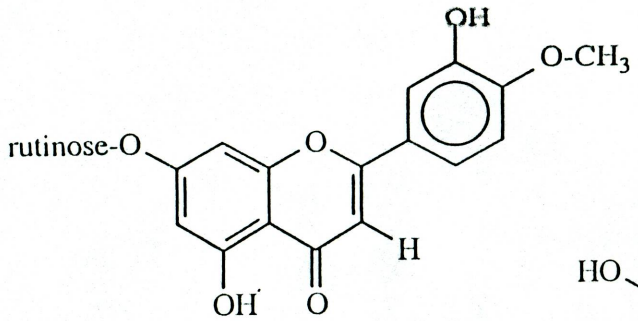

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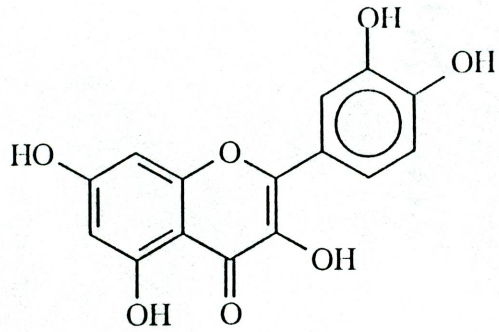

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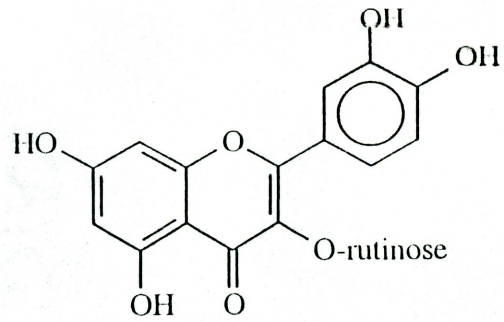


rutin

the phytochemicals or foods containing phytochemicals that may be beneficial in preventing the onset of disease in such persons. It is known that various phytochemicals have the ability to block the initiation step in carcinogenesis by blocking CYP-dependent activation of procarcinogens like NNK (11-14). We report herein the effects of five citrus flavonoids (Figure 1) on the in vitro metabolism of NNK by hamster liver and lung microsomes and on CYP-dependent O-dealkylation of methoxyresorufin and pentoxyresorufin which is linked to CYP isoforms that activate NNK.

Materials and Methods

Chemicals

Methoxyresorufin was obtained from Molecular Probes (Eugene, OR). Pentoxyresorufin, DMSO, diosmin, naringin, naringenin, quercetin, rutin, NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). [5-H^3] NNK was acquired from Chemsyn Science (Lenexa, KS). NNK metabolites prepared as described previously (15) were used as reference standards.

Preparation of microsomes

Male Syrian golden hamsters, 130-150 g, (Charles River Laboratories, Wilmington, ME) were fed Purina rodent laboratory chow and given tap water ad libitum. After

asphyxiation with CO_2, the livers and lungs were removed and homogenized in 0.1 M Tris-HCl, pH 7.4. Differential centrifugation of the homogenate was used to isolate the microsomes (16). Microsome fractions were resuspended in Tris-HCl: EDTA buffer and stored in cryovials in one ml aliquots at -80° C. Protein concentrations were determined (17).

Assays of NNK metabolism

A modification of a procedure described by Smith, et al, (18) was employed to study the effects of diosmin, naringin, naringenin, quercetin, and rutin on the in vitro metabolism of NNK. Incubation mixtures contained 5 mM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 1 mM EDTA, 3 mM $MgCl_2$, 3 μCi [5-H^3] NNK, 0.5 μmole of one of the citrus flavonoids, and 5 mM sodium bisulfite in a final volume of 1 ml. The mixtures were incubated in triplicate for 10 minutes at 37° C followed by the addition of 0.5 mg liver or lung microsomal protein to start the reaction. After an additional 30 minutes of incubation the reaction was arrested by adding 200 μl each of 25% zinc sulfate and saturated barium hydroxide. After centrifugation (14,000 x g) for 20 minutes, the mixtures were filtered through 0.45 μm filters (MSI, Westboro, MA). One hundred μl of the filtrate and 7 μl of NNK metabolite standards were coinjected onto a reversed-phase HPLC system. NNK metabolites were eluted from a Waters C18 μBondapak column (3.9 mm x 300 mm, Millipore Waters, Milford, MA) using sodium acetate buffer (pH 6) and methanol (15). The inclusion of 1 mM sodium bisulfite in the incubation and elution buffers stabilized the keto aldehyde-bisulfite

adducts. One ml fractions of the eluate were collected and combined with scintillation cocktail. Liquid scintillation spectroscopy (Beckman LS 5801 counter, Beckman Instruments Division, Berkeley, CA) was employed to measure the radioactivity of the fractions. NNK metabolite formation was expressed as pmol/mg microsomal protein/min.

Alkoxyresorufin O-dealkylation

O-dealkylation activity was measured using modifications of previously described methods (19,20). Methoxyresorufin or pentoxyresorufin (0.5 μM), 0.5 mg liver or lung microsomal protein and 0.5 μmole citrus flavonoid were mixed in 0.05 M Tris-HCl (pH 7.4) in a final volume of 1 ml in triplicate. The reaction was started by the addition of 0.5 μM NADPH. The incubation at 37° C was terminated after 10 minutes by the addition of 2 ml methanol. The mixture was then centrifuged at 700 x g for 5 minutes. The fluorescence of the supernatant (λ_{ex} = 522 nm, λ_{em} = 586 nm) was measured using a Perkin-Elmer fluorescence spectrophotometer (Model MPF-3C). The control tubes contained the equivalent volume of DMSO used as solvent for the phytochemical.

Statistical Analysis

Both the alkoxyresorufin O-dealkylation and the NNK metabolism assays were carried out in triplicate. NNK metabolism data were analyzed using an Excel® software program and applying the Student's paired two-tailed t-test. Alkoxyresorufin O-

dealkylation activity data were analyzed using the same software and applying the Student's unpaired t-test. Statistical significance was defined as $p \leq 0.05$ compared to control values.

Results and Discussion

The effects of five citrus flavonoids on NNK metabolism by the liver microsomes are shown in Figure 4 and those by the lung microsomes in Figure 5. Naringenin, quercetin, and rutin each significantly inhibited the α-hydroxylation pathway mediated by the liver microsomes. Naringin, naringenin, and quercetin significantly inhibited α-hydroxylation by the lung microsomes. Diosmin, naringin, naringenin, and quercetin significantly inhibited the NNK reduction pathway in liver microsomes, whereas this pathway was inhibited significantly by all five flavonoids in the lung microsomes. All five flavonoids displayed significant inhibition of the N-oxidation in both hamster liver and lung microsome-mediated reactions. Naringenin and quercetin were the most potent inhibitors of all three pathways. α-Hydroxylation (pathway c) results in the methylation and pyridyloxobutylation of DNA (Figure 2). NNK reduction to NNAL and N-oxidation of NNK and NNAL are detoxification pathways (3). The inhibition of the α-hydroxylation of NNK by phytochemicals is desirable whereas inhibition of detoxification pathways is not. The citrus flavonoids used in this study did not selectively inhibit only the α-hydroxylation pathway.

Studies of the effects of phytochemicals on NNK metabolism include acetylsalicylic acid, isothiocyanates from cruciferous vegetables, green tea polyphenols, and pycnogenol

Figure 4. Effects of five citrus flavonoids on the in vitro metabolic activation of NNK by hamster liver microsomes. Procedural details are given in Materials and Methods. Values are mean ± SEM of triplicate samples from ten animals. Asterisks indicate a statistically significant difference from control values, $p \leq 0.05$.

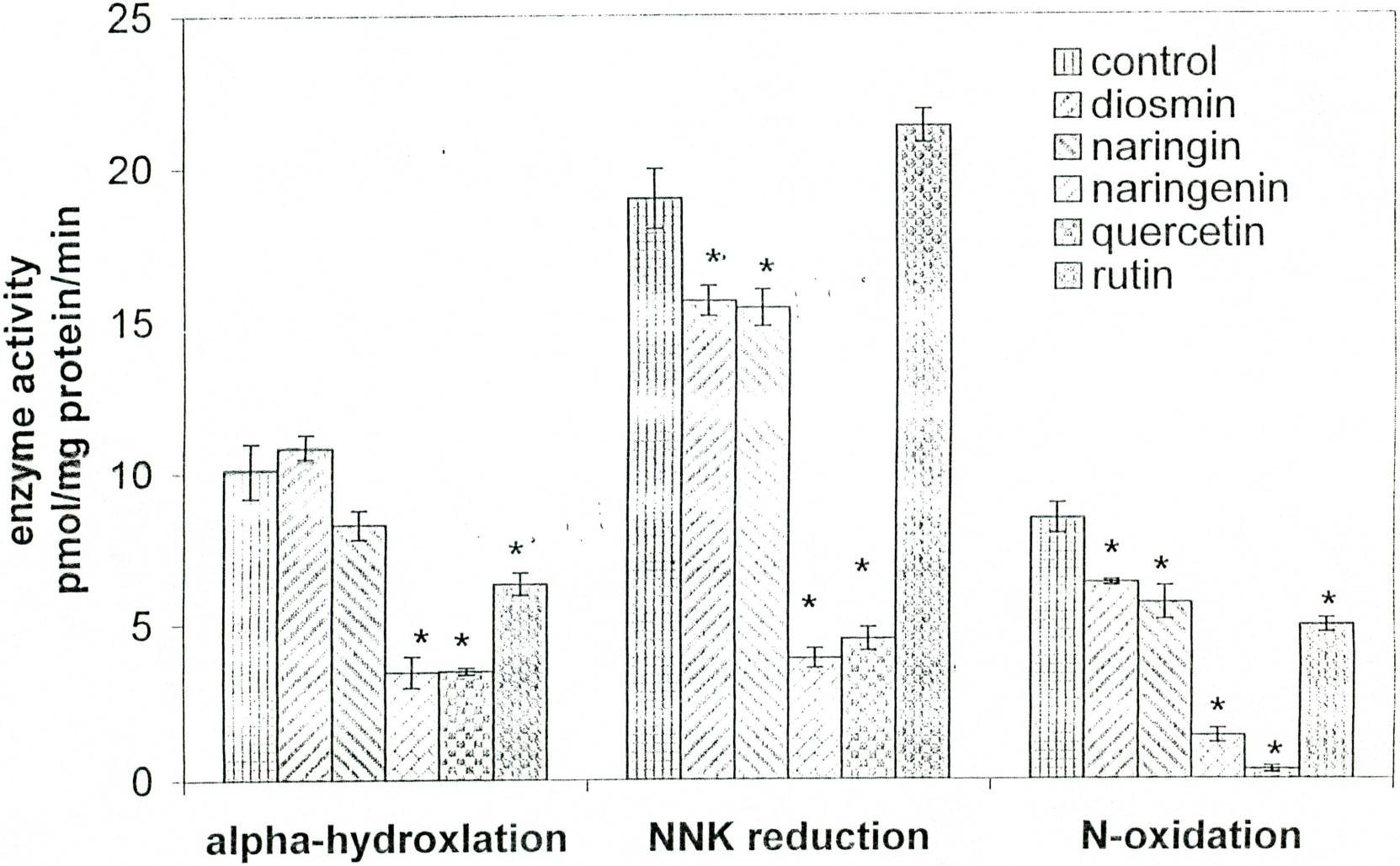
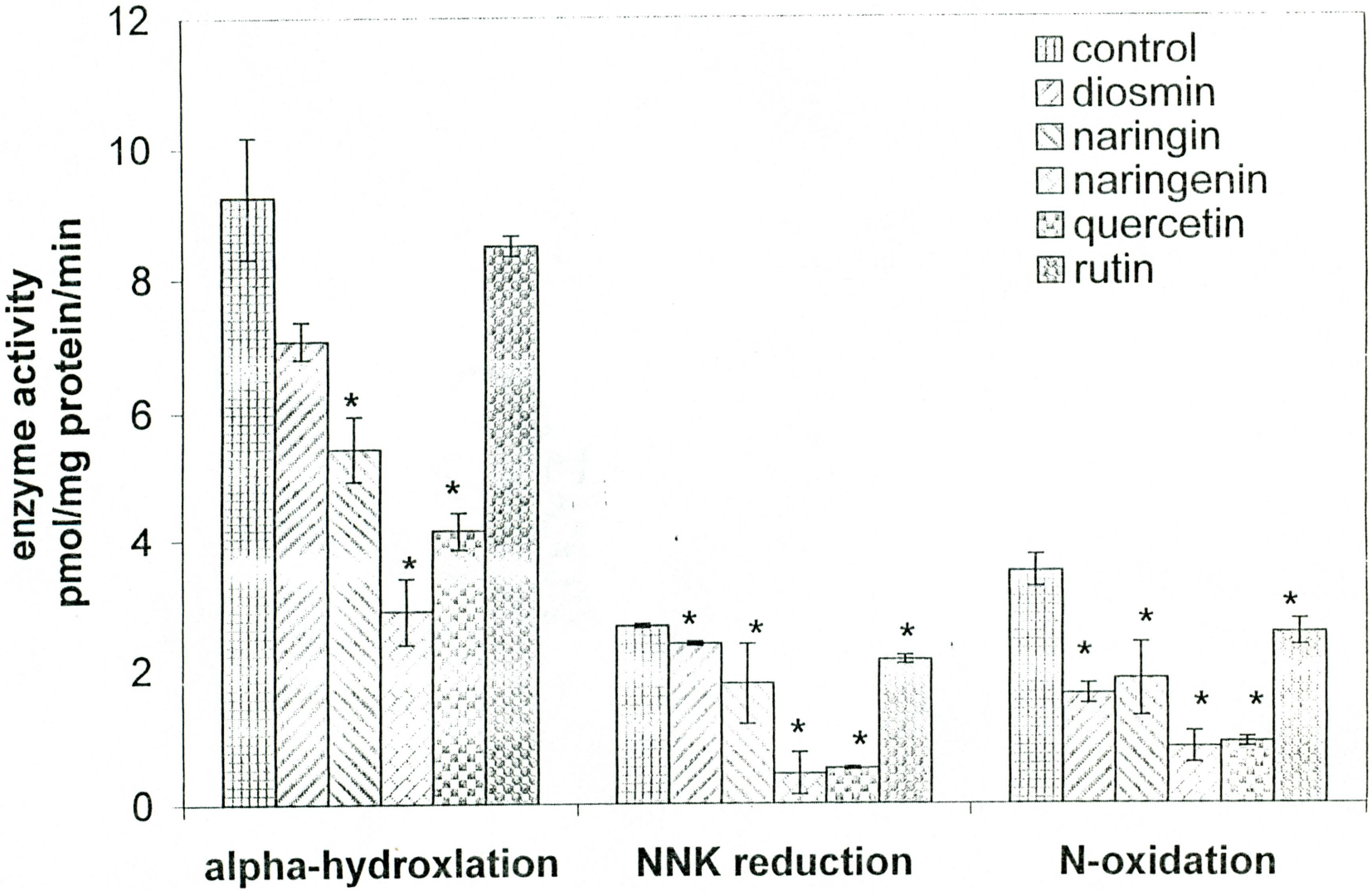

enzyme activity
pmol/mg protein/min
25
20
15
10
5
0
control
diosmin
naringin
naringenin
quercetin
rutin
alpha-hydroxlation
NNK reduction
N-oxidation

Figure 5. Effects of five citrus flavonoids on the in vitro metabolic activation of NNK by hamster lung microsomes. Procedural details are given in Materials and Methods. Values are mean ± SEM of triplicate samples from ten animals. Asterisks indicate a statistically significant difference from control values, $p \leq 0.05$.


enzyme activity
pmol/mg protein/min
12
10
8
6
4
2
0
control
diosmin
naringin
naringenin
quercetin
rutin
alpha-hydroxlation
NNK reduction
N-oxidation

from maritime pine trees. In A/J mice one study has shown that acetylsalicylic acid reduces lung tumor multiplicity induced by NNK (21). The results of an extended diet containing phenethyl isothiocyanate show that this phytochemical inhibits NNK-induced tumorigenesis in the lungs of rats (22). The inhibitory potency of an isothiocyanate on NNK metabolism varies directly with its alkyl chain length (23). Chung, et al (13) showed that green tea polyphenols protect against NNK-induced tumorigenesis in the lungs of A/J mice. Intragastrically administered pycnogenol to F344 rats suggests that this phytochemical mixture inhibits NNK activation in lung microsomes but not in liver microsomes (24).

O-dealkylation of alkoxyresorufins is CYP-dependent. Although these reactions have limited specificity for CYP isoforms, it is reported that CYP1A2 is linked to methoxyresorufin O-dealkylation (MROD) and pentoxyresorufin O-dealkylation (PROD) is linked to CYP2B isoforms (25-27). Figure 6 shows the inhibitory effects of the five citrus flavonoids on MROD and PROD activity in the liver microsomes in our study. Diosmin, naringenin, and quercetin were the most potent inhibitors. Figure 7 shows the effects of the citrus flavonoids on MROD and PROD activity in hamster lung microsomes. MROD activity was inhibited to a small degree by all five flavonoids, whereas PROD activity was significantly inhibited only by rutin. We cannot at this time account for the greater inhibition of MROD and PROD activity by the citrus compounds in liver microsomes compared to the lung microsomes.

CYP enzyme activity is modulated by phytochemicals. Chlorophyllin, curcumin, tannic acid, (-)-epigallocatechin gallate and isothiocyanates decrease MROD activity

Figure 6. Effect of five citrus flavonoids on the in vitro O-dealkylation of methoxyresorufin and pentoxyresorufin in hamster liver microsomes. Procedural details are given in Materials and Methods. Values are mean ± SEM of triplicate samples from each of four separate experiments from eight animals. Asterisks indicate a statistically significant difference from control values, $p \leq 0.05$.

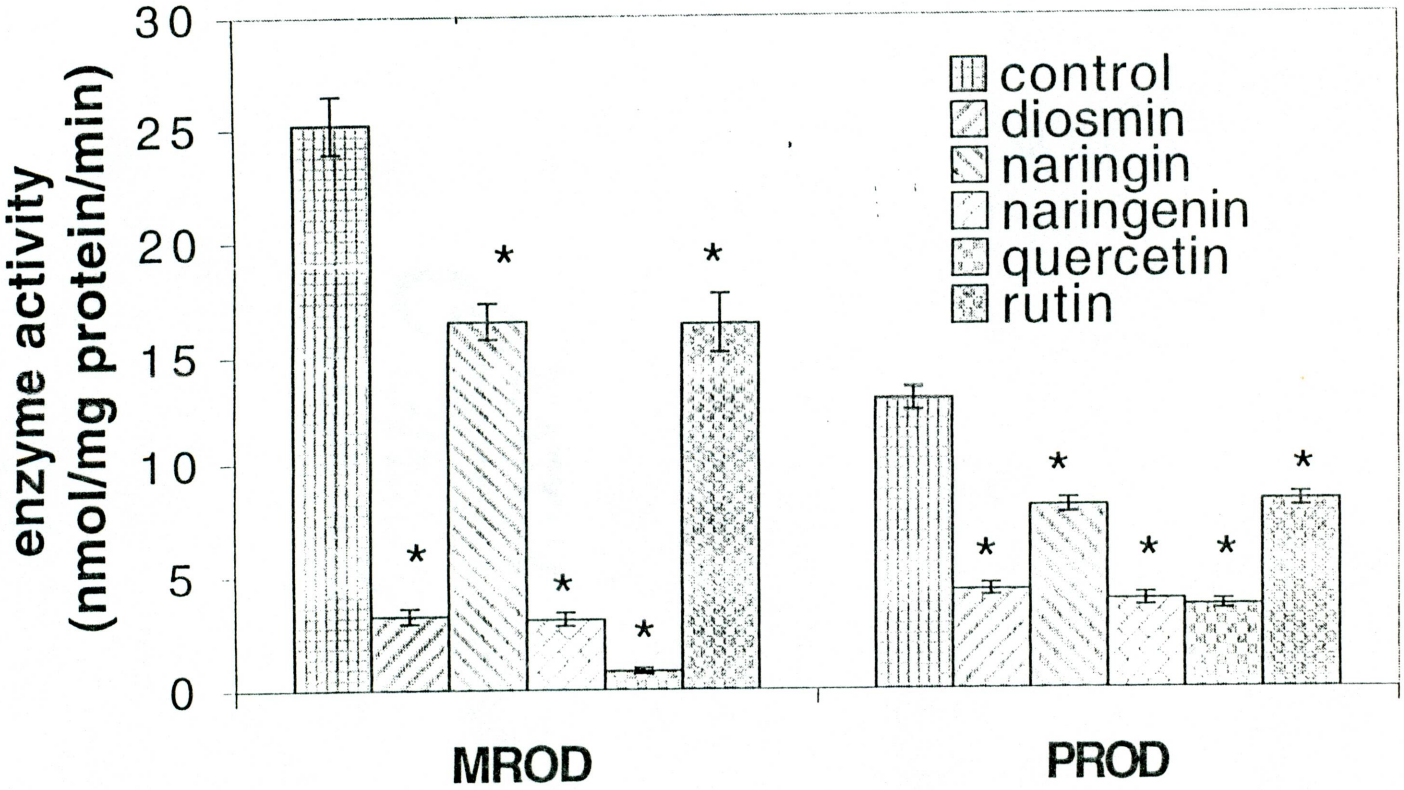
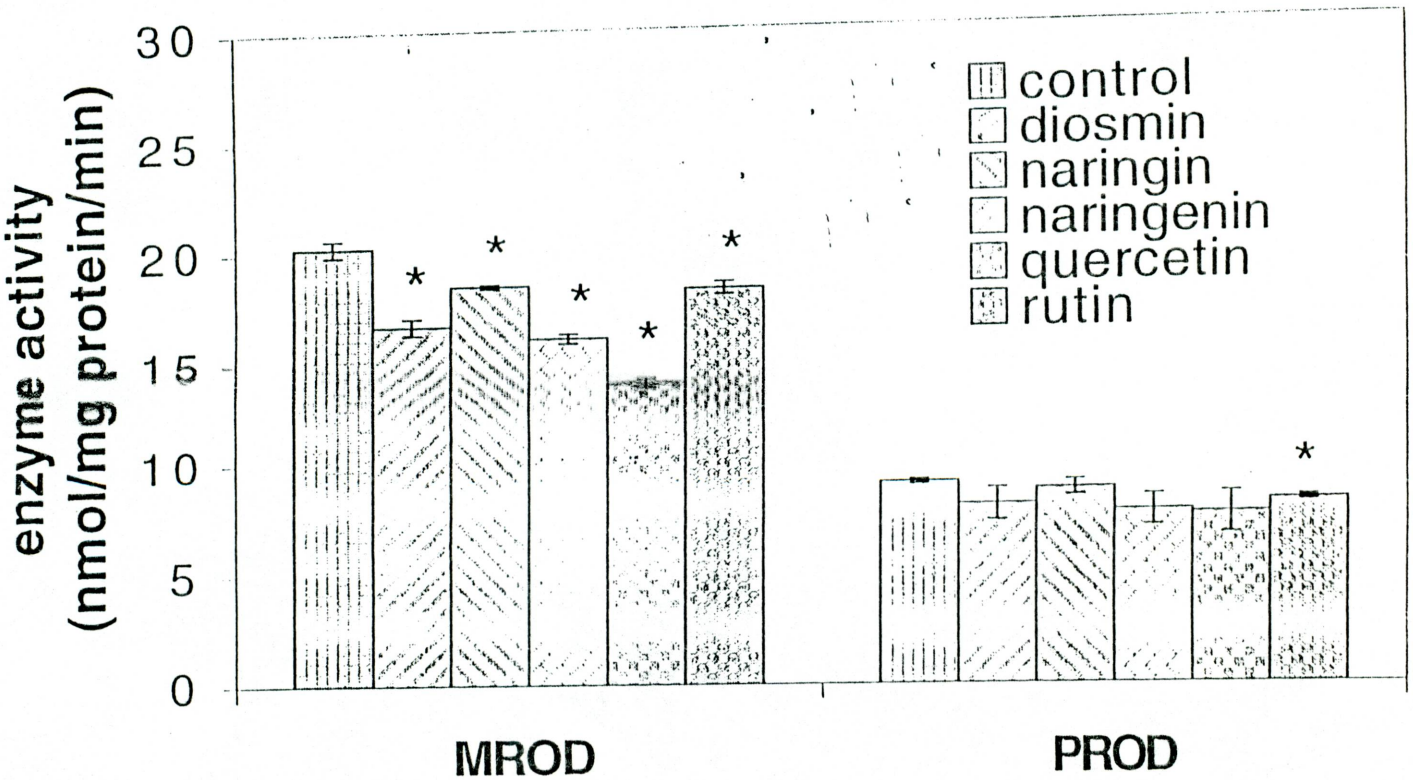

enzyme activity
(nmol/mg protein/min)
30
25
20
15
10
5
0
control
diosmin
naringin
naringenin
quercetin
rutin
*
MROD
PROD

Figure 7. Effect of five citrus flavonoids on the in vitro O-dealkylation of methoxyresorufin and pentoxyresorufin in hamster lung microsomes. Procedural details are given in Materials and Methods. Values are mean ± SEM of triplicate samples from nine animals. Asterisks indicate a statistically significant difference from control values, $p \leq 0.05$.


enzyme activity
(nmol/mg protein/min)
30
25
20
15
10
5
0
control
diosmin
naringin
naringenin
quercetin
rutin
*
MROD
PROD

(25,28). PROD activity increased in rats administered tangeretin, flavone, and flavanone in the diet (29).

The citrus flavonoids luteolin and natsudaidain promote differentiation in HL-60 cell lines (30). Diosmin (concentrated in the peels of oranges) is reported to greatly increase CYP1A1 transcription and activity in MCF-7 human breast epithelial cancer cells (31). Diosmin has also been shown to effectively decrease N-butyl-N-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced urinary-bladder carcinogenesis in male ICR mice (32) and esophogeal tumorigenesis in rats induced by N-methyl-N-amylnitrosamine (33).

Naringin (the most abundant flavonoid in grapefruit) and its aglycone, naringenin, are not the primary CYP3A inhibitors in grapefruit juice (34). However, another study documented the CYP3A binding and inhibiting ability of naringenin (8). In the human body naringin is in part converted to naringenin. Naringenin can be metabolized to genistein (35). Naringenin is an estrogen receptor antagonist (36).

Rutin and it aglycone quercetin are mutagenic toward *Salmonella typhimurium* TA98 (37). Quercetin is present in apples as well as oranges. It is estimated to make up about 5% of the total mass of daily dietary intake of flavonoids in humans and is a potent inhibitor of CYP 3A4 and 1A catalyzed reactions (8).

In conclusion, our results show that the strongest inhibition of NNK metabolism in hamster liver microsomes in vitro occurred in the presence of the citrus flavonoids naringenin and quercetin. This correlated with the inhibition of MROD and PROD activity by these phytochemicals. The effect of rutin on α-hydroxylation and N-oxidation pathways of NNK metabolism in liver microsomes also correlated with its inhibitory

action toward MROD and PROD activity in vitro. Although naringenin and quercetin strongly inhibited the NNK metabolic pathways in lung microsomes, the effects did not correlate with inhibition of MROD and PROD activity in these microsomes. These results suggest that the inhibition of NNK-metabolism in vitro by naringenin and quercetin is linked to the inhibition of CYP1A2 and CYP2B1/2 isoforms in hamster liver microsomes but not in lung microsomes of the hamster. Since other CYP isoforms are involved in NNK metabolism (3,4), the inhibition of NNK metabolism in hamster lung microsomes by naringenin and quercetin may be linked to the inhibition of CYP isoforms other than CYP1A2 and CYP2B1/2. As research support for the efficacy of citrus flavonoids and other phytochemicals as chemopreventive agents increases it may become desirable to establish recommended daily allowances of phytochemicals for disease prevention and to genetically modify staple food crops to contain higher concentrations of desirable phytochemicals (38).

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CHAPTER THREE

EFFECTS OF CITRUS FLAVONOIDS ON THE MUTAGENICITY OF HETEROCYCLIC AMINES AND ON CYTOCHROME P450 1A2 ACTIVITY

Wayne L. Bear and Robert W. Teel

Anticancer Research *20:* 3609-3614, 2000.

Abstract

Heterocyclic amines (HCA's) are promutagens produced by high temperature cooking of meat products and are activated by cytochrome P450 (CYP) 1A2. Using Aroclor 1254 induced rat liver S9 we tested four citrus flavonoids diosmin, naringenin, naringin and rutin for their effects on the mutagenicity of HCA's MeIQx*, Glu-P-1*, IQ* and PhIP* in *Salmonella typhimurium* TA98. The effects of the citrus flavonoids on CYP1A2 activity was determined by measuring demethylation of methoxyresorufin (MROD). MeIQx induced mutagenesis in *S. typhimurium* was significantly inhibited by all four flavonoids in a concentration dependent manner at 0.25, 0.5 and 1.0 μmole. Glu-P-1 induced mutagenesis was inhibited by rutin and naringenin. IQ induced mutagenesis was significantly inhibited by each flavonoid except diosmin at all three doses. With the exception of diosmin and naringin at 0.25 μmole all four flavonoids at all three doses significantly inhibited PhIP induced mutagenesis. The inhibition of MROD activity by the citrus flavonoids correlated best with the inhibition of MeIQx induced mutagenesis but also correlated with the inhibition of IQ induced mutagenesis except for diosmin and with the inhibition of PhIP induced mutagenesis except for the 0.25 μmole dose of diosmin and naringin. Our data suggest a chemopreventive potential for diosmin, naringin, naringenin and rutin towards CYP1A2 mediated mutagenesis of HCA's.

*HCA abbreviations are 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-6-methyldipyrido[1,2-a:3,'2'-d]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

1. Introduction

The initial steps of carcinogenesis for some cancers include enzymatic activation of procarcinogenic agents to their carcinogenic counterparts. Some cytochrome P450 (CYP) isoforms are known to carry out such a mechanism. CYP 1A1, 1A2, 2B1, 2D6 and 2E1 isoforms are reported to activate the procarcinogen NNK to carcinogenic metabolites (1,2). CYP1A2 is acknowledged as the primary activating enzyme in the induced mutagenesis of the four heterocyclic amines (HCA's) MeIQx, IQ, Glu-P-1 and PhIP employed in our study (3-7). Chemoprevention is defined in part as the inhibition of such enzymatic activities by phytochemicals found in foods that are common in the human diet. Recent phytochemical chemopreventive studies include the phytoalexin resveratrol and its role in prostate cancer prevention (8), the ability of the flavonoid genistein to decrease breast cancer risk as well as inhibit breast tumor cell growth (9,10), suppression of pancreatic tumor growth by the isoprenoids farnesol and geraniol (11), and the preventive role of the monoterpene perillyl alcohol in colon carcinogenesis (12).

The HCA's MeIQx, IQ, Glu-P-1 and PhIP are known promutagens/procarcinogens, each having been demonstrated to cause mutagenesis in the Ames Test and tumor induction in mouse liver as well as other major organ sites in the mouse and rat (13). High temperature and/or long duration cooking (e.g., broiling, frying or grilling) of muscle meats generates HCA's. The combination of creatine or creatinine, amino acids and sugars found in the muscle flesh with cooking can form different HCA's according to the animal type (14). PhIP is predominantly found in fish and chicken whereas the primary source of MeIQx is beef (14). Metabolic activation of HCA's resulting in DNA

Figure 8. Metabolic bioactivation of HCA's to DNA adduct formation (adapted from Schut and Snyderwine, 1999).

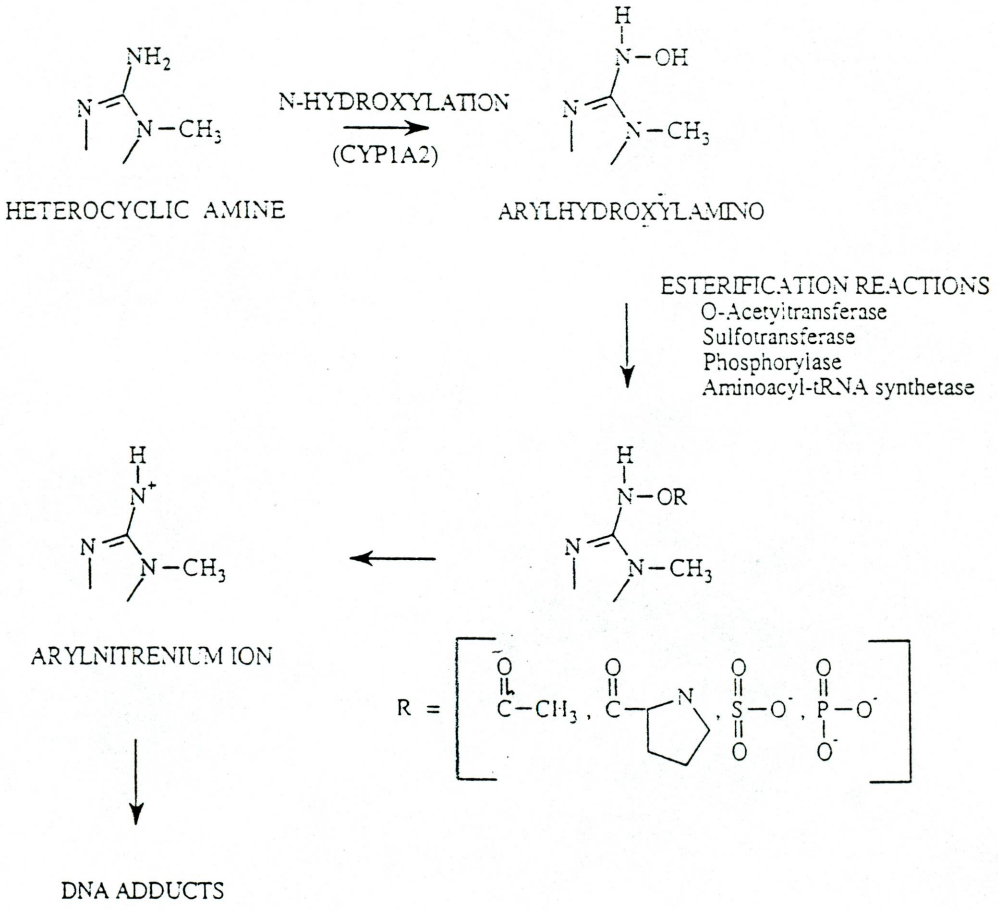
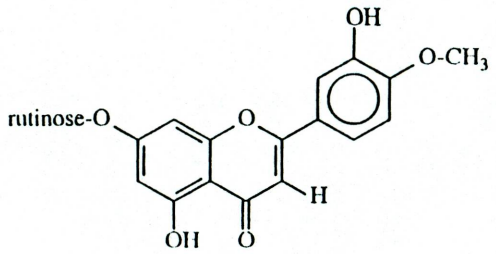
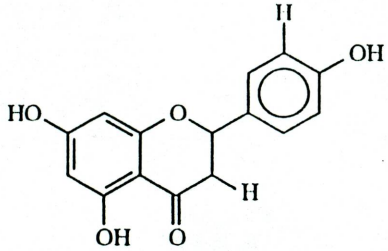

N-HYDROXYLATION
(CYP1A2)
HETEROCYCLIC AMINE
ARYLHYDROXYLAMINO
ESTERIFICATION REACTIONS
O-Acetyltransferase
Sulfotransferase
Phosphorylase
Aminoacyl-tRNA synthetase
ARYLNITRENIUM ION
R =
DNA ADDUCTS

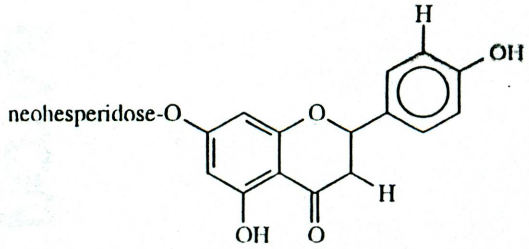
Figure 9. Chemical structures of citrus flavonoids diosmin, naringenin, naringin and rutin and HCA's MeIQx, IQ, Glu-P-1 and PhIP.



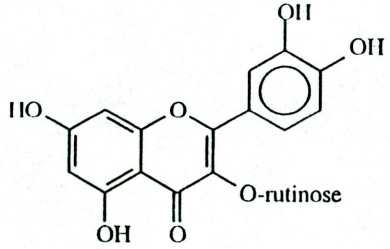
diosmin



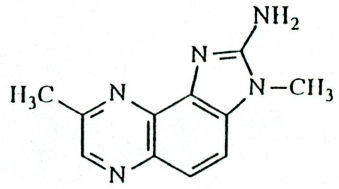
naringenin



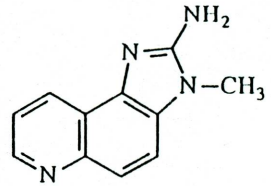
naringin



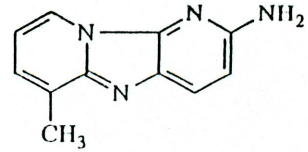
rutin



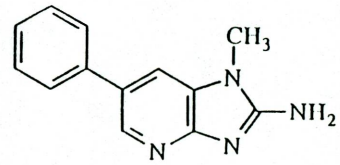
MeIQx



IQ



Glu-P-1



PhIP

the antimutagenic capabilities and chemopreventive potentials of the citrus flavonoids diosmin, naringin, naringenin and rutin.

2. Materials and Methods

2.1. Chemicals

Methoxyresorufin was obtained from Molecular Probes (Eugene, OR). Diosmin, naringin, naringenin, rutin, DMSO, NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). The HCA's were acquired from Toronto Research Chemicals (Downsview, Ontario). *Salmonella typhimurium* TA98 was a gift from Dr. Bruce Ames, University of California at Berkeley. Bacto™ Agar was obtained from Difco (Becton Dickinson, Sparks, MD) and nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Hants., England).

2.2. Mutagenesis Assays

Sterile tubes containing 500 µl Tris-HCl cofactor buffer (pH 7.4); 60 ng IQ, 250 ng MeIQx, 500 ng Glu-P-1, or 3 µg PhIP; 0.2 mg Aroclor 1254 induced rat liver S9 protein; 100 µl *Salmonella typhimurium* TA98; and either 0, 0.25, 0.5 or 1.0 µmole of citrus flavonoid were incubated in a 37° C shaking H_2O bath for 20 minutes. Aroclor 1254 induced Sprague Dawley rat liver S9 was purchased from BioReliance-Tox Labs (Rockville, MD). Two ml of molten soft agar was then added to each tube, mildly

vortexed, poured onto minimal glucose agar plates, and then incubated for 48 hours at 37° C. The number of His^+ colonies on the plates were scored. Blank plates (without HCA or flavonoid) were part of each assay to determine the number of spontaneous revertants which were subtracted from both the control and experimental groups. Assays were done in triplicate determinations from two separate experiments. Mutagenesis assays were modified from Maron and Ames (25).

2.3. Methoxyresorufin O-dealkylation

Enzyme activities were measured using modifications of previously described methods (26,27). Methoxyresorufin (0.5 μM), 0.2 mg rat liver S9 protein, and 0.25, 0.5 or 1.0 μmole flavonoid were mixed in 0.05 M Tris-HCl, pH 7.4, in a final volume of 1 ml. The reaction was started by the addition of 0.5 μM NADPH. Incubations were for 10 minutes at 37° C and were terminated by the addition of 2 ml methanol. The mixture was then centrifuged at 700 x g for 5 minutes. Using a Perkin-Elmer fluorescence spectrophotometer (Model MPF-3C), the fluorescence of the supernatant was measured (λ_{ex} = 522 nm, λ_{em} = 586 nm). Incubations were performed in triplicate. The control tubes (without citrus flavonoid) contained an equivalent volume of DMSO (the flavonoid solvent).

2.4. Statistical analysis

Both the alkoxyresorufin O-dealkylation and the HCA mutagenesis assays were done in triplicate and repeated at least once. Both sets of data were analyzed using an Excel® software program and applying the Student's t-test. Statistical significance was defined as $p \leq 0.05$ compared to control values.

3. Results and discussion

Figure 10 shows the effects of diosmin, naringenin, naringin and rutin at 0, 0.25, 0.5 and 1.0 μmoles/plate towards the mutagenicity of MeIQx, IQ, Glu-P-1 and PhIP in *S. typhimurium* TA98 with Aroclor 1254 induced rat liver S9 protein. MeIQx induced mutagenesis was significantly inhibited by all four flavonoids at all three doses. Naringin, naringenin and rutin each inhibited IQ induced mutagenesis at all three doses. Glu-P-1 induced mutagenesis was inhibited by naringenin at all three doses and by rutin at the 0.5 and 1.0 μmole doses. PhIP induced mutagenesis was significantly inhibited by naringenin and rutin at all three doses and by diosmin and naringin at the 0.5 and 1.0 μmole doses. Dose-dependent inhibition by all four flavonoids in all four HCA induced mutagenesis experiments was observed except for diosmin in Glu-P-1 and IQ induced mutagenesis assays. Naringenin was the most antimutagenic flavonoid in our study.

The effects of the citrus flavonoids at 0, 0.25, 0.5 and 1.0 μmole towards Aroclor 1254 induced rat liver S9-mediated MROD activity are given in Figure 11. Diosmin and naringenin each significantly inhibited MROD activity at all three doses. Rutin and

Figure 10. Effects of citrus flavonoids on the rat liver S9 activated mutagenesis of HCA's. The y-axis represents the number of induced revertants per plate; the x-axis represents the concentration of flavonoid used in μmoles per plate. Procedural details are given in Materials and Methods. Values are means ± SEM of triplicate samples from each of two separate experiments. Asterisks indicate a statistically significant difference from control values, $p \leq 0.05$. (-—-◇-—- diosmin; - - -□- - - naringenin; — — —△— — — naringin; ——×—— rutin.)

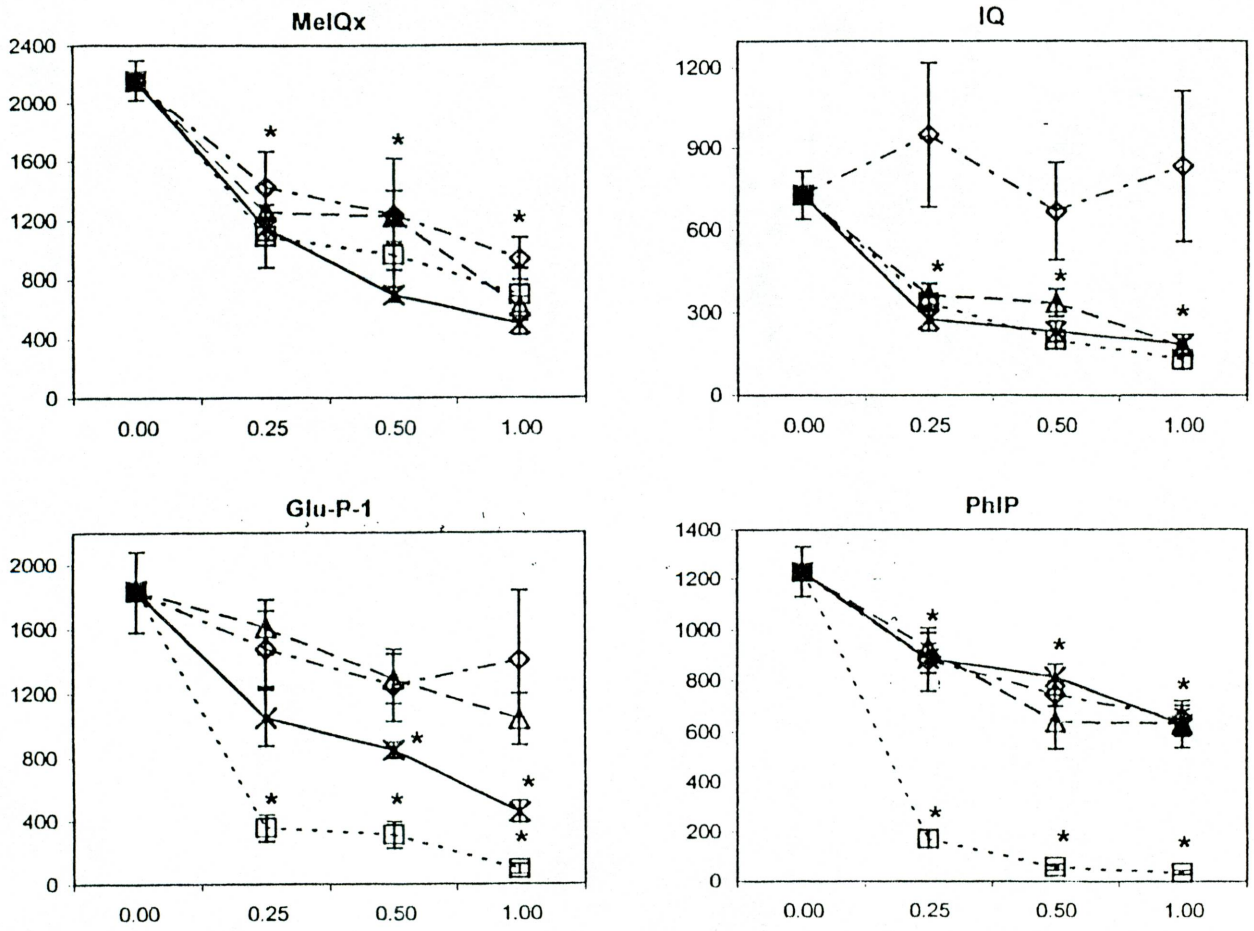
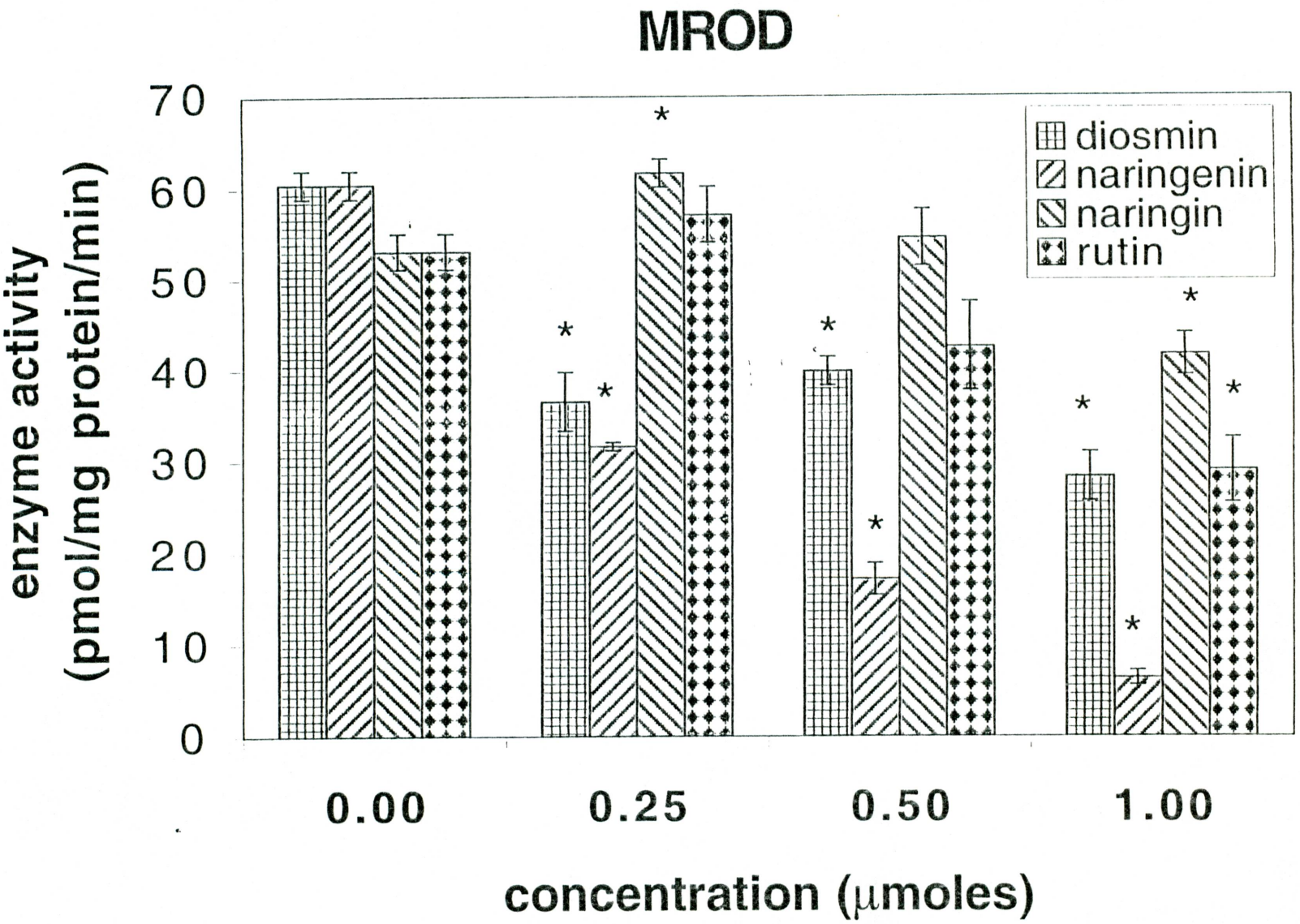

MeIQx
2400
2000
1600
1200
800
400
0
0.00
0.25
0.50
1.00
IQ
1200
900
600
300
0
0.00
0.25
0.50
1.00
Glu-P-1
2000
1600
1200
800
400
0
0.00
0.25
0.50
1.00
PhIP
1400
1200
1000
800
600
400
200
0
0.00
0.25
0.50
1.00

Figure 11. Effects of citrus flavonoids on the in vitro O-dealkylation of methoxyresorufin by rat liver S9. Procedural details are given in Materials and Methods. Values are means ± SEM of triplicate samples from each of two separate experiments. Asterisks indicate a statistically significant difference from control values, $p \leq 0.05$.


MROD
enzyme activity
(pmol/mg protein/min)
70
60
50
40
30
20
10
0
diosmin
naringenin
naringin
rutin
0.00
0.25
0.50
1.00
concentration (μmoles)

naringin displayed significant inhibition only at the 1.0 μmole dose. Naringin, at the lowest dose tested (0.25 μmole), caused a significant increase in MROD activity. Naringenin showed the greatest inhibitory activity of the four citrus flavonoids tested.

The inhibitory activity of diosmin towards MROD correlates with its antimutagenic activity toward MeIQx and PhIP but not toward IQ and Glu-P-1. The inhibition of MROD activity by naringenin showed a strong correlation with its inhibition of mutagenesis by all four HCA's. Naringin and rutin displayed antimutagenic and MROD inhibiting properties similarly.

Studies show that flavonoids which lack a sugar group (i.e., the aglycones) are better CYP enzyme inhibitors than their corresponding glycones (28). Results from MROD assays comparing the inhibitory properties of rutin and its aglycone quercetin in our lab have revealed similar findings (data not shown). Steric hindrance caused by the sugar group neohesperidose may cause naringin to be generally less effective in inhibiting CYP than its aglycone, naringenin.

The observed inconsistency of inhibition of MROD activity and inhibition of HCA induced mutagenesis by diosmin may relate to its effect on phase II enzymes present in rat liver S9. Use of microsomes rather than S9 could perhaps resolve this. Induction of phase II enzymes, e.g., glutathione S-transferase and quinone reductase, may be a significant factor in chemoprevention (29). The esterification reactions in the DNA adduct formation pathway (Figure 1) may be inhibited or activated by phytochemicals. Naringin, naringenin and rutin may inhibit this esterification step more than diosmin does. The methoxy group on the diosmin molecule may limit its effective antimutagenicity towards IQ and Glu-P-1. Polymethoxylated flavonoids, e.g., tangeretin,

in contrast to diosmin which has only one methoxy group, are somewhat unique to citrus fruits and display a wider range and greater degree of biological activity than the hydroxylated forms (30).

The flavonols morin, fisetin and rutin have each been demonstrated to inhibit aflatoxin B_1 induced mutagenesis in *S. typhimurium* TA98 in a dose-dependent pattern (31). Likewise, the flavonoids myricetin, morin and quercetin greatly decreased IQ and MeIQx induced mutagenesis in *S. typhimurium* TA98 (20). Experiments with female BALB/c mice fed diets containing equimolar amounts of quercetin and rutin revealed that the microsomes from the mice on the quercetin and rutin diets were significantly more potent activators of the HCA's MeIQ and Trp-P-2 (32). It has also been shown that naringin, naringenin and rutin significantly inhibited mutagenesis in *S. typhimurium* TA100 NR by the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (33).

Because some chemopreventive studies show mixed results with in vivo and in vitro experiments (e.g., flavonoids inducing and increasing phase I enzyme activities in vivo contrasting with inhibition of these enzymes in vitro), future research should focus on defining phytochemical mechanisms of action and better assessment of defined stucture-activity relationships. Phytochemicals administered singly and in combination should be tested in lab animals and eventually in humans. As the epidemiological and experimental data increasingly argue in favor of the benefits of phytochemicals, the consumption of phytochemicals in fruits, nuts, seeds and vegetables should be considered key to dietary prevention of cancer.

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CHAPTER FOUR

DISCUSSION

From our experiments we have found quercetin and naringenin to possess the overall greatest anticarcinogenic potential amongst the five citrus flavonoids examined. We have demonstrated that quercetin and naringenin can block or decrease some of the initial steps required by some carcinogenesis pathways. They may accomplish this by inhibiting the activity of phase I enzymes such as CYP1A2 and CYP2B1.

More specifically, the results of our first study presented in chapter two suggest that naringenin, quercetin and rutin may prevent the initial stages of NNK-induced carcinogenesis. The second study described in chapter three supports the chemopreventive potential of diosmin, naringin, naringenin and rutin against HCA-induced mutagenesis and carcinogenesis. Chapter Two results showed that naringenin, quercetin and rutin each significantly inhibited the α-hydroxylation pathway of NNK metabolic activation mediated by the hamster liver microsomes while naringin, naringenin and quercetin each significantly inhibited the same pathway mediated by the lung microsomes. Inhibition of this pathway decreases the methylation and pyridyloxobutylation of DNA and thus decreases the mutagenic effects of NNK. The most potent inhibitors of MROD and PROD activity in the liver microsomes were diosmin, naringenin and quercetin. All five flavonoids had a small but statistically significant inhibitory effect on MROD activity in lung microsomes whereas PROD activity was significantly inhibited only by rutin. PROD activity is linked to CYP2B isoforms (1-3) and had less activity than MROD in both hamster liver and lung microsomes. As indicated in the discussion in chapter two the inhibition of NNK

metabolism in hamster lung microsomes by naringenin, quercetin and rutin may be connected with the inhibition of CYP isoforms other than CYP1A2 and CYP2B1/2.

As described in chapter three naringenin and rutin displayed the greatest overall antimutagenic potential against HCA-induced mutagenesis with naringenin being the most potent by far. In the MROD assays, naringenin and diosmin were significant inhibitors at all three doses whereas rutin and naringin only displayed significant inhibition at the highest concentration used. Thus, the citrus flavonoid naringenin expressed the best correlation between the inhibition of HCA-induced mutagenesis and inhibition of MROD activity.

Certain phytochemicals possess anticarcinogenic properties (4-6). Identifying phytochemicals along with their mechanisms has been the focus of nutrition researchers interested in chemopreventive/anticarcinogenic agents in the human diet.

Various types of cancer are preventable (7,8). Prevention of cancer with subpharmacological doses, i.e., micronutrient levels, of phytochemicals parallels homeopathic treatment of patients. Micronutrient levels of combinations of phytochemicals can be obtained by normal consumption of foods of plant origin categorized as fruits, nuts, grains, herbs, legumes or vegetables. The aspects of homeopathic medicine that deal with chemoprevention can be subjected to careful scientific scrutiny to determine whether homeopathic treatment regimens are beneficial; and if they are, research can focus on explanations of how subpharmacological doses of combinations of phytochemicals inhibit various steps of carcinogenesis, tumor growth or metastasis.

Chemoprevention of cancer in the 21st century can focus on identified phytochemicals being singly administered at pharmacological doses to persons at high risk for certain types of cancer, or identified phytochemicals being administered in combination at subpharmacological doses as currently practiced by many homeopathic physicians. With the first approach, phytochemical treatment strategies and risks can be formulated (9). Upon identification of persons at high risk for various cancers, phytochemicals with known chemopreventive properties against specific cancer type(s) may be directly administered similar to chemotherapeutic treatments. However, this method of chemoprevention is contrary to the chemoprevention supported by epidemiological studies which focus on natural or normal consumption of a variety of phytochemicals each at micronutrient levels. Because chemotherapeutic treatment of cancer patients involves serious adverse side effects, an ever-increasing number of patients—particularly in the U.S.—are seeking homeopathic or alternative treatments. These treatments lie within the largely unregulated herbal industry. In order to prevent potential risks of consuming large amounts of herbs combined with chemotherapeutic agents, herbal quality control must be greatly increased along with the patient's pharmacist and/or physician being aware of the patient's total drug intake (10). Phytonutrition (health supporting roles of phytochemicals) and phytotherapeutics (modifications in human physiological function, i.e., a phytochemical's pharmacodynamics) must be maintained as active areas of research as science explores the potential benefits of phytochemicals in the human diet (11). Before phytochemicals are to be administered at pharmacological levels, in contrast to subpharmacological levels, they should pass the four phases of clinical research testing that the FDA requires of prescription drugs (11).

Cancer prevention in the 21st century should not only include reducing exposure to environmental carcinogens like HCA's and tobacco byproducts like NNK, but should encourage eating diets of fruits, nuts, grains, herbs, legumes and vegetables that contain natural phytochemicals. As epidemiological evidence and experimental data increasingly argue in favor of the health benefits of phytochemicals, the natural consumption of phytochemicals should be considered an important key to preventing cancer in humans.

Overall conclusions regarding chemopreventive mechanisms of phytochemical action as well as future clinical usefulness may be postulated given the results of such expanded in vitro and in vivo testing. These studies will provide us with better and more advanced cancer preventive knowledge and will help in formulating new phytochemically based cancer treatment regimens for increased efficacy with fewer and less harmful side effects as compared to currently administered therapeutic cancer treatment strategies.

The involvment of citrus flavonoids as potential anti-cancer agents include a correlation between the antiproliferative potency of quercetin for human colon cancer cells and its ability to inhibit cellular accumulation of ascorbic acid (12); inhibition of apoptosis in normal glomerular mesangial cells by quercetin while inducing apoptosis in tumor cells (13); supra-additive anticancer properties with chemically combined rutin and tempace (14); inhibition of lung metastasis induced by B16F10 melanoma cells in mice by rutin, naringin and naringenin (15); dose-dependent antiproliferative activity of diosmin against human colon cancer cell lines (16); and inhibition of MCF-7 human breast cancer cell proliferation by naringenin (17). Quercetin and rutin have demonstrated antitumor activity towards NK/Ly ascites tumor cells in mice (18) in

addition to inhibiting the formation of transformed rat tracheal epithelial cell colonies caused by exposure to benzo(a)pyrene (19).

A possible role for quercetin in the treatment of various human cancers is supported by experiments exploring or involving quercetin's interaction with type II estrogen binding sites. These cancers include melanoma (20), non-small-cell lung cancer (21), meningioma (22), colon (23) and ovarian cancers (24,25) and acute lymphoid and myeloid leukemias (26,27). In combination with tiaofurin quercetin worked synergistically to inhibit growth of human ovarian carcinoma cells possibly through the inhibition of 1-phosphatidylinositol 4-kinase activity in the signal transduction pathway to reduce inositol 1,4,5-triphosphate concentration (28). Quercetin was shown to be a potentially effective anticancer agent alone or in combination with adriamycin against multidrug-resistant human breast cancer possibly by decreasing P-glycoprotein concentrations (29). In the human breast cancer cell line MDA-MB468 quercetin had no effect on P-glycoprotein concentrations, but strongly inhibited, in a time and dose-dependent fashion, mutated p53 expression and cell proliferation (30). Quercetin and rutin each significantly reduced azoxymethanol-induced hyperproliferation of colonic epithelial cells and focal areas of displasia in mice (31) thus decreasing tumor incidence and multiplicity. Acting at the initiation phase of carcinogenesis, quercetin suppressed N-nitrosodiethylamine-induced lung tumorigenesis in mice (32) and induced terminal differentiation of human promyelocytic leukemia cells (HL-60) as measured by nitro blue tetrazolium reducing activity, nonspecific and specific esterase activity and phagocytic activity (33).

Some flavonoids may be suitable for coadministering with anticancer agents currently being used to blunt the adverse effects of the anticancer drugs. Antioxidative action of a rutin-based agent, alpha G-rutin, protected against doxorubicin-induced cardiotoxicity (34) suggesting that the ingestion of foods containing flavonoids may be protective. Doxorubicin is an anticancer agent commonly used to treat acute leukemias, Hodgkin's lymphoma and breast carcinoma with known cardiotoxicity. Quercetin affords concentration dependent protection against cisplatin toxicity in cultured renal tubular cells (35). Cisplatin is used in treating testicular and ovarian cancer but poses adverse effects on the kidneys.

The data cited including those we report in Chapters 2 and 3 support the potential clinical usefulness of citrus flavonoids as chemopreventive agents including the reduction of the adverse effects of anticancer drugs. Phytonutrition and phytopharmacology are emerging areas of research into the efficacy of phytochemicals as chemopreventive agents of human disease.

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