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大蒜精油免疫調控活性之研究

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主持人：李宗貴

參與人員：劉承慈、吳志中、陳暉雯、蔡佳文、張景泓

執行單位：中山醫學大學營養科學系

緣由與目的

大蒜(garlic, Allium sativum L.)數千年來即被廣泛栽植於中東及遠東地區，作為日常蔬菜與香辛調味料，此外，大蒜保健功效亦受到人們高度重視(Obrien, 1990)。近年來，已有相當多證據指出大蒜具有多種生理活性可應用於預防保健上，比如預防心血管疾病(Abramovitz et al., 1999; Bordia et al., 1996)、預防癌症(Takezaki et al., 1999; Yang et al., 1994)、調節血糖(Bordia et al., 1996)、調節免疫機能(Salman et al., 1999; Liu et al., 1998)、調節抗氧化活性(Wu et al., 2001)與抗菌(Klein et al., 1999)等，其中在預防癌症上一般認為與大蒜調節藥物解毒能力有關(Hu et al., 1997; Guyonnet et al., 1999; Yang et al., 1994)。已知大蒜生理活性與大蒜所含有的有機硫成分(organic sulforganic compounds)有密切關係，而且這些硫成分種類與數量受到大蒜加工方法與條件的影響，一般市售大蒜精即是利用水蒸汽蒸餾法萃取自新鮮大蒜的揮發性含硫成分，又稱為大蒜精油，二烯丙基硫化物(diallyl sulfide, DAS)、二烯丙基二硫化物(diallyl disulfide, DADS)、二烯丙基三硫化物(diallyl trisulfide, DATS)即是大蒜精油中三種主要成分。大蒜生理活性除與這些成分種類及含量有關外，目前也有證據顯示大蒜硫成分預防腫瘤或調控抗氧化活性與硫原子數目或烯丙基數目有關(Sparnins et al., 1988; Wu et al., 2001)。然而探討大蒜精油及其硫成分的免疫調節效應卻不多見，因此本實驗首先利用動物飼養模式探討不等劑量大蒜精油、DAS、DADS 或 DATS 對頸淋巴結淋巴細胞增生率之影響；並利用 macrophage Raw 264.7 細胞，在 0.1 μg/ml LPS 誘發下探討不同濃度大蒜精油、DAS、DADS 或 DATS 對 inducible nitric oxide synthase (iNOS)蛋白質及 mRNA 表現之影響。

材料與方法

一．實驗材料

大蒜精油參考 Sheen 等(1992)方法利用水蒸汽蒸餾法萃取自新鮮大蒜，大蒜精油之產率約為 0.25-0.3%，氣象層析質譜儀(G1800A GCD, Hewlett Packard, USA)分析指出其中 DAS、DADS 及 DATS 分別約為 10%、39%及 35%。純化 DAS 購自 Fluka Chemical Co. (Buchs, Switzerland)，DADS 購自 Tokyo Kasei Chemical Co. (Tokyo, Japan)，DATS 則購自 KLT Laboratories (St. Louis, MN)。

二．動物實驗與分析

4 週齡雄性 Sprague-Dawley 大鼠購自國科會實驗動物物中心，經一週 AIN76A 飲食適應後，依體重隨機分組，每組 5 隻，分別經口灌食大蒜精油及大蒜含硫成分，灌食劑量分別是：大蒜精油，200 mk/kg bw；DAS, 0.5 and 2.0 mmol/kg bw; DADS, 0.5 mmol/kg bw; DATS, 0.5 mmol/kg bw，控制組則是灌食 1ml/kg bw 玉米油。每週灌食三次，為期六週。

隔夜禁食，利用二氧化碳窒息法犧牲，由肝門脈採取血液樣本，並加入 sodium citrate 為抗凝血劑，500 g 離心 10 分鐘取得血漿；同時，分別取出肝臟、心臟、肺、胸腺、脾臟、腎臟等臟器並稱重，頸部淋巴結取出稱重後迅速分離淋巴細胞，培養後利用 Con A 作為分裂原刺激細胞分裂，以閃爍計數儀計數 ^{3}H-thymidine incorporation rate (Liu et al., 1998)；血漿將以自動生化分析儀分析 alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, glucose, creatinine。

實驗數據以平均值加減一個標準差(means ± SD)表示，一元變方分析後再以 Tukey's test 進行事後檢定，$P<0.05$ 表示處理組平均值間具有顯著差異性。

三、細胞培養與分析

Macrophage Raw264.7 細胞生長至 85-90%細胞密度後，加入 0.1 μg/ml LPS 及不同濃度 Garlic Oil (36.5 μg/ml)、DAS (100、300 μmol)、DADS (100、300 μmol) 及 DATS (50、100 μmol)，培養 16 小時後，取樣進行下列各項分析；以 ELISA 讀取器分析 nitrite 濃度；利用西方墨點法及北方墨點法分析 iNOS 蛋白質及 mRNA 表現(Chen et al., 2001)。

結果與討論

大蒜精油與三種含硫成分在六週灌食後，除了相對脾重及相對腎重外，最後體重、相對肝重、相對肺重、相對心重、相對胸腺重、相對頸部淋巴結重在各處理組間均無差異(Table 1)，200 mg/kg bw 大蒜精油組相對脾重顯著高於(除了 DATS 組)其他組別，相對腎重則是除了 50 mg/kg bw 大蒜精油組，其他大蒜處理組均顯著高於對照組。

血液生化值分析結果如 Table 2 所示，Alanine aminotransferase (ALT)活性、尿素氮濃度、肌酸酐濃度均不受大蒜精油及其三種主要硫成分灌食影響，但是 aspartate aminotransferase (AST)與血糖濃度則因大蒜成分的處理而變化；DADS 處理組的 AST 顯著高於對照組($P<0.05$)，其他處理組則與對照組無差別；血中葡萄糖濃度則是各大蒜處理組均低於對照組，其中 0.5 及 2.0 mmol/kg bw DAS 與 200 mg/kg bw 大蒜精油處理達到顯著差異水準。

Con A 誘發下頸部淋巴結淋巴細胞增生率如表三，各組細胞增生均呈現 Con A 劑量依賴關係，最高增生率出現在 ConA 濃度為 5 μg/mL 時，當濃度高於 10 μg/ml 時，細胞增生率則明顯下降，至於大蒜硫成分灌食對淋巴細胞增生的影響，由結果可見僅 DADS 與 DATS 具有提高淋巴細胞增生的效果，當 Con A 是 5 μg/mL 或 10 μg/mL 時細胞增生率均顯著高於對照組($P<0.05$)，DAS 相較對照組則無效果。

在 LPS 0.1 μg/ml 誘發巨噬細胞條件下，NO 生成能力如圖一所示，結果顯示 DAS、DADS、DATS 均呈劑量抑制效應，其中又以 DATS 抑制效果最強，DADS 次之，DAS 最弱，在與控制組比較之下，100 及 300 μM DADS 與 50 及 100 μM

DATS 均達顯著抑制水準($P<0.05$)，其中 300 μM DADS 與 50 或 100 μM DATS 分別減少 63 %、56%、64 %的亞硝酸生成濃度。

西方墨點法分析如圖二顯示大蒜精油及精油中的三種主成分對 iNOS 蛋白質表現影響與與前述 nitrate 濃度分析結果一致，DADS 在 100、300 μM 及 DATS 在 50、100 μM 濃度下，巨噬細胞 iNOS 蛋白質表現均受到明顯抑制，且呈劑量關係。至於 36.5 μg/ml 大蒜精油及 100 μM 或 300 μM DAS，對巨噬細胞 iNOS 蛋白質表現則無影響。iNOS mRNA 表現情形也與蛋白質結果相似，DADS 與 DATS 均顯著抑制 iNOS mRNA 的表現，且兩者抑制效果較 GO 及 DAS 強。另外；在 300 μM DAS 及 100 μM DADS 處理下，IκB-α 含量相較對照組明顯減少，而 IκB-α 含量則是 DADS 與 DATS 處理組則較 DAS 組高，且 DADS 呈現劑量關係。

以上結果可以得知：大蒜精油中三種含硫成分具有不等的免疫調控活性，就增加 CoA 誘發頸部淋巴細胞增生及抑制巨噬細胞 iNOS 表現而言，DATS 的調控效果最佳，DADS 次之，但 DAS 並無效果或僅有微弱效果。

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Table 1 Effects of garlic oil (GO), diallyl sulfide (DAS), diallyl disulfide (DADS), or diallyl trisulfide (DATS) on body weight and the percentage of organ weight to body weight.

Group	Dose*	Final bw	Liver	Lung	Spleen	Heart	Kidney	Thymus	Jugular lympho node
		g	%	%	%	%	%	%	%
Control	----	391±20	2.94±0.47	0.45±0.10	0.19±0.01^{b}	0.34±0.03	0.71±0.03^{a}	0.14±0.02	0.040±0.006
DAS	0.5	365±35	2.86±0.18	0.48±0.07	0.19±0.07^{b}	0.37±0.09	0.84±0.06^{b}	0.15±0.03	0.044±0.008
DAS	2.0	357±18	3.04±0.24	0.49±0.10	0.20±0.04	0.33±0.02	0.86±0.04	0.12±0.01	0.062±0.022
DADS	0.5	356±22	3.15±0.21	0.48±0.02	0.20±0.02^{b}	0.34±0.01	0.84±0.04^{b}	0.13±0.02	0.040±0.004
DATS	0.5	360±27	3.14±0.09	0.47±0.05	0.25±0.02^{ab}	0.32±0.02	0.83±0.07^{b}	0.13±0.01	0.048±0.012
GO	50	376±33	2.94±0.21	0.43±0.06	0.22±0.02	0.33±0.01	0.79±0.05	0.12±0.03	0.043±0.004
GO	200	348±25	2.87±0.20	0.46±0.08	0.31±0.02^{a}	0.34±0.06	0.85±0.08^{b}	0.12±0.02	0.042±0.007

*Rats were orally administered with 50 or 200 mg/kg bw of garlic oil (GO) or 0.5 or 2.0 mmol/kg bw of DAS, or 0.5 mol/kg bw of DADS or DATS for 6 weeks. Values are means±SD (n=5). ^{ab} Group means not sharing a same letter are significantly different by Tukey's test ($P<0.05$).

Table 2 Blood biochemical parameters in rats treated with garlic oil and organosulfur compounds for 6 weeks.

Group	Dose*	ALT	AST	Glucose	BUN	Creatinine	White Blood Cells
		U/L	U/L	mg/dl	mg/dl	mg/dl	10^6/ml
Control	----	28± 4	88±19^{b}	186±55^{a}	10.8±1.3	0.50±0.04	13.2±4.1
DAS	0.5	36± 7	100±20^{ab}	101±22^{b}	10.8±3.8	0.48±0.02	14.1±2.3
DAS	2.0	32± 3	80±17	104±30	12.4±1.5	0.45±0.10	9.9±2.9
DADS	0.5	72±46	165±60^{a}	111±17^{ab}	13.6±0.9	0.49±0.07	11.2±2.0
DATS	0.5	49±22	124±37^{ab}	149±46^{ab}	14.6±2.6	0.50±0.09	13.5±4.2
GO	50	29± 4	87±20	136±58	11.8±1.5	0.46±0.05	12.3±2.2
GO	200	33± 5	96±19^{b}	85±13^{b}	11.4±2.3	0.47±0.03	12.7±2.6

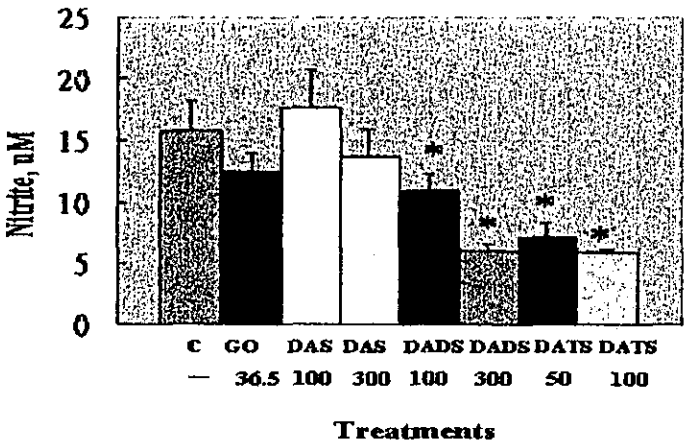
*Rats were orally administered with 50 or 200 mg/kg bw of garlic oil (GO) or 0.5 or 2.0 mmol/kg bw of DAS, or 0.5 mol/kg bw of DADS or DATS for 6 weeks. Values are means±SD (n=5). ^{ab} Group means not sharing a same lette are significantly different by Tukey's test ($P<0.05$). AST, aspartate aminotransferase; ALT, alanine amiotransferase; BUN, blood urea nitrogen.

Table 3 Effect of various garlic organosulfur components on Con A-induced lymphocyte proliferation.

	Con A, μg/mL					
Groups	0	1	2.5	5	10	50
	cpm, $x10^3$					
Control	1.09±0.59	4.22±1.39	6.64±2.25	7.26±1.77b	6.62±1.72b	0.40±0.14ab
DAS	1.11±0.18	3.57±0.97	7.36±1.50	7.36±1.67b	6.54±0.86b	0.58±0.26a
DADS	1.42±0.60	3.97±1.73	8.36±2.21	9.43±2.41a	9.44±3.32a	0.26±0.08b
DATS	1.47±0.41	4.47±1.62	8.26±1.52	10.98±1.24a	8.85±1.08a	0.53±0.33a

Rats were orally administered 0.5 mmol/kg body weight of DAS, DADS, or DATS for 6 weeks. Lymphocytes isolated from jugular lymph nodes were incubated with various concentration of Con A (0-50 μg/mL) for 48 h before the addition of ^{3}H-thymidine. Values are means±SD of three rats with four replications in each preparation. Groups not sharing the same letter (a,b) are significantly different by Tukey's test ($P<0.05$).

Fig1. Effect of various garlic organosulfur components on LPS-induced macrophage RAW264.7 nitrite concentration.



Values are means±SD (n=3).
*與控制組比較具顯著差異 (P<0.05)
大蒜成分處理濃度 : 0.1 μg/ml LPS
GO : 36.5 μg/ml DAS : 100, 300 μM
DADS : 100, 300 μM DATS : 50, 100 μM

Fig 2. Effect of various garlic organosulfur components on LPS-induced macrophage RAW264.7 iNOS protein expression.

(0.1 μg/ml LPS)

	Control	GO	DAS	DAS	DADS	DADS	DATS
μM	____	(36.5)	100	300	100	300	100

Fig 3. Effect of various garlic organosulfur components on LPS-induced macrophage RAW264.7 iNOS mRNA expression.

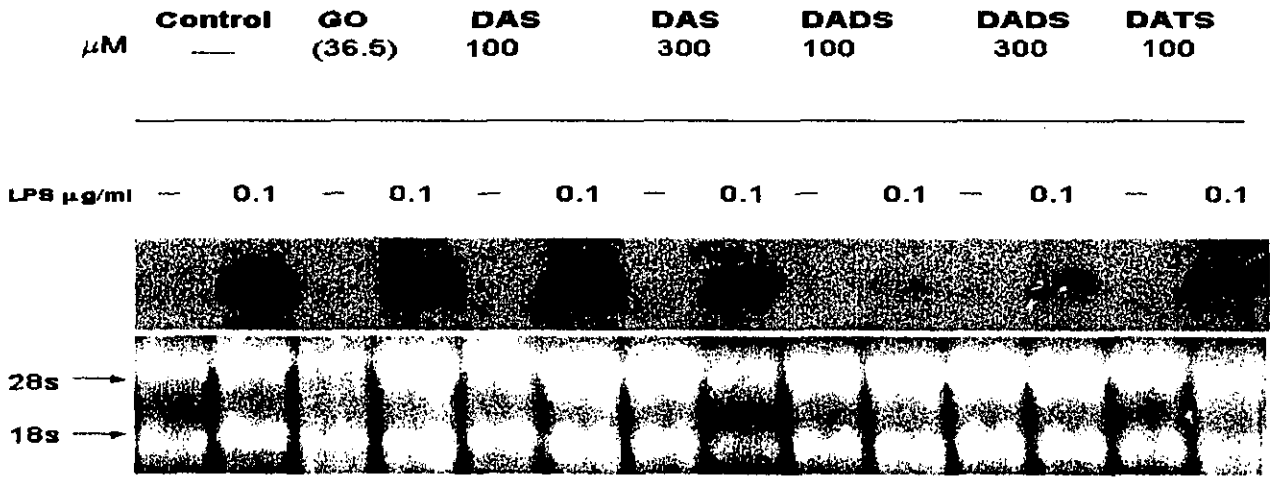
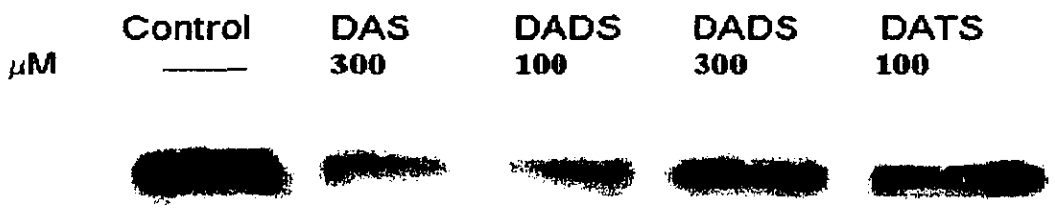


Fig 4. Effect of various garlic organosulfur components on LPS-induced macrophage RAW264.7 IκB-α protein expression.



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Research Section

Effects of organosulfur compounds from garlic oil on the antioxidation system in rat liver and red blood cells

C.C. Wu [a], L.Y. Sheen [b], H.-W. Chen [c], S.-J. Tsai [a], C.-K. Lii [c,*]

[a] *Department of Food Science, National Chung-Hsing University, Taichung, Taiwan*
[b] *Department of Nutrition, China Medical College, Taichung, Taiwan*
[c] *Department of Nutrition, Chung Shan Medical College, Taichung, Taiwan*

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Abstract

The modulation of garlic oil (GO) and three allyl compounds, diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), on the antioxidation system in rat livers and red blood cells was examined. Rats were orally administered GO (200 mg/kg body weight), DAS (20, 80 mg/kg body weight), DADS (80 mg/kg body weight) or DATS (70 mg/kg body weight) three times a week for 6 weeks. Control rats received corn oil (2 ml/kg body weight) alone. GO, DADS and DATS treatment significantly increased the glutathione (GSH) content (48–84%) in red blood cells ($P < 0.05$). DATS displayed a greater enhancement than GO and DADS ($P < 0.05$). Hemolysis induced by *tert*-butyl hydroperoxide was not suppressed by GO or allyl compound treatment although higher GSH content was evident. Hepatic GSH was not influenced by garlic components. In rat livers, DADS and DATS significantly increased the activity of GSH reductase (46 and 54%, respectively) and of GSH *S*-transferase (GST) (63 and 103%, respectively), but decreased the GSH peroxidase activity (27 and 28%, respectively). In contrast, GSH reductase and GST activities in the DAS group, either 20 or 80 mg/kg body weight, were similar to the control group. A decrease of GSH peroxidase activity was observed in rats dosed with 80 mg/kg body weight ($P < 0.05$). An increase in GST activity and a decrease in GSH peroxidase activities were also noted in GO-treated rats ($P < 0.05$). In red blood cells, three GSH-related antioxidant enzyme activities were not affected by garlic oil and its organosulfur components. Immunoblot assay showed that, accompanying the increase in hepatic GST activity, GO, DADS, DAS (80 mg/kg body weight) and DATS increased the expression of GST Ya, Yb1 and Yc proteins. Results indicate that GO and three allyl compounds play a differential role in modulation of the GSH-related antioxidant system in rat livers and red blood cells.

Keywords: Garlic oil; Allyl compounds; Antioxidation; Rats

1. Introduction

Garlic (*Allium sativum* L.) has been widely used as a foodstuff since antiquity, and has been known to play a diverse role in biological activities including hypolipidemia, antithrombosis, antiatherosclerosis, antimutagenesis, anticarcinogenesis and antibacterial (Agarwal, 1996). A variety of organosulfur compounds (OSCs) have been demonstrated to be active components in garlic (Lau and Tadi, 1990; Yang et al., 1994; Bordia et al., 1996; Drouin, 1999; Salman et al., 1999).

The methods used to manufacture garlic products include steam distillation, water extraction, drying and pulverizing or alcohol immersion. Products prepared by these different methods vary in their physiological activities. The steam distillation method is widely used to extract and condense volatile OSCs and the final oily product is called garlic oil (GO). More than 20 OSCs have been identified in GO by gas chromatography (Yu et al., 1989). Among these OSCs, diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS) are the three major components (Fig. 1). GO sold as health food is generally diluted with various vegetable

Abbreviations: DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; GO, garlic oil; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; OSCs, organosulfur compounds; PBS, phosphate buffered saline; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

* Corresponding author. Tel.: +886-4-473-0022, ext. 1747; fax: +886-4-473-9030.

E-mail address: cklii@mercury.csmc.edu.tw (C.-K. Lii).

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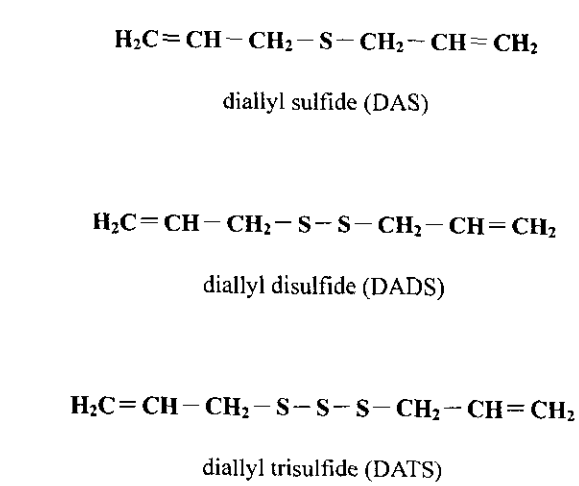
oils; these products have little difference in the percentage composition of OSCs, but they may vary in the total sulfide contents, reflecting various degrees of dilution (Lawson et al., 1991).

The anticarcinogenic/antitumorigenic effects of garlic have been attributed, at least in part, to modulation of the antioxidant and/or drug-metabolizing enzyme system (Yang et al., 1994). Among OSCs, DAS and DADS are two garlic components that have been studied extensively. In various animal studies, evidence indicated that these two allyl compounds were not only effective in modulating phase I and phase II metabolizing enzymes (Pan et al., 1990; Reicks and Crankshaw, 1996; Jeong and Lee, 1998; Singh et al., 1998), but also the antioxidant system capacity (Dwivedi et al., 1998; Chen et al., 1999). Recently, we reported that after a 7-week feeding, DAS and DADS changed rat hepatic glutathione (GSH)-related antioxidant enzyme activities by increasing the GSH reductase and decreasing the GSH peroxidase (GPx) activities (Sheen et al., 1999). The effects of DATS on the antioxidant system, however, are limited.

It has been suggested that the number of sulfur atoms and allyl groups may play a determining factor on the biological activities of garlic OSCs (Sumiyoshi and Wargovich, 1990). Sparnins et al. (1988) indicated that garlic components (such as DADS) which contain disulfide as well as allyl groups are more potent in inhibiting benzo[*a*]pyrene-induced forestomach neoplasia than those containing monosulfide (such as DAS) and propyl groups (such as dipropyl disulfide). Later, Wang et al. (1996) reported that OSCs having the allyl-S-allyl structure produced strong protection against acetaminophen-induced hepatic toxicity compared to those with the S-allyl structure, such as the methyl allyl sulfide and allyl mercaptan. However, few studies have examined the structural effect of active garlic components on the antioxidant system.



diallyl sulfide (DAS)

diallyl disulfide (DADS)

diallyl trisulfide (DATS)

Fig. 1. Chemical structures of diallyl sulfide, diallyl disulfide and diallyl trisulfide.

In this study, rats were orally administered GO, DAS, DADS or DATS, and the effects of these active garlic components on the level of GSH and the GSH-related antioxidant enzyme activity in liver tissues and red blood cells were investigated.

2. Materials and methods

2.1. Materials

GO was prepared by steam distillation. The GO constituents were analyzed and identified with the GC–MS system (G1800 GCD, Hewlett Packard, USA), and consisted of DAS (10%), DADS (40%), DATS (35%) and many minor volatile components. DAS, DADS and DATS were purchased from Fluka Chemical Co. (Buchs, Switzerland), Tokyo Kasei Chemical Co. (Japan) and LKT Laboratories, Inc. (St Paul, MN, USA), respectively. Antiserum against GST Ya, Yb1 and Yc were purchased from Biotrin Co. (Dublin, Ireland).

2.2. Animals and treatments

Four-week-old male Sprague–Dawley rats were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). Rats were randomly assigned to each experimental group by weight and housed with a 12-h light cycle. Animals had free access to water and diet. The diet contained (g/kg) 200 casein, 50 corn oil, 500 sucrose, 150 corn starch, 50 cellulose, 10 AIN-76 vitamin mix, 35 AIN-76 mineral mix, 3 methionine and 2 choline bitartrate. All diet ingredients were supplied by Harlan Teklad (Madison, WI, USA). The treated dose of garlic oil was 200 mg/kg body weight. The dosage of DAS, DADS and DATS was based on their relative amount in the garlic oil and was 20, 80 and 70 mg/kg body weight, respectively. An 80 mg/kg body weight dose of DAS, close to the DADS and DATS dosage, was also tested. All garlic components were diluted in corn oil and administered by oral intubation three times each week. Rats treated with corn oil alone (2 ml/kg body weight) were regarded as the control.

After 6 weeks of treatment, rats were fasted overnight and sacrificed by carbon dioxide euthanasia. Blood samples were drawn with sodium citrate (0.5 mg/ml) as an anticoagulant. After centrifugation, red blood cells were washed twice with cold phosphate buffered saline (PBS), pH 7.0, and finally resuspended in PBS containing 10 mM glucose. Red blood cells were used for GSH level and hemolysis assays immediately or stored at −80°C for later enzyme activity assay. The livers and spleens were removed and weighed immediately. Fresh livers were used for lipid peroxidation and GSH determination, or quickly freeze-clamped in liquid nitrogen and stored at −80°C until analysis.

2.3. GSH determination

Fresh liver tissues and red blood cells were used for GSH redox status analysis. Intracellular GSH and oxidized GSH (GSSG) were determined using HPLC (Reed et al., 1980). Owing to the existence of high concentrations of heme iron, red blood cells were pretreated with 100 mM iodoacetic acid in PBS for 15 min before perchloric acid precipitation (Lii and Hung, 1997). This pretreatment avoided GSH autooxidation, which yields high level of GSSG during sample preparation.

2.4. Antioxidant enzyme activity assays

Livers were homogenized in 4× of a buffer (pH 7.4) containing 10 mM potassium phosphate and 1.5 % KCl, and centrifuged at 10,000 *g* for 30 min at 4°C. The resultant supernatant was further ultracentrifuged at 105,000 *g* for 1 h and the final cytosol fraction was used for activity measurement. Hepatic GPx activity was determined spectrophotometrically with the coupled method using hydrogen peroxide (H_2O_2) as a substrate (Lawrence and Burk, 1976). In the red blood cells, the cell lysates were diluted first with 10-fold 1× PBS, and then with an equal volume of Drabkin's reagent (double strength) to block the peroxidase activity of hemoglobin (Andersen et al., 1997). GSH reductase activity was measured as described by Bellomo et al. (1987). GSH *S*-transferase (GST) activity was determined according to the method of Habig et al. (1974) using 2,4-chloro-dinitrobenzene as substrate. The superoxide dismutase (SOD) activity in the red blood cells was determined with a RANDOX SOD kit according to the manufacturer's instructions (RANDOX Laboratories, Antrim, UK).

2.5. Lipid peroxidation assay

Fresh livers were homogenized in 4× of a buffer (pH 7.4) containing 10 mM potassium phosphate and 1.5% KCl. The lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS) in a fluorescence spectrophotometer (Hitachi F4500, Tokyo, Japan) as described by Fraga et al. (1988).

2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

GST isoenzyme expression was determined by SDS–PAGE and immunoblot assay. Equal amounts of cytosol proteins were subjected to 10% polyacrylamide gels. Following electrophoresis, proteins separated on SDS–polyacrylamide gels were transferred to polyvinylidene difluoride membranes. To block the non-specific binding, membranes were incubated at 4°C overnight with 5% skim milk, followed by a 30-min incubation at 37°C with a mixture of antiserum containing antibodies against GST Ya, Yb1 and Yc isoforms. Peroxidase-conjugated secondary antibody, H_2O_2 and tetrachloride diaminobenzidine were used to detect the immunoreactive bands.

2.7. Hemolysis assay

Fresh red blood cells were suspended in PBS/10 mM glucose to a final hematocrit value of 5%. Cell suspensions were then incubated with 0.5 mM *tert*-butyl hydroperoxide at 37°C to initiate hemolysis. For various time intervals, the cell suspensions were sampled and red blood cells were quickly spun down at 500 *g*. The extent of hemoglobin released was spectrophotometrically determined at 540 nm.

2.8. Statistical analysis

Statistical analysis was performed using ANOVA (SAS Institute Inc., Cary, NC, USA). Tukey's multiple comparison was used to determine significant differences among group means ($P < 0.05$).

3. Results

3.1. Animal characteristics

Effects of GO and three allyl compounds on the growth characteristics are shown in Table 1. After 6 weeks of treatment, the body weight gain of the rats was not significantly different among the study groups. Liver weight (both crude and as a percentage of body weight) did not change by garlic components compared to the control. This hepatic parameter, however, in the DADS group was significantly higher than those in the 20 mg/kg body weight DAS group ($P < 0.05$). The percentage of spleen weight to body weight in rats administered GO was significantly higher than the control rats (data

Table 1
Effects of garlic oil, diallyl sulfide, diallyl disulfide and diallyl trisulfide on growth[a,b]

Groups	Body weight gain (g)	Liver weight/ body weight (%)
Control ($n=6$)	285±27	3.4±0.4a,b
GO 200 ($n=6$)	277±26	3.3±0.2a,b
DAS 20 ($n=6$)	273±24	3.2±0.1b
DAS 80 ($n=6$)	286±31	3.3±0.2a,b
DADS 80 ($n=6$)	295±45	3.7±0.4a
DATS 70 ($n=5$)	270±29	3.5±0.3a,b

[a] Data are expressed as means ±S.D. The average initial body weights among these groups were in the range from 154 to 158 g. Values following garlic components in the 'Groups' column represent the administered dosage (mg/kg bw).

[b] Numbers within a column not sharing the same letter are significantly different from one another ($P < 0.05$).

not shown). The enlargement of spleen tissues, however, was not noted in rats treated with either DAS, DADS or DATS.

3.2. GSH redox status and TBARS levels

As shown in Table 2, hepatic GSH levels in rats administered GO, DAS, DADS or DATS were not different from that in the control rats. Rats treated with DATS had significantly higher GSH content than the 80 mg/kg body weight DAS group ($P < 0.05$). In contrast to the lack of changes in hepatic GSH content, with the exception of the DAS group, either 20 or 80 mg/kg body weight, GSH levels in the red blood cells were significantly increased by GO, DADS and DATS ($P < 0.05$). The increase in GSH content was in the order of DATS (84%) > GO (50%) > DADS (47%) > DAS (2%). The GSSG and TBARS levels in liver tissues and the GSSG level in red blood cells were similar among all groups.

3.3. Antioxidant enzyme activities

Hepatic GPx, GSH reductase and GST activities in rats administered GO or allyl compounds are shown in Table 3. With the exception of 20 mg/kg body weight DAS, rat treated with GO, DADS, DATS and 80 mg/kg body weight DAS caused a significant decrease in GPx activity ($P < 0.05$). The GST activity, however, was significantly increased by GO, DADS and DATS but not by DAS, either 20 or 80 mg/kg body weight, as compared to the control rats. The activity of GST in rats treated with DATS was significantly higher than rats treated with GO. Regarding GSH reductase activity, rat administered DADS and DATS also resulted in higher enzyme activity as compared to the control rats ($P < 0.05$). In contrast to the effects of garlic allyl compounds on these GSH-related antioxidant enzyme activities in liver tissues, GO, DAS, DADS or DATS did not affect GPx, GSH reductase and GST activities in red blood cells (Table 4). However, the SOD activity was significantly higher in the GO group than the other groups ($P < 0.05$).

3.4. GST isozyme expression

To further examine whether GST expression was modulated by GO and three allyl compounds, an immunoblotting analysis was performed (Plate 1). Results showed that the levels of three GST isozymes, Ya, Yb1 and Yc, in liver tissues were simultaneously modulated by garlic treatments. Rat treatment with GO, DAS (80 mg/kg body weight), DADS and DATS increased the expression of all three GST isozymes. Accompanying the increase in GST activity, the effectiveness of induction of allyl compounds was DATS > DADS > DAS.

3.5. Hemolysis assay

Hemolysis analysis was used to test whether GO-, DADS- and DATS-induced cellular GSH content protects the membrane lysis of red blood cells in the presence of *tert*-butyl hydroperoxide (Fig. 2). As shown, hemolysis was noted after 60 min of *tert*-butyl hydroperoxide

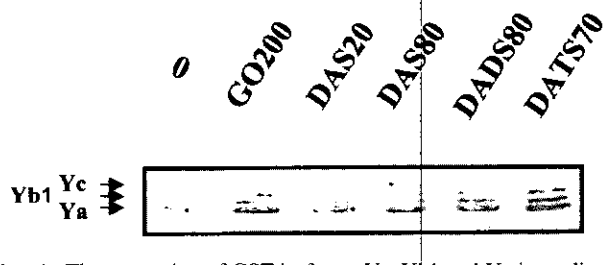


Plate 1. The expression of GST isoforms Yc, Yb1 and Ya in rat livers. 3 μg cytosol proteins were electrophoresized on a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Immunostaining was performed as described in Materials and Methods. The number in each lane represents the dosage (mg/kg bw) of the garlic components. GO, garlic oil; DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide.

Table 2
Effects of garlic oil and its organosulfur components on thiobarbituric acid-reactive substances (TBARS) and glutathione (GSH) levels in livers and red blood cells[a,b]

Groups	Hepatic			Red blood cells	
	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	TBARS (nmol/mg protein)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)
Control ($n=6$)	74.3±16.1a,b	2.3±0.3	0.22±0.08	8.2±1.5b	0.10±0.05
GO 200 ($n=6$)	72.6±5.7a,b	2.5±0.4	0.17±0.04	12.3±0.7c	0.11±0.03
DAS 20 ($n=6$)	75.1±8.4a,b	2.6±1.0	0.18±0.06	8.4±1.1b	0.11±0.05
DAS 80 ($n=6$)	62.6±19.3b	2.7±0.7	0.21±0.06	8.4±0.9b	0.11±0.02
DADS 80 ($n=6$)	92.6±19.7a,b	3.5±1.4	0.20±0.02	12.1±1.2c	0.10±0.03
DATS 70 ($n=5$)	101.0±27.0a	3.6±1.5	0.20±0.04	15.1±2.0a	0.11±0.05

[a] Data are expressed as means ±S.D. GO, garlic oil; DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide. Values following garlic components in the 'Groups' column represent the administered dosage (mg/kg body weight).

[b] Numbers within a column not sharing the same letter are significantly different from one another ($P < 0.05$).

Table 3
Hepatic GSH peroxidase, GSH reductase and GSH *S*-transferase (GST) activities in rats treated with garlic oil and its organosulfur components[ab]

Groups	GSH peroxidase (nmol/min/mg protein)	GST (nmol/min/mg protein)	GSH reductase (nmol/min/mg protein)
Control ($n=6$)	254±33a	2155±538c	31.4±7.9b
GO 200 ($n=6$)	191±18b	3137±532b	39.0±3.3a,b
DAS 20 ($n=6$)	260±21a	2165±387c	39.2±7.0a,b
DAS 80 ($n=6$)	205±30b	2990±492b,c	36.9±5.1a,b
DADS 80 ($n=6$)	185±33b	3565±631a,b	45.7±5.7a
DATS 70 ($n=5$)	191±22b	4243±447a	48.4±7.5a

[a] Data are expressed as means ±S.D. GO, garlic oil; DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide. Values following garlic components in the 'Groups' column represent the administered dosage (mg/kg body weight).

[b] Numbers within a column not sharing the same letter are significantly different from one another ($P<0.05$).

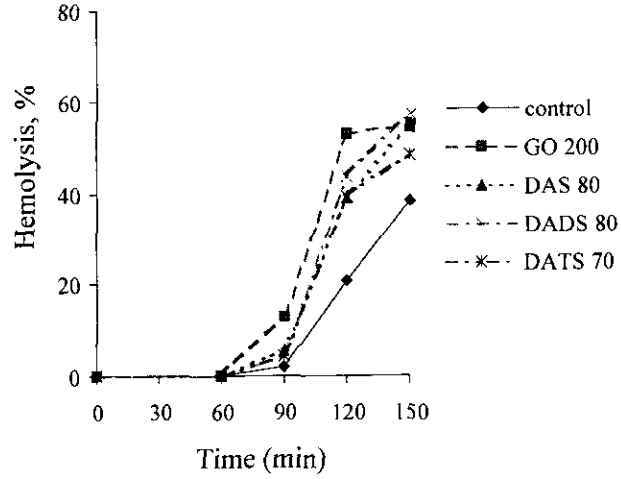


Fig. 2. Effects of garlic and allyl compounds on *tert*-butyl hydroperoxide-induced hemolysis. Fresh red blood cells were resuspended in PBS/10 mM glucose with a hematocrit value of 5% and were then incubated with 0.5 mM *tert*-butyl hydroperoxide at 37°C for 0, 60, 90, 120 and 150 min, respectively. Values are means from two separate experiments.

Table 4
Effects of garlic oil and its organosulfur compounds on the activities of GSH peroxidase, GSH reductase , GSH *S*-transferase (GST) and superoxide dismutase (SOD) in red blood cells[ab]

Groups	GSH peroxidase (nmol/min/mg protein)	GSH reductase (nmol/min/mg protein)	GST (nmol/min/mg protein)	SOD (U/min/mg protein)
Control ($n=6$)	37.4±3.9	8.6±1.1	17.1±3.6	0.087±0.028b
GO 200 ($n=6$)	39.6±6.6	10.7±2.3	21.0±3.6	0.132±0.011a
DAS 20 ($n=6$)	35.9±5.3	9.0±1.4	20.8±5.9	0.087±0.020b
DAS 80 ($n=6$)	34.4±5.4	9.3±1.9	19.5±5.2	0.072±0.016b
DADS 80 ($n=6$)	43.9±4.8	10.8±1.8	21.0±4.2	0.072±0.023b
DATS 70 ($n=5$)	43.2±2.1	11.2±2.1	22.0±2.8	0.074±0.013b

[a] Data are expressed as means ±S.D. GO, garlic oil; DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide. Values following garlic components in the 'Groups' column represent the administered dosage (mg/kg body weight).

[b] Numbers within a column not sharing the same letter are significantly different from one another ($P<0.05$).

treatment and then increased rapidly up to 150 min. The extent of hemolysis, however, was higher in GO, DADS and DATS-treated groups than in the control group. Higher hemolysis was also found in rats dosed with 80 mg/kg body weight DAS.

4. Discussion

This study showed that, after 6 weeks of treatment, GO and three major OSCs of GO did not change the body weight gain, the percentage of liver weight to body weight in treated rats. However, the dramatic increase of spleen weight by GO indicates that the spleen appears to be the target of GO. This suggests that GO may act on the host defense system by enhancement of the splenocyte proliferation rate (Liu et al., 1998). The reason the spleen was enlarged is not yet clear. It is also interesting to note that the effect of GO on the spleen was not seen in rats treated with DAS, DADS or DATS, which accounts for 85% of the total volatile OSCs in GO. This suggests that the existence of other minor active components in GO or the synergetic effect among three OSCs may contribute to the spleen enlargement.

GSH plays an important role in hepatic antioxidation and drug metabolism (Kosower and Kosower, 1978). The higher intracellular GSH content promotes reduced damage and better survival under oxidative stress (Meister, 1988). In this study, it was noted that GO significantly increases GSH content in red blood cells, and this increase is attributed to the effect of DADS and DATS but not DAS. However, three GSH-related enzyme activities measured in red blood cells were not changed by these garlic components. *tert*-Butyl hydroperoxide is an organic hydroperoxide, which is metabolized by GPx accompanying the oxidation of GSH to GSSG. The resultant GSSG is then reduced to GSH by GSH reductase with the consumption of NADPH. If this defense system is overwhelmed, *tert*-butyl hydroperoxide generates free radicals and initiates a free

radical chain reaction (Sies and Summer, 1975; Shertzer et al., 1994). In contrast to our hypothesis, the extent of hemolysis was higher in all garlic-treated groups than in the control group. This result indicates that factors other than GSH content may also be involved in determining the cell damage. GO has been reported to alter the fatty acid profile in membrane phospholipids in rat liver tissues (Liu et al., 1998).

Antioxidant enzymes are known to play a crucial role in the development of certain diseases, such as the cancer and atherosclerosis (Gaziano, 1999; Hayes and McLellan, 1999; Mates et al., 1999). Higher enzyme activity prevents the generation and accumulation of a variety of reactive oxygen species and produces better protection against oxidative damage. In this study, GSH peroxidase, GSH reductase and GST activities in the red blood cells were not changed by either GO or three allyl compounds. However, these GSH-related enzyme activities in the rat livers were modulated by these garlic components, but in different ways. In contrast to the inhibition of GSH peroxidase activity towards H_2O_2, GO, DADS and DATS significantly increased GST (Se-independent GSH peroxidase) activity ($P < 0.05$). The activity of GSH reductase was also noted to significantly increase with DADS and DATS ($P < 0.05$). In comparisons of the structure–function relationship among these allyl compounds, DATS showed the greatest effect, either induction or inhibition, followed by DADS and then by DAS. Such a finding suggests that the number of sulfur atoms determine, at least in part, their modulatory activities on the GSH related antioxidant enzymes. The relationship between structure and bioactivity has been reported in the inhibition of neoplasm formation (Sparnins et al., 1988; Hu et al., 1996; Singh et al., 1998). Results showed DADS and DATS are more effective in suppressing benzo[*a*]pyrene-induced forestomach tumorigenesis than DAS. Later, the differences in the chemopreventive efficacy of garlic OSCs were attributed to their differential ability to modulate GST activity toward anti-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, which is the active carcinogen of benzo[*a*]pyrene (Srivastava et al., 1997). In addition to the sulfur atom numbers, garlic sulfides which carry allyl groups, such as DAS and DADS, are more potent in the increase of GST activity than those carrying propyl groups, such as dipropyl sulfide and dipropyl disulfide (Srivastava et al., 1997).

GST not only acts as a selenium-independent GSH peroxidase, which reduces a variety of organic hydroperoxides to alcohols, but also detoxifies various xenobiotics. The GST family is composed of several isozymes in a homo- or hetero-dimer (Pickett and Lu, 1989). It is known that GST expression is highly inducible in the presence of various drugs and toxicants (Vos and Van Bladeren, 1990). In this study, along with the increase in GST activity towards CDNB, GO and its three major allyl compounds increased the level of GST Ya, Yb1 and Yc, and the extent of induction of three allyl compounds was in the order of DATS > DADS > DAS. Increase in GST expression and activity has been contributed to the anticarcinogenic/antitumorigenic activity of garlic OSCs (Chasseaud, 1979; Sparnins et al., 1982; Yang et al., 1994).

In conclusion, GO and its three major allyl sulfides effectively and differentially modulate the antioxidant system in rat livers and red blood cells. The effectiveness of DAS, DADS and DATS relates to the number of sulfur atoms.

Acknowledgements

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Dietary Fat and Garlic Oil Independently Regulate Hepatic Cytochrome P_{450} 2B1 and the Placental Form of Glutathione S-Transferase Expression in Rats[1]

Haw-Wen Chen, Jaw-Ji Yang,* Chia-Wen Tsai, Jyh-Jong Wu,† Lee-Yan Sheen,** Chu-Chyn Ou and Chong-Kuei Lii[2]

*Department of Nutrition, Chung Shan Medical College; †Department of Food Science, National Chung-Hsing University; **Department of Nutrition, China Medical College, Taichung 402; and *Institute of Molecular Biology, National Chung Cheng University, Min-Hsiung 621, Taiwan*

ABSTRACT The individual and combined effects of dietary fat and garlic oil on two drug-metabolizing enzymes, cytochrome P_{450} 2B1 and the placental form of glutathione (GSH) S-transferase (PGST), in rat liver were examined in this study. Rats were fed a low corn oil, high corn oil or high fish oil diet and received various amount of garlic oil (0, 30, 80, 200 mg/kg body) orally three times per week for 6 wk. The fat energy in the low and high fat diets accounted for 11.6 and 45.7% of total energy, respectively. Final body weights did not differ among the three dietary fat groups and were not affected by garlic oil treatment. The fatty acid profile in hepatic phospholipids revealed higher eicosapentaenoic acid [20:5(n-3)] and docosahexaenoic acid [22:6(n-3)] levels in the fish oil–fed group than in the low and high corn oil–fed groups ($P < 0.05$). In contrast, the corn oil–fed groups had greater hepatic phospholipid arachidonic acid [20:4(n-6)] levels ($P < 0.05$). Both dietary fat and garlic oil significantly affected hepatic cytochrome 7-pentoxyresorufin O-dealkylase (PROD) activity and GST activity toward ethacrynic acid. Rats fed the high fish oil diet had 85 and 51% higher PROD activity compared with those fed the low or the high corn oil diet, respectively ($P < 0.05$). The GST activity in the high fish oil and the high corn oil groups was 33 and 18% higher than that in the low corn oil group ($P < 0.05$), respectively, and the GST activity in rats fed the high fish oil diet was higher than in those fed the high corn oil diet ($P < 0.05$). Garlic oil dose-dependently increased GST activity. No interaction between dietary fat and garlic oil on PROD or GST activity was noted. Northern and Western blot analysis revealed that dietary fish oil increased both cytochrome P_{450} 2B1 and PGST mRNA and protein levels. Cytochrome P_{450} 2B1 and PGST mRNA and protein levels were also dose-dependently increased by garlic oil treatment. The effects of garlic oil and dietary fat on P_{450} 2B1 and PGST mRNA and protein expression were independent. These results indicate that dietary fat and garlic oil independently modulate P_{450} 2B1 and PGST expression at transcriptional and/or post-transcriptional stages. *J. Nutr.* 131: 1438–1443, 2001.

KEY WORDS: • *fish oil* • *garlic oil* • *cytochrome P_{450} 2B1* • *glutathione S-transferase* • *rats*

The hepatic bioactivation and detoxification system plays an important role in carcinogenesis. This system is composed of phase I and phase II enzymes. Phase I enzymes, mainly the cytochrome P_{450}, are involved in the bioactivation of chemical carcinogens, the biotransformation of many endogenous compounds and the detoxification of numerous xenobiotics (1,2). The physiologic function of phase II enzymes such as glutathione (GSH)[3] (3) S-transferases (GST) is to catalyze the conjugation of small water-soluble molecules to xenobiotics and facilitate their excretion. There is a strong inverse association between tissue levels of detoxifying enzymes and susceptibility to chemical carcinogenesis (4–6).

GST are a family of dimeric enzymes composed of at least seven gene products (3). Overexpression of the placental form of GST (PGST) has attracted great interest because of its relationship to carcinogenesis and human cancers (7,8). GST and cytochrome P_{450} are highly inducible in animals and humans, and their expression is affected by nutritional as well as nonnutritional factors. The dietary factors include lipid (9–11), vitamin E (12,13), water-soluble vitamins (14), garlic components (15) and green tea polyphenols (16). Recently, animal studies showed that rats fed a high fish oil diet (20.5 g/100 g) had significantly higher 7-pentoxyresorufin O-dealkylase (PROD) activity than rats fed a low or high corn oil diet (17). In addition to the source of dietary lipid, the amount of dietary lipid also plays an important role in the modulation of hepatic N-nitrosodimethylamine demethylase activity and cytochrome P_{450} 2E1 protein expression (10). A higher degree of unsaturation of polyunsaturated fatty acids present in liver

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[2] To whom correspondence should be addressed.
E-mail: cklii@mercury.csmc.edu.tw

[3] Abbreviations used: AFB_1, aflatoxin B_1; ARE, antioxidant-responsive element; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione S-transferase; HCO, high corn oil diet, HFO, high fish oil diet; LCO, low corn oil diet; PCR, polymerase chain reaction; PGST, placental GSH S-transferase; PROD, 7-pentoxyresorufin O-dealkylase.

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microsomal membranes may be associated with the increases in the rate of drug metabolism and drug oxidation (18).

The role of natural foods in disease prevention has been studied extensively in recent years. Among these natural foods, garlic has attracted a great deal of attention. During the past 15 y, most studies on garlic have been in the fields of cardiovascular and cancer research (19). Previous studies have found that ingestion of garlic is inversely related to the incidence of hyperlipidemia, atherosclerosis and thrombosis (19). Among the active garlic components, the organosulfur compounds are considered to be the most potent agents in chemoprevention (15), and their chemopreventive capabilities have been shown to be related to their modulation of drug-metabolizing enzymes involved in the activation or detoxification of carcinogens (20,21). Garlic oil is rich in numerous active organosulfur compounds such as diallyl sulfide, diallyl disulfide and diallyl trisulfide. Diallyl sulfide has been shown to increase the activities of cytochrome P_{450} 2B1 and GST (22–24).

Although dietary lipid and garlic modulate the activity of drug-metabolizing enzymes, the combined effect of garlic oil and fish oil on the drug metabolism system has not been studied. Recently, coadministration of garlic and fish oil was shown to ameliorate hyperlipidemia in hypercholesterolemic men (25). This study was designed to investigate the individual and combined action of fish oil and garlic oil on cytochrome P_{450} 2B1 and GST, particularly the placental form (PGST). The results of this study may help to clarify the extent of interaction between fish oil and garlic oil in carcinogen bioactivation and xenobiotic detoxification.

MATERIALS AND METHODS

Materials. 2,4-Chloro-dinitrobenzene (CDNB), 7-pentoxyresorufin and other biochemical reagents were purchased from Sigma Chemical (St. Louis, MO). Trizol was ordered from Gibco BRL (Grand Island, NY). Anti-P_{450} 2B1 polyclonal antibody was purchased from Oxford Biomedical Research (Oxford, MI). Antibody against PGST was purchased from Biotrin (Dublin, Ireland).

Garlic oil preparation. Garlic cloves were purchased from a local market. In each preparation, 1.5 kg of garlic cloves was blended with 3 L of distilled water in a Waring blender. Volatile components were extracted for 4 h with boiling distilled water. The extract was dried with anhydrous Na_2SO_4 and then filtered through nitrocellulose acetate membranes (26). The oily product was designated garlic oil. An average of 2.5 g of garlic oil was extracted from 1 kg of garlic cloves. The garlic oil constitutes were analyzed by a gas chromatography-mass spectrometry system (G1800, Hewlett Packard, Palo Alto, CA); the levels of the four major organosulfur compounds, diallyl sulfide, diallyl disulfide, diallyl trisulfide and allyl methyl trisulfide, were 5, 39, 34, and 10 g/100 g, respectively.

Animals and treatments. Male Sprague-Dawley rats (4 wk old) were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). After 1 wk of acclimation, rats were assigned to each experimental group by weight and housed in stainless steel wire cages, on a 12-h light:dark cycle. Rats were fed a diet containing 5 g/100 g corn oil (low corn oil, LCO) or 23.5 g/100 g corn oil (high corn oil, HCO) or 20.5 g/100 g fish oil + 3.0 g/100 g corn oil (high fish oil, HFO) (**Table 1**) as described by Reddy and Sugie (27). The composition of all experimental diets was adjusted so that rats in all of the dietary groups received the same dosage of vitamins, minerals and fiber (27). The addition of corn oil (3%) to the HFO diet was to prevent essential fatty acid deficiency. Diets were prepared every 2 wk and stored at −4°C. The diets provided to rats were changed every other day. Throughout the experiment, rats were administered 0, 30, 80 or 200 mg/kg body garlic oil (corn oil as a vehicle, 1 mL/kg body) by oral intubation three times per week. Rats were allowed free access to water and food. Body weight was measured weekly. Rats were treated in compliance with NIH guidelines (28).

TABLE 1

Formulation of the three diets[1]

Ingredients	LCO	HCO	HFO
		g/100 g	
Caseine	20.0	23.5	23.5
Sucrose	49.5	25.6	25.6
Cornstarch	15.0	15.0	15.0
Corn oil	5.0	23.5	3.0
Fish oil	—	—	20.5
Cellulose	5.0	5.9	5.9
AIN76 mineral mix[2]	3.5	4.11	4.11
AIN76 vitamin mix[2]	1.0	1.18	1.18
L-Methionine	0.3	0.35	0.35
Choline bitartrate	0.2	0.24	0.24
Cholesterol	0.5	0.59	0.59

[1] All diet ingredients except for fish oil were purchased from Harlan Teklad (Madison, MI). Fish oil, which contained 49 α-tocopherol equivalents/100 g, was purchased from Tama Biochemical (Tokyo, Japan). LCO, low corn oil; HCO, high corn oil; HFO, high fish oil.

[2] Source: (45).

After 6 wk, rats were deprived of food overnight and killed by carbon dioxide asphyxiation. The livers were removed and weighed immediately. Fresh livers were used for microsomal preparations and RNA extraction, or they were quickly freeze-clamped in liquid nitrogen and stored at −80°C until analysis.

Hepatic phospholipid fatty acid composition. Liver lipids were extracted according to the method of Folch et al. (29); total phospholipids were then isolated by TLC with hexane/diethyl ether/formic acid (80:20:2, v/v/v). After visualizing by spraying with 2′,7′-dichlorofluorescein (1 g/L methanol) and marking under UV light (366 nm), spots were scraped off and collected into glass tubes for fatty acid analysis. Fatty acid analysis was performed as described by Lepage and Roy (30) using a Supelco fused silica column with an i.d. of 0.25 mm (Bellefonte, PA). The integration of the peak area of each individual fatty acid was determined and its relative percentage of the sum of the peak area of all detectable fatty acids was calculated.

Hepatic PROD and GST activity assays. Livers were homogenized in 4 volumes of a buffer (pH 7.4) containing 10 mmol/L potassium phosphate and 150 mmol/L KCl, and centrifuged at 10,000 × g for 30 min at 4°C. The resultant supernatant was further ultracentrifuged at 105,000 × g for 1 h, and the final cytosolic supernatant was stored at −80°C until analysis. The microsomal pellets were resuspended in 50 mmol/L potassium phosphate, 1 mmol/L EDTA buffer (pH 7.6), and the activity of PROD was measured with a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) as previously described (31). Cytosolic GST activity was assayed by the method of Habig et al. (32), with CDNB as the substrate. Ethacrynic acid, which shows higher substrate specificity for PGST, was used as an alternative GST substrate (32,33).

cDNA probes. Two pairs of oligonucleotide primers were designed on the basis of the published sequences of 2B1 (forward: 5′-GGATGGGAAAGAGGAGTGTGGA-3′, backward: 5′-CTGGAGGAT GGTGGTGAAGAAG-3′) and PGST (forward: 5′-TTCAAGGCTCGCTCAAGTCCAC-3′, backward: 5′-CTTGATCTTGGGGCGGGCACTG-3′). mRNA obtained from rat liver tissues was used as the template for reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR conditions were set as follows: denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, for 35 cycles followed by a 7-min extension at 72°C. Bands corresponding to the DNA fragment of 2B1 and PGST were labeled with α-^{32}P-dCTP through NEBlot kit (New England Biolabs, Beverly, MA) and used as probes for Northern blot analysis.

RNA preparation and Northern blot analysis. Fresh liver (100 mg) was homogenized in 1 mL Trizol reagent using a power homogenizer. The homogenates were allowed to react at room temperature

for 5 min; 0.2 mL of chloroform was then added followed by incubation for an additional 3 min. The samples were centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by adding 0.5 mL isopropyl alcohol. The RNA samples remained at room temperature for 10 min followed by centrifugation at 12,000 × g for 10 min at 4°C. The resultant RNA pellets were washed twice with ice-cold ethanol.

For Northern blot analysis, 20 μg of each RNA sample was electrophoretically separated by 1% agarose gel containing 6% formaldehyde and transferred to HyBond membrane as previously described (34). For hybridization with cDNA, the membrane was prehybridized for 2 h at 42°C in a solution containing 10X Denhardt's reagent (0.2% Ficoll, 2 g/L polyvinylpyrolidone, 2 g/L bovine serum albumin), 5X SSPE (750 mmol/L NaCl, 50 mmol/L NaH_2PO_4, 5 mmol/L EDTA), 20 g/L SDS, 50% foramide, and 100 mg/L of single-strand sheared salmon sperm DNA. The membrane was then hybridized in the same solution with ^{32}P-labeled 2B1 cDNA probe at 4°C overnight. The membrane was washed 4 times with 2X SSC (300 mmol/L NaCl and 30 mmol/L sodium citrate)-0.5 g/L SDS at room temperature and twice with 0.1X SSC-1 g/L SDS at 52°C. Autoradiography was performed by exposing the membrane to Kodak SuperRx X-ray film at −80°C with an intensifying screen. For rehybridization with PGST cDNA probe, the membrane was deprobed by washing twice with boiling 1 g/L SDS. The bands on the X-ray film were measured with an Alphalmager 2000 (Alpha Innotech, San Leandro, CA).

SDS-polyacrylamide gel electrophoresis and immunodetection. Equal amounts of liver microsomal or cytosolic fractions were applied to 10% SDS-polyacrylamide gels. After electrophoresis, proteins separated on gels were transferred to polyvinylidiene difluoride membranes and were immunostained as described by Towbin et al. (35). The membranes were incubated with 50 g/L nonfat dry milk in 15 mmol/L Tris, 150 mmol/L NaCl buffer, pH 7.4, at 4°C overnight to block nonspecific binding. The membranes were then incubated with anti-P_{450} 2B1 and PGST antibodies at 37°C for 1 h followed by peroxidase-labeled goat anti-rabbit immunoglobulin G. Hydrogen peroxide and tetrahydrochloride diaminbenzidine were used for color development.

Statistical analysis. Data were analyzed by means of one-way ANOVA and Tukey's test was used to test the significance of the effect of garlic oil treatments in each dietary fat–fed group. Two-way ANOVA was used to test the effects of both dietary fat and garlic oil and their interaction. When variances were heterogeneous, data were log-transformed before ANOVA. All statistical analyses were performed with commercially available software (SAS Institute, Cary, NC). A value of $P < 0.05$ was considered significant.

RESULTS

Differences in dietary lipid amount and source had no effect on the growth of rats (**Table 2**). Final body weight of rats fed different dosages of garlic oil was also not significantly different. Differences in dietary lipid amount had a significant effect on liver weight and relative liver weight of rats ($P < 0.05$). Rats fed the HCO and HFO diets had significantly greater absolute and relative liver weights than rats fed the LCO diet ($P < 0.05$) (Table 2). However, garlic oil did not affect liver weight.

Dietary lipid amount and source did not affect hepatic phospholipids 14:0, 16:0, 18:0, 18:1 or 18:2 (**Table 3**). Rats fed the LCO and HCO diets had significantly greater hepatic phospholipid 20:4(n-6) than rats fed the HFO diet ($P < 0.05$). Rats fed the HFO diet had significantly more hepatic phospholipid 20:5(n-3) and 22:6(n-3) than rats fed the corn oil diets ($P < 0.05$).

Dietary lipid amount and source and garlic oil treatment significantly affected hepatic PROD and GST activities (**Table 4**). Rats fed the HFO diet had significantly greater hepatic PROD activity than rats fed the corn oil diets ($P < 0.05$). Rats fed the HFO and HCO diets had significantly greater hepatic GST activity than rats fed the LCO diet ($P < 0.05$). Rats fed the HFO diet had significantly higher PGST activity toward ethacrynic acid than rats fed the HCO diet, and the HCO group had significantly higher PGST activity than the LCO group. Within the LCO and HFO groups, rats treated with 200 mg/kg garlic oil had significantly greater hepatic PROD activity than those not treated with garlic oil ($P < 0.05$). Within all dietary lipid groups, garlic oil dose-dependently increased GST and PGST activities. Rats treated with 200 mg/kg garlic oil had significantly greater hepatic GST and PGST activities

TABLE 2

Effect of garlic oil on the body and liver weights of rats fed low corn oil (LCO), high corn oil (HCO) or high fish oil (HFO) diets for 6 wk[1]

Diet	Garlic oil	Initial wt	Final wt	Liver wt	Relative liver wt
	mg/kg	g			g/100 g body
LCO	0	167 ± 14	432 ± 32	17.3 ± 1.9	4.00 ± 0.27
	30	163 ± 14	412 ± 41	16.5 ± 2.0	4.01 ± 0.34
	80	164 ± 16	399 ± 40	16.5 ± 2.5	4.14 ± 0.39
	200	165 ± 4	424 ± 19	16.9 ± 1.5	3.98 ± 0.30
HCO	0	163 ± 13	445 ± 34	19.0 ± 1.9	4.26 ± 0.23
	30	163 ± 10	447 ± 21	19.9 ± 1.3	4.44 ± 0.36
	80	165 ± 9	436 ± 21	19.1 ± 0.6	4.40 ± 0.27
	200	155 ± 20	401 ± 24	17.4 ± 1.2	4.45 ± 0.30
HFO	0	157 ± 12	442 ± 49	19.4 ± 2.9	4.36 ± 0.36
	30	164 ± 14	433 ± 44	19.4 ± 2.3	4.47 ± 0.27
	80	167 ± 10	419 ± 41	18.5 ± 3.0	4.40 ± 0.40
	200	153 ± 19	407 ± 46	17.8 ± 2.1	4.40 ± 0.55
				P-value (2-way ANOVA)	
Dietary fat		NS	NS	0.0035[a]	0.0015[a]
Garlic oil		NS	NS	NS	NS
Dietary fat × garlic oil		NS	NS	NS	NS

[1] Rats were fed diets containing low (LCO) or high (HCO) amounts of corn oil, or a high amount of fish oil (HFO) for 6 wk. During the feeding period, rats were orally administered various amounts of garlic oil three times per week. Values are means ± SD, $n = 5$. [a] HFO and HCO groups are significantly greater than LCO group ($P < 0.05$); NS, $P \geq 0.05$.

TABLE 3

Fatty acid profiles in hepatic phospholipids in rats fed low corn oil (LCO), high corn oil (HCO) or high fish oil (HFO) diets for 6 wk[1,2]

Fatty acids	LCO	HCO	HFO
	g/100 g fatty acids		
14:0	0.27 ± 0.18	0.33 ± 0.13	0.38 ± 0.20
16:0	17.56 ± 1.83	15.12 ± 1.11	17.08 ± 2.46
18:0	22.65 ± 2.02	22.64 ± 1.42	21.97 ± 1.93
18:1(n-6)	6.16 ± 1.56	5.46 ± 0.93	6.01 ± 1.49
18:2(n-6)	13.16 ± 2.55	15.00 ± 1.69	12.92 ± 1.79
20:4(n-6)	37.24 ± 2.17[b]	37.75 ± 2.16[b]	16.80 ± 1.62[a]
20:5(n-3)	0.11 ± 0.18[b]	0.32 ± 0.12[b]	10.10 ± 1.48[a]
22:6(n-3)	2.84 ± 0.55[b]	3.39 ± 0.33[b]	15.36 ± 2.89[a]

[1] Values are means ± SD, $n = 5$. Means in a row that do not share a letter are significantly different, $P < 0.05$.
[2] Rats treated with no garlic oil.

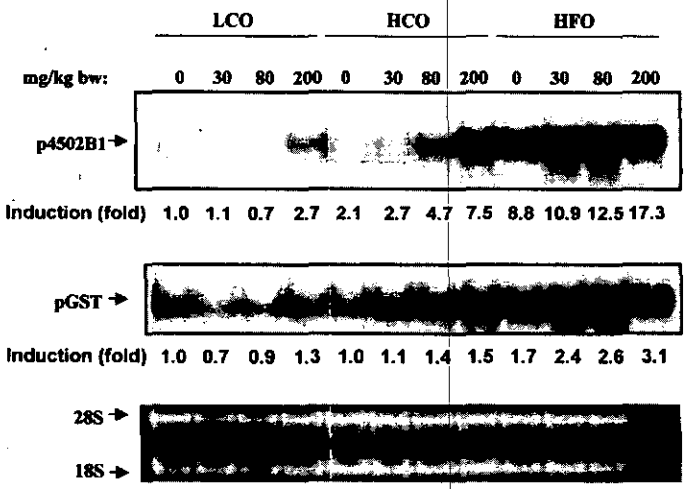

FIGURE 1 Hepatic P_{450} 2B1 and placental glutathione S-transferase (PGST) mRNA level in rats fed low corn oil (LCO), high corn oil (HCO) or high fish oil (HFO) diets and treated with 0, 30, 80 or 200 mg/kg garlic oil for 6 wk. Total RNA was extracted by Trizol as described in Materials and Methods. An equal amount of RNA was subjected to Northern blot analysis (20 μg/lane). The filter was first hybridized with a P_{450} 2B1 cDNA and then rehybridized with a PGST cDNA after deprobing. The fold of induction was quantitated by densitometry based on the relative amount of 18S RNA. A representative experiment is shown. The average values of P_{450} 2B1 mRNA induction of three separate experiments were (from left to right) 1.0 ± 0, 1.47 ± 0.57, 1.92 ± 1.07, 3.39 ± 0.70, 1.54 ± 0.55, 2.24 ± 0.40, 3.63 ± 1.00, 9.96 ± 2.91, 7.17 ± 3.20, 9.07 ± 3.33, 14.06 ± 1.65, 17.91 ± 2.06, respectively, and those of PGST mRNA were 1.0 ± 0, 0.93 ± 0.31, 1.48 ± 0.52, 2.05 ± 0.93, 1.08 ± 0.41, 1.38 ± 0.51, 1.76 ± 0.37, 1.98 ± 0.65, 1.88 ± 0.41, 2.65 ± 0.84, 2.70 ± 0.63, 3.61 ± 1.0, respectively.

than rats not treated with garlic oil ($P < 0.05$). Although both dietary fat and garlic oil significantly affected hepatic PROD, GST and PGST activities, no interaction was found between dietary fat and garlic oil on these three enzyme systems.

The modulation of mRNA and protein levels of hepatic P_{450} 2B1 and PGST by dietary fat and garlic oil is shown in **Figures 1** and **2**. The transcripts of hepatic P_{450} 2B1 were greater in rats fed the HFO diet than in those fed the corn oil diets. This expression was also greater in rats fed the HCO diet compared with rats fed the LCO diet. The 200 mg/kg garlic oil treatment increased the steady state of P_{450} 2B1 mRNA level in all diet groups, and a dose-dependent increase of mRNA by garlic oil was noted in both the HCO and the HFO groups. The transcripts of hepatic PGST were also affected by dietary lipid. As shown in Figure 1, the HFO diet increased the mRNA level of PGST compared with either corn oil diet. Garlic oil affected PGST expression in both the HCO and the HFO groups, but not in the LCO group. The induction of PROD and GST activities by garlic oil was accompanied by

TABLE 4

Effect of garlic oil on 7-pentoxyresorufin O-dealkylase (PROD) and glutathione S-transferase (GST) activities in rats fed low corn oil (LCO), high corn oil (HCO) or high fish oil (HFO) diets for 6 wk[1]

Diet	Garlic oil	PROD	GST	PGST
	mg/kg	*pmol/(min · mg protein)*	*nmol/(min · mg protein)*	
LCO	0	6.2 ± 2.3†	1959 ± 173‡	287 ± 24†
	30	7.4 ± 3.9*†	2251 ± 258†‡	325 ± 34*†
	80	12.4 ± 6.3*†	2654 ± 358*†	333 ± 53*†
	200	14.1 ± 5.7*	2905 ± 472*	378 ± 64*
HCO	0	11.3 ± 3.4	2666 ± 350‡	356 ± 12†
	30	11.2 ± 5.9	3162 ± 265†‡	373 ± 8†
	80	11.8 ± 2.6	3253 ± 244†	391 ± 32†
	200	15.0 ± 0.9	3993 ± 304*	443 ± 27*
HFO	0	13.3 ± 2.8†	2686 ± 219‡	421 ± 46†
	30	22.1 ± 6.1*	3100 ± 279†	406 ± 16†
	80	16.7 ± 3.1*†	3112 ± 115†	437 ± 27*†
	200	22.1 ± 5.0*	3620 ± 123*	492 ± 37*
			P-value (2-way ANOVA)	
Dietary fat		0.0001[a]	0.0001[b]	0.0001[c]
Garlic oil		0.0001	0.0001	0.0001
Dietary fat × garlic oil		NS	NS	NS

[1] Values are means ± SD, $n = 4$–5. PGST, placental form of GST. *†‡ Groups receiving the same diet that do not share a symbol are significantly different, $P < 0.05$. [a] HFO diet significantly greater than LCO and HCO diets, $P < 0.05$. [b] HFO and HCO diets significantly greater than LCO diet, $P < 0.05$. [c] HFO diet significantly greater than HCO diet and HCO diet greater than LCO diet, $P < 0.05$; NS, $P \geq 0.05$.

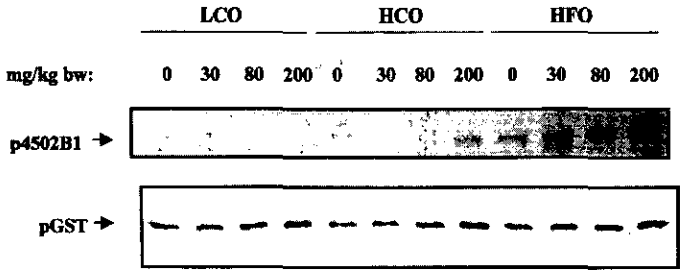

FIGURE 2 Immunoblot analysis of P_{450} 2B1 and placental glutathione S-transferase (PGST) in rats treated with 0, 30, 80 or 200 mg/kg garlic oil and fed low corn oil (LCO), high corn oil (HCO) or high fish oil (HFO) diets for 6 wk. Proteins were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes. The amount of protein in each lane for P_{450} 2B1 and PGST immunostaining was 6 and 3 μg, respectively. Protein level was quantitated by densitometry and the value in rats fed the LCO diet and receiving no garlic oil was regarded as 1.0. A representative experiment is shown. The average fold inductions of P_{450} 2B1 of three separate experiments were (from left to right) 1.0 ± 0, 1.50 ± 0.36, 1.91 ± 0.36, 3.98 ± 0.37, 1.75 ± 0.77, 3.29 ± 0.96, 4.33 ± 1.99, 6.99 ± 1.72, 5.70 ± 1.46, 10.0 ± 3.80, 9.60 ± 3.20, 14.3 ± 5.19, respectively, and those of PGST were 1.0 ± 0, 1.43 ± 0.19, 1.46 ± 0.14, 1.76 ± 0.35, 1.03 ± 0.18, 1.23 ± 0.20, 1.56 ± 0.17, 1.69 ± 0.23, 1.33 ± 0.19, 1.71 ± 0.38, 1.94 ± 0.21, 2.30 ± 0.40, respectively.

inductions of P_{450} 2B1 and PGST protein levels as determined by immunoblot assay. Regardless of dietary lipid amount and source, the induction of P_{450} 2B1 and PGST protein expression by garlic oil occurred in a dose-dependent manner. The protein expression of P_{450} 2B1 was greater in rats fed the HFO diet than in those fed corn oil diets. This expression was also greater in rats fed the HCO diet compared with those fed the LCO diet. The translational expression of PGST was higher in the HFO-fed group than in the corn oil–fed groups.

DISCUSSION

After 6 wk of treatment, the hepatic phospholipid fatty acid profile of rats was significantly affected by the dietary lipid source. Eicosapentaenoic acid and docosahexaenoic acid were incorporated into hepatic phospholipids of rats fed fish oil at the expense of arachidonic acid (Table 3). At the same time, the activity, protein level and mRNA expression of hepatic P_{450} 2B1 and PGST were affected by dietary regimen. In addition, garlic oil affected both detoxifying enzyme activity and expression. However, there was no interaction between dietary lipid and garlic oil (Table 4).

Cytochrome P_{450} 2B1 protein and mRNA levels were affected by both the amount and source of dietary lipid with an order of influence of HFO > HCO > LCO. Hepatic P_{450} 2B1 is one of the microsomal enzymes, and microsomal enzymes are embedded within the microsomal membranes. Any changes in the fatty acid composition in membrane phospholipids affects the fluidity of the microsomal membrane matrix and possibly alters the activity of cytochrome P_{450} via electron transfer from NADPH to cytochrome P_{450} (36). In addition to the microsomal P_{450} 2B1, changes in protein and mRNA levels of the cytosolic PGST suggest that, other than the alteration of enzyme conformation in membrane matrix, regulation of protein expression at the transcriptional and/or translational stages by the amount and source of dietary fat is possible. Numerous studies have suggested that dietary fatty acids can directly and indirectly modulate receptor-mediated signaling pathways at multiple levels and therefore the gene expression [see review in Hwang and Rhee (37)]. Elucidating the molecular and cellular mechanisms of such responses requires further study. Hepatic P_{450} 2B1 is inducible by phenobarbital, a hepatopromoter. The role of hepatic P_{450} 2B1 in carcinogenesis is not fully understood, but previous studies in rats indicate that it is involved in the activation of aflatoxin B_1 (AFB_1) to AFB_1-8,9-epoxide (38). In this study, we noted that the increase of P_{450} 2B1 protein and mRNA levels by garlic oil in rats fed the HCO and HFO diets was not consistent with the changes of PROD activity. This discrepancy was attributed in part to the characteristic wide substrate spectrum of P_{450}. Thus, PROD activity may not be fully representative of P_{450} 2B1.

In this study, GST activities toward CDNB and ethacrynic acid were affected by dietary lipid amount and source, and garlic oil treatment. However, no interaction that affected hepatic GST activity was found among these factors (Table 4). Among the GST isoforms, the expression of the placental form, which is highly inducible in hepatocarcinogenesis, is of particular interest (Figs. 1, 2). Similar to P_{450} 2B1, a HFO diet showed the greatest effect on PGST transcripts and protein levels. In this study, CDNB and ethacrynic acid were used as the substrates for GST activity assay, and PGST protein and mRNA expression patterns were more consistent with enzyme activity toward ethacrynic acid rather than CDNB. PGST is one of the GST isoenzmes, and GST activity toward CDNB may not truly reflect the PGST protein level due to the limited substrate specificity (33). The difference in enzyme activities toward CDNB, ethacrynic acid and PGST expression suggests that GST isoforms other than the placental form, such as Ya, Yb and Yc, may also be modulated in a different pattern by dietary lipid and/or garlic oil (39).

Recent studies found that the molecular mechanisms involved in the regulation of PGST gene are mediated by an antioxidant-responsive element (ARE) and the activator protein-1-responsive element; both are located on PGST gene promoter and/or enhancer regions (40,41). The role of garlic in chemoprevention has been attributed to its modulation of bioactivation and/or detoxification systems (20,21). However, the molecular mechanism of garlic effect is not clear, and it is compelling to investigate whether garlic oil mediation of the PGST gene is through the ARE pathway and/or the Fos/Jun binding to AP-1 binding site.

The growth of rats was not significantly affected by dietary lipid amount or source or by garlic oil treatment (Table 2). This result is not consistent with our previous study (42), which showed that oral intubation of 200 mg/kg garlic oil three times per week for 7 wk significantly decreased body weight gain of rats compared with oral intubation of 2 mL/kg corn oil as the control in both the high fat and low fat groups. This discrepancy may have been due to the age of rats used in the experiments and the duration of the experiments. The age of rats used in the present study was 5 wk, whereas that in the previous study was 4 wk. The experimental period in this study was 6 wk and that in the previous study was 7 wk. In a previous study (43), soft feces were found in rats treated with 200 mg/kg garlic oil; however, this phenomenon was not observed in the present study. This difference may have been due to the greater maturity of the gastrointestinal system of rats in the present study because the gastrointestinal systems of younger rats are more susceptible to the irritant effect of garlic oil. Lower intestinal mucosa protein contents noted in rats receiving the high dose of garlic oil indicated the possible effect of garlic oil on the gastrointestinal tract function (data not shown). In this study, absolute and relative liver weights of rats were significantly affected by dietary lipid amount; those re-

sults are consistent with those of a previous study (42) and were related to the difference in the hepatic bioactivation/detoxification system.

The aim of the present study was to investigate the individual and combined effects of fish oil and garlic oil on the bioactivation and/or detoxification systems in rats. Both of these compounds are involved in the regulation of P_{450} 2B1 and PGST. An increase in phase II enzyme systems enhances the detoxification potential of animals. However, an increase in phase I enzyme systems may not always be beneficial because induction of pathways protective against one group of compounds may potentiate the toxic effects of another class of toxins. The balance between activation and detoxification determines the net effect (44). In this study, both fish oil and garlic oil were active modulators and no interaction between these two factors on P_{450} 2B1 and PGST was identified.

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