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Dietary Diacetylene Falcarindiol Induces Phase 2 Drug-Metabolizing Enzymes and Blocks Carbon Tetrachloride-Induced Hepatotoxicity in Mice through Suppression of Lipid Peroxidation

Tomokazu Ohnuma, Eisaburo Anan, Rika Hoashi, Yuika Takeda, Takahito Nishiyama, Kenichiro Ogura, and Akira Hiratsuka*

Department of Drug Metabolism and Molecular Toxicology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan. Received November 5, 2010; accepted December 4, 2010; published online December 10, 2010

Falcarindiol is a diacetylenic natural product containing unique carbon-carbon triple bonds. Mice were orally administrated falcarindiol (100 mg/kg), and drug-metabolizing and antioxidant enzymes were monitored in several tissues of mice. Treatment with falcarindiol was found to increase glutathione S-transferase (GST) and NAD(P)H: quinone oxidoreductase 1 activities in liver, small intestine, kidney, and lung. No changes were observed in cytochrome P450 (CYP) 1A known to activate procarcinogens. Western blot analysis revealed that various GST subunits including GSTA4, which plays an important role in the detoxification of alkenals produced from lipid peroxides, were induced in liver, small intestine, and kidney of falcarindiol-treated mice. Additionally, we investigated the protective effects of falcarindiol against hepatotoxicity induced by carbon tetrachloride (CCl₄) and the mechanism of its hepatoprotective effect. Pretreatment with falcarindiol prior to the administration of CCl₄ significantly suppressed both an increase in serum alanine transaminase/aspartate transaminase (ALT/AST) activity and an increase in hepatic thiobarbituric acid reactive substance levels without affecting CCl_4 -mediated degradation of CYP2E1. Formation of hexanoyl-lysine and 4-hydroxy-2(E)-nonenal-histidine adducts, lipid peroxidation biomarkers, in homogenates from the liver of CCL₄-treated mice was decreased in the group of mice pretreated with falcarindiol. These results suggest that the protective effects of falcarindiol against CCl₄ toxicity might, in part, be explained by anti-lipid peroxidation activity associated with the induction of the **GSTs including GSTA4.**

Key words falcarindiol; drug-metabolizing enzyme; induction; glutathione S-transferase; lipid peroxidation; carbon tetrachloride

Falcarindiol (heptadeca-1,9(Z)-diene-4,6-diyne-3,8-diol) is a diacetylenic natural compound commonly occurring in the families Apiaceae and Araliaceae and is found in the edible parts of plants commonly used for food in the Apiaceae family such as carrot, celery, and parsnip.¹⁾ Falcarindiol has been shown to have antibacterial $activity^{2-4)}$ and an anti-inflammatory effect.^{5,6)} These beneficial effects occur at non-toxic concentrations and thus represent pharmacologically useful properties. Recently, we firstly found that falcarindiol has the ability to induce phase 2 drug-metabolizing enzymes (DMEs) and antioxidant enzymes in a liver-derived cell line and protect such cells against cytotoxicity caused by electrophilic stress.⁷⁾ Moreover, we elucidated the mechanism of phase 2 DME induction by falcarindiol at both cellular and molecular levels and emphasized that a conjugated diacetylene in the chemical structure of falcarindiol played an important role in the induction mechanism.⁸⁾

Phase 2 DMEs, such as glutathione *S*-transferase (GST) and UDP-glucuronosyltransferase (UGT), and quinone-reducing enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) are involved in the detoxification of carcinogens and function to facilitate their elimination. Therefore, induction of phase 2 DMEs and NQO1 is an effective mechanism for protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by chemical compounds. A wide variety of dietary and synthetic compounds that function as inducers of those DMEs have been shown to exert a chemopreventive effect. For instance, sulforaphane, an isothiocyanate compound found in broccoli, is known as a potent GST and NQO1 inducer and has been reported to suppress carcinogen-induced tumorigenesis in rodent organs, including colon, skin, and stomach. $^{9-11)} \,$

The expression of GST and NQO1 is up-regulated by nuclear factor-E2-related factor 2 (Nrf2) binding to the antioxidant response element (ARE), a *cis*-acting sequence located in the 5'-flanking region of these genes.^{12,13} Most GST and NQO1 inducers, including falcarindiol and sulforaphane, interact with critical cysteines in Kelch-like ECH-associated protein 1 (Keap1) through alkylation, allowing Nrf2 to escape proteasomal degradation and to accumulate in the nucleus.^{8,14—17)} Accumulated Nrf2 activates the expression of multiple categories of genes, including genes for phase 2 DME, anti-inflammatory responses, and molecular chaperones as well as stress response genes such as heme oxygenase-1 (HO-1).^{18,19)} Thus, the Keap1/Nrf2/ARE signaling pathway plays a central role in cytoprotection against various stress conditions.

Based on amino acid sequence similarities, seven classes (alpha, mu, pi, sigma, theta, omega, and zeta) of cytosolic GSTs are recognized in mammalian species.²⁰⁾ GSTs catalyze the nucleophilic addition of the thiol of glutathione (GSH) to a variety of electrophiles. Metabolites after glutathionylation are more hydrophilic and normally less reactive. They are readily excreted in bile and/or urine as conjugates. This action is believed to be a major mechanism for the detoxification of reactive ultimate carcinogens. In addition to being able to catalyze the formation of a thioether bond between GSH and electrophiles, a number of GST isozymes also exhibit GSH peroxidase activity and catalyze the reduction of hydroperoxides of fatty acids, phospholipids,

and DNA bases to their corresponding alcohols. For example, GSTs A1-1(2), A1(2)-3, and A3-3 have GSH peroxidase activity toward cholesterol 7-hydroperoxides, linoleic acid 13-hydroperoxide, linolenic acid 13-hydroperoxide, and arachidonic acid 15-hydroperoxide.²¹⁾ 4-Hydroxy-2(*E*)-nonenal (HNE), an end product of lipid peroxidation, is specifically conjugated with GSH by GSTA4-4.²²⁾

Our previous in vitro study demonstrated that phase 2 DMEs and antioxidant enzymes were induced by falcarindiol.⁷⁾ However, it is not known whether the induction can be reproduced in vivo. In the present study, we measured phase 2 DME and antioxidant enzyme activities in hepatic and extrahepatic tissues of mice treated with falcarindiol. Moreover, we investigated protective effects of falcarindiol on the hepatotoxicity of carbon tetrachloride (CCl₄), a potent lipid peroxidation inducer. The mechanism of CCl₄-induced hepatotoxicity is well studied in various models.²³⁻²⁶⁾ Metabolic activation of CCl, by cytochrome P450 (CYP) 2E1 to free radicals, namely trichloromethyl and trichloromethyl peroxy radicals, is reported to enhance heme degradation and lipid peroxidation in liver, resulting in hepatocyte necrosis.^{25,26)} In the present study, we investigated whether falcarindiol affects CYP2E1 degradation and lipid peroxidation caused by the free radicals. To examine the effect of falcarindiol on hepatic lipid peroxidation, the contents of hexanoyl-lysine adduct derived from modifications of lipid hydroperoxides such as linoleic acid 13-hydroperoxide and arachidonic acid 15-hydroperoxide and Michael adducts predominantly formed by reacting histidine residues with HNE were determined by Western blotting.

MATERIALS AND METHODS

Materials CCl₄, corn oil, ethoxyresorufin, 2,6-dichloroindophenol (DCIP), and 4-methylumbelliferone (4-MU) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). 1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and diagnostic kits to measure serum alanine transaminase (ALT) and aspartate transaminase (AST) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,1,3,3-Tetramethoxypropane was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Antibodies against HO-1 (Stressgen Biotechnologies Co., San Diego, CA, U.S.A.), CYP2E1, β -actin (Abnova Co., Taipei City, Taiwan), hexanoyl-lysine adduct, and HNE-histidine adduct (Nikken Seil Co., Ltd., Shizuoka, Japan) were all purchased. Antibodies against various GST subunits were prepared in our laboratory.²⁷⁾ Falcarindiol was isolated from Japanese hornwort kindly provided by Okubo Engei (Shizuoka, Japan) and purified by HPLC. Based on HPLC analysis, it was confirmed that the purity of falcarindiol was approximately 96%.

Animals and Treatment Male C57BL/6J mice (5 weeks old) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Ibaraki, Japan). Mice were housed in a lightcontrolled room at a constant temperature, and acclimatized for one week prior to use. All animal experiments were approved by the Animal Ethics Committee of Tokyo University of Pharmacy and Life Sciences. Mice were given water and laboratory rodent chow ad libitum throughout the entire experiment. In experiments on enzyme induction, mice were divided into four groups of three each. Falcarindiol was dissolved in corn oil and administered intragastrically (i.g.) at 0, 1, 10, or 100 mg/kg for 4 consecutive days in a volume of 10 ml/kg. Twenty-four hours after the last dose, mice were sacrificed under ether anesthesia, and tissues were excised. In experiments on CCl₄ hepatotoxicity, mice were divided into two groups of six each. Corn oil (vehicle control) or falcarindiol (100 mg/kg) was administered i.g. for 4 consecutive days. Twenty-four hours after the last dose, the mice were further divided into two groups of three each and were treated with corn oil or CCl_4 (4 g/kg, i.g.) dissolved in corn oil. Twenty-four hours after administration of vehicle or CCl₄, mice were sacrificed under ether anesthesia, blood samples were collected for assays of ALT/AST, and tissues were excised. Cytosols and microsomes from each tissue were prepared by ultracentrifugation at 105000 *a* for 60 min.

Enzyme Assay GSH content was measured according to the procedures described previously.²⁸⁾ The following assays were performed using cytosolic fractions. GST activity was measured using CDNB and DCNB as substrates.²⁹⁾ NQO1 activity was measured using DCIP as a substrate.³⁰⁾ In measurements of catalase and selenium-dependent GSH peroxidase (Se-GPx) activities, H_2O_2 was used as a substrate.^{31,32)} GSH reductase (GR) activity was measured with oxidized GSH as a substrate.³³⁾ The following assays were performed using microsomal fractions. UGT activity was measured with 4-MU as a substrate.⁷⁾ CYP1A and CYP2E1 activities were determined using ethoxyresorufin and aniline, respectively.^{34,35)}

Western Blot Analysis Cytosolic and microsomal fractions prepared from liver, small intestine, and kidney were separated by gel electrophoresis in the presence of 2-mercaptoethanol and were transferred onto a Hybond ECL nitrocellulose membrane (GE Healthcare U.K. Ltd., Buckinghamshire, England). Immunoreactive proteins were detected with the use of horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) and ECL blotting reagents (GE Healthcare). Photographs were representative immunoblots of the same sample loaded in duplicate.

Hepatotoxicity Assay Serum ALT and AST activities and the level of hepatic lipid peroxidation were measured to assess hepatotoxicity. Measurements of ALT and AST activities were done with a commercial kit according to the manufacturer's protocol. Lipid peroxidation products in the liver were assayed according to a thiobarbituric acid fluorometric method at an excitation wavelength of 515 nm and an emission wavelength of 553 nm using 1,1,3,3-tetramethoxypropane as a standard.³⁶

Histological Examinations Liver tissues of experimental and control mice were fixed with 4% formaldehyde in phosphate-buffered saline for 24 h. For examination by light microscopy, tissues were embedded in paraffin, sectioned at $3 \mu m$, and stained with hematoxylin and eosin (H&E). The extent of CCl₄-induced necrosis was evaluated by assessing morphological changes in liver sections stained with H&E.

Determination of Free Radical Scavenging Activity Free radical scavenging activity of falcarindiol was determined by a method involving the bleaching of stable DPPH. A reaction mixture containing test samples $(10 \,\mu l$ dissolved in methanol or water) and 190 μ l of a 200 μ M DPPH ethanolic solution was incubated at 37 °C for 20 min in a 96-well microplate. The absorbance of the free radical DPPH was measured at 515 nm using a microplate reader.

Induction of Hepatic Microsomal Lipid Peroxidation *in Vitro* Hepatic microsomal lipid peroxidation was induced by CCl_4 according to the procedure³⁷⁾ described previously with minor modifications. A reaction mixture was composed of 100 μ l of normal mouse hepatic microsomes (10 mg/ml), 869.5 μ l of 150 mM potassium phosphate (pH 7.2), 20 μ l of 10 mM NADPH, 0.5 μ l of CCl₄, and 10 μ l of various falcarindiol concentrations. After the reaction mixture was incubated for 20 min at 37 °C, levels of thiobarbituric acid reactive substances (TBARS) in microsomal membrane proteins were measured according to the same procedure as described above.

Statistical Analysis Data are expressed as mean \pm S.E.M. The statistical significance of differences was calculated using Student's *t* and Dunnett's tests and Bonferroni's method. Values of *p*<0.05 were considered significant.

RESULTS

Elevation of Phase 2 DME, NQO1, and Antioxidant Enzyme Activities by Falcarindiol None of the falcarindiol and corn oil treatments influenced body weight gain (data not shown). We investigated whether falcarindiol affected GST and NQO1 activities, which are often utilized as indicators of Nrf2 activation, in liver, small intestine, and kidney of mice. CDNB and DCNB were used to measure total GST and mu-class GST activities, respectively. As shown in Table 1, treatment of mice with falcarindiol (100 mg/kg) significantly increased GST and NQO1 activities in all three tissues. Interestingly, although there was no detectable GST activity against DCNB in small intestine of control mice, significant activity was observed in mice treated with 100 mg/kg of falcarindiol (Table 1).

Next, we investigated the effect of falcarindiol (100 mg/ kg) on DME and antioxidant enzymes in various tissues including lung, stomach, and heart. GST and NQO1 activities in the lung were significantly greater in falcarindiol-treated mice than in those not treated (Table 2). GST and NOO1 activities in the stomach of falcarindiol-treated mice were moderately (but non-significantly) 1.4-fold and 1.5-fold, respectively, higher compared with controls (Table 2). Treatment of mice with falcarindiol resulted in a significant increase in UGT activity in the small intestine and kidney, but not in the liver, stomach, and heart in comparison with control animals. In all tissues tested, CYP1A activity, which can convert promutagens such as polycyclic aromatic hydrocarbons and heterocyclic amines to proximate reactive metabolites, was not affected by falcarindiol (Table 2). Catalase, GSH, and GSHlinked antioxidant enzymes, including Se-GPx and GR, protect cells from oxidative stress through the detoxification of peroxides such as H2O2. We assayed these antioxidant enzyme activities. In comparison with controls, catalase and Se-GPx activities were significantly greater in the kidney and heart, respectively, of falcarindiol-treated mice, while significantly higher levels of intestinal GR and GSH were observed in falcarindiol-treated mice (Table 3).

Induction of GST Subunit Protein by Falcarindiol Expression levels of GST proteins in each tissue was examined by Western blotting to determine whether the elevations in GST activity in the liver, small intestine, and kidney of fal-

Table 1. Dose Dependent Changes in GST and NQO1 Activities in Liver, Small Intestine, and Kidney of Mice Treated with Falcarindiol

Enzyme activity	Treatment	Dose (mg/kg)	Liver	Small intestine	Kidney
GST activity	Control	0	1.31 ± 0.09	0.17±0.03	0.29±0.01
(µmol CDNB conjugated/min/mg)	Falcarindiol	1	1.41 ± 0.12	0.15 ± 0.00	$0.30 {\pm} 0.03$
	Falcarindiol	10	1.54 ± 0.04	0.22 ± 0.02	$0.36 {\pm} 0.06$
	Falcarindiol	100	2.45±0.18*	$0.35 \pm 0.09 *$	$0.63 \pm 0.04*$
GST activity	Control	0	6.38 ± 0.42	n.d.	1.95 ± 0.19
(nmol DCNB conjugated/min/mg)	Falcarindiol	1	5.90 ± 0.51	n.d.	2.36 ± 0.35
	Falcarindiol	10	$8.44 \pm 0.21*$	n.d.	3.16±0.23*
	Falcarindiol	100	14.0±1.39*	1.73 ± 0.31	$4.53 \pm 0.54 *$
NQO1 activity	Control	0	7.43 ± 0.69	51.1 ± 2.97	93.2±27.0
(nmol/min/mg)	Falcarindiol	1	7.64 ± 2.40	51.1±11.4	95.2±14.7
	Falcarindiol	10	10.3 ± 2.01	68.7 ± 5.06	149 ± 49.3
	Falcarindiol	100	22.0±1.78*	130±15.3*	219±6.90*

Data are expressed as mean \pm S.E.M. (n=3 mice in each treatment group). * Significantly different from each vehicle control at p<0.05 (Dunnett's test). n.d., not detectable (less than 1 nmol/min/mg).

Table 2. Drug-Metabolizing Enzyme Activities in Various Tissues of Mice Treated with Falcarindiol (100 mg/kg)

Enzyme activity	Treatment	Liver	Small intestine	Kidney	Lung	Stomach	Heart
GST activity	Control	1.31 ± 0.09	$0.17 {\pm} 0.03$	0.29 ± 0.01	0.20 ± 0.02	$0.34 {\pm} 0.06$	0.13 ± 0.01
(µmol/min/mg)	Falcarindiol	$2.45 \pm 0.18*$	$0.35 \pm 0.09 *$	$0.63 \pm 0.04 *$	$0.31 \pm 0.03*$	0.47 ± 0.04	0.15 ± 0.00
NQO1 activity	Control	7.43 ± 0.69	51.1 ± 2.97	93.2 ± 27.0	14.5 ± 0.73	547 ± 32.8	58.4 ± 11.3
(nmol/min/mg)	Falcarindiol	22.0±1.78*	130±15.3*	219±6.90*	31.1±2.57*	818 ± 130	58.3 ± 6.25
UGT activity	Control	23.6 ± 0.75	13.8±0.78	5.31 ± 0.58	7.84 ± 3.06	10.3 ± 2.68	$0.37 {\pm} 0.03$
(nmol/min/mg)	Falcarindiol	25.7±1.37	42.4±5.71*	$9.52 \pm 0.27 *$	12.9 ± 2.18	9.33 ± 2.57	0.29 ± 0.10
CYP1A activity	Control	56.1 ± 5.31	2.27 ± 0.17	3.77 ± 1.64	2.39 ± 0.34	5.36 ± 1.77	3.29 ± 0.99
(pmol/min/mg)	Falcarindiol	41.5 ± 6.65	1.96 ± 0.27	3.10 ± 1.20	2.57 ± 0.66	4.96 ± 1.30	3.33 ± 0.81

Data are expressed as mean±S.E.M. (n=3 mice in each treatment group). * Significantly different from each vehicle control at p<0.05 (Student's t test).

Enzyme activity	Treatment	Liver	Small intestine	Kidney	Lung	Stomach	Heart
Catalase activity	Control	75.1±13.7	2.48±0.55	58.8±3.41	3.20±0.95	7.14 ± 1.50	2.78 ± 0.83
(µmol/min/mg)	Falcarindiol	100 ± 8.22	2.12 ± 0.84	95.8±3.59*	3.78 ± 0.22	8.73 ± 0.30	3.91 ± 0.43
Se-GPx activity	Control	48.4 ± 1.47	38.7±13.2	68.4 ± 6.29	23.1±2.19	n.d.	14.2 ± 0.11
(nmol/min/mg)	Falcarindiol	46.7 ± 2.34	36.7 ± 9.76	82.0 ± 4.22	29.2 ± 1.93	n.d.	17.4±6.25*
GR activity	Control	76.7 ± 7.26	332 ± 8.96	212 ± 14.1	67.5 ± 3.49	199 ± 7.18	$32.8 {\pm} 2.05$
(nmol/min/mg)	Falcarindiol	94.1 ± 7.48	517±26.9*	229 ± 20.9	72.1±11.2	223 ± 10.5	36.9 ± 4.58
GSH content	Control	6.62 ± 0.12	2.30 ± 0.04	2.18 ± 0.17	1.90 ± 0.04	2.64 ± 0.05	1.05 ± 0.01
(μ mol/g tissue)	Falcarindiol	7.09 ± 0.17	$2.98 \pm 0.07 *$	2.62 ± 0.07	1.92 ± 0.04	2.74 ± 0.04	$1.07 {\pm} 0.01$

Table 3. Antioxidant Enzyme Activities and GSH Contents in Various Tissues of Mice Treated with Falcarindiol (100 mg/kg)

Data are expressed as mean \pm S.E.M. (n=3 mice in each treatment group). * Significantly different from each vehicle control at p<0.05 (Student's t test).

carindiol-treated mice were associated with induction of GST protein expression. Western blot analysis showed that falcarindiol treatment led to significant induction of alpha-class GSTA4 in all three tissues and marked induction of mu-class GSTM1 in small intestine (Fig. 1). Additionally, the expression level of pi-class GSTP1/2 was enhanced in small intestine, but not in liver and kidney. All GST subunits were found to be induced in the small intestine of falcarindiol-treated mice. HO-1 expression levels in mice treated with falcarindiol were greater in all three tissues in comparison with controls.

Protective Effect of Falcarindiol against CCl₄-Induced Hepatotoxicity To assess the falcarindiol-induced cytoprotective enzymes such as GSTs and HO-1, CCl₄, a lipid peroxidation and hepatotoxicity inducer, was given to two groups of mice pretreated with or without falcarindiol. Figure 2A shows the effect of falcarindiol on a CCl₄-induced elevation in serum ALT and AST activities. Pretreatment with falcarindiol resulted in no differences in serum ALT and AST activities compared to control animals not pretreated. A significantly higher serum level of ALT and AST was seen in mice administered CCl₄. However, the elevation in serum AST and ALT activities in the falcarindiol pretreatment groups was significantly lowered in comparison with CCl₄ alone. Liver injury was evaluated by a histological approach (Fig. 2B). Examination of H&E stained tissue revealed clear cytoplasm in those animals administered CCl₄. On the other hand, the hepatic lesions induced by CCl₄ were fewer by pretreatment with falcarindiol, which was in good agreement with the observed results in aminotransferase activity. Falcarindiol pretreatment alone did not cause a change in the liver histology.

To better understand the role of the DMEs induced by falcarindiol in protection against CCl_4 -induced hepatotoxicity in mice, we next determined the effect of orally administering falcarindiol following CCl_4 administration on activities of GST and NQO1 in the liver (Fig. 3). The group treated with falcarindiol alone showed a significantly higher both activities when compared with the control group. While the administration of CCl_4 lowered GST and NQO1 activities as compared with the control group, this decrease in both activities was significantly suppressed by falcarindiol pretreatment.

It is generally believed that CCl_4 toxicity results from the bioactivation of the CCl_4 molecule to the trichloromethyl free radical by CYP2E1.²⁶⁾ Because the trichloromethyl free radical degrades the heme moiety of microsomal CYP, CCl_4 is known as a suicide substrate of CYP2E1. Further experi-



Fig. 1. Induction of GSTs and HO-1 in Liver, Small Intestine, and Kidney by Treatment with 100 mg/kg Falcarindiol

GSTs in cytosolic fractions $(20 \,\mu\text{g})$ and HO-1 in microsomal fractions $(20 \,\mu\text{g})$ were detected by Western blotting. All samples were loaded in duplicate.



Fig. 2. Effect of Falcarindiol Pretreatment on CCl₄-Induced Hepatotoxicity in Mice

(A) Serum AST and ALT activities in each treatment group. Data represent means \pm S.E.M. (n=3 mice in each treatment group). * Significantly different from control (Bonferroni's method). # Significantly different from CCl₄ alone (Bonferroni's method). FAL: falcarindiol. (B) Representative H&E stained liver sections from mice administered vehicle or CCl₄ following pretreatment with vehicle or falcarindiol. Hepatic lobules in mice treated with CCl₄ alone were less eosinophilic than those not so treated. Scale bar in lower corner represents 200 μ m.



Fig. 3. Effect of Falcarindiol on GST and NQO1 Activities in CCl₄-Induced Hepatic Damage in Mice

Data represent means \pm S.E.M. (*n*=3 mice in each treatment group). * Significantly different from control (Bonferroni's method). # Significantly different from CCl₄ alone (Bonferroni's method).

ments were performed to investigate whether falcarindiol could affect CYP2E1 activity and the expression level of CYP2E1 in individual mice from all treatment groups. Figure 4A shows aniline hydroxylase activity of CYP2E1 in liver microsomes. Compared with control animals, CYP2E1 activity in mice receiving CCl₄ alone was significantly lower. In the mice pretreated with falcarindiol prior to the administration of CCl₄, CYP2E1 activity was similar to that observed in the CCl₄-treated mice. In contrast, in the mice receiving falcarindiol alone this activity was not altered. Although we investigated whether or not falcarindiol directly inhibits CYP2E1 activity in vitro, aniline hydroxylase activity in mouse hepatic microsomes was not inhibited in the presence of $10 \,\mu\text{M}$ falcarindiol (data not shown). Immunoblot analysis was performed to examine the effect of falcarindiol on CYP2E1 protein expression. As shown in Fig. 4B, pretreatment with falcarindiol did not affect the basal expression level of CYP2E1, while CCl₄ administration following pretreatment with falcarindiol resulted in a significantly lower CYP2E1 expression level in comparison with control animals. This result is consistent with microsomal aniline hydroxylase activity in each treatment group (Fig. 4), suggesting that falcarindiol was not able to inhibit heme degradation caused by the trichloromethyl radical.

In order to determine whether falcarindiol has the ability to directly scavenge free radicals, we performed a DPPH assay, which is commonly used as a simple test of free radical scavenging ability. Although ascorbic acid used as a positive control showed potent scavenging activity toward the DPPH radical, radical scavenging activity of falcarindiol was not detected in the DPPH assay (Fig. 5A). In addition, we investigated whether falcarindiol can directly suppress hepatic microsomal lipid peroxidation caused by trichloromethyl and trichloromethyl peroxy radicals derived from CCl₄. CCl₄-elevated levels of TBARS were not suppressed in the presence of falcarindiol (Fig. 5B). These data indicate that falcarindiol does not act as a radical scavenging compound and is not able to suppress lipid peroxidation.

To investigate the effect of pretreatment with falcarindiol on CCl_4 -induced lipid peroxidation in mouse liver, the levels of TBARS were measured as a marker of lipid peroxidation. As shown in Fig. 6A, the TBARS level in the CCl_4 -treated group was significantly higher than the control group. In contrast, pretreatment with falcarindiol significantly reduced the CCl_4 -elevated TBARS level to the control level. In addition, we detected hexanoyl–lysine and HNE–histidine adducts in liver homogenates by Western blotting using monoclonal an-



Fig. 4. Effect of Falcarindiol Pretreatment on CYP2E1 Activity and Protein Expression

(A) Hepatic microsomal CYP2E1 activity in each treatment group. Data represents means \pm S.E.M. (n=3 mice in each treatment group). * Significantly different from control (Bonferroni's method). (B) Western blot analysis of CYP2E1 expression in the liver of mice. Microsomal fractions (20 μ g) were examined by Western blotting using an antibody against CYP2E1. All samples were loaded in duplicate.



Fig. 5. Assessment of Radical Scavenging Capacity and Lipid Peroxidation-Inhibiting Capacity of Falcarindiol

(A) DPPH radical scavenging activity of falcarindiol. The DPPH radical scavenging assay was carried out as described in Materials and Methods. Ascorbic acid was used as a positive control. Data represent means of duplicate samples. (B) Direct inhibitory effect of falcarindiol on CCl₄-induced lipid peroxidation *in vitro*. Data represent means of duplicate samples.

tibodies. The amide-type hexanoyl–lysine adduct is a useful marker for the lipid hydroperoxide-derived modification of biomolecules.³⁸⁾ The Michael addition-type HNE–histidine adduct is useful for the evaluation of proteins modified by HNE, a major cytotoxic end product of lipid peroxidation.³⁹⁾ Western blot analysis revealed that the levels of both adducts generated by CCl₄-induced lipid peroxidation were reduced in the falcarindiol pretreatment group (Fig. 6B).



Fig. 6. Effect of Falcarindiol Pretreatment on CCl₄-Induced Lipid Peroxidation

(A) TBARS levels in liver homogenates of mice in each treatment group. Data represent means \pm S.E.M. (n=3 mice in each treatment group). * Significantly different from control (Bonferroni's method). * Significantly different from CCl₄ alone (Bonferroni's method). (B) Hexanoyl–lysine and HNE–histidine adducts in liver homogenates of mice in each treatment group. Liver homogenates ($20 \, \mu$ g) were analyzed by Western blotting using monoclonal antibodies for the detection of lipid peroxidation-derived protein modifications. All samples were loaded in duplicate.

DISCUSSION

The present study demonstrated that phase 2 DMEs and NQO1 in liver and extrahepatic tissues of mice were induced by falcarindiol, a diacetylenic natural compound. Previous studies reported that sulforaphane, a well-known GST and NQO1 inducer, protected against carcinogen-induced tumorigenesis in various experimental models.^{9–11} It is now widely accepted that induction of phase 2 DMEs, NQO1, and antioxidant enzymes by natural compounds leads to prevention of chemical carcinogenesis. Our previous studies have reported that the potency of falcarindiol to induce phase 2 DMEs *in vitro* is almost equivalent to that of sulforaphane.^{7,40} We believe that this is the first study to demonstrate the induction of phase 2 DMEs by falcarindiol *in vivo*.

Dose-dependent treatment with falcarindiol revealed that falcarindiol significantly induced GSTs and NQO1 in liver, small intestine, and kidney when administered orally to mice for 4 d at 100 mg/kg/d (approximately 7.7 μ mol/mouse/d) (Table 1). Zhang *et al.* showed the significant induction of GSTs and NQO1 in liver and small intestine of mice that were orally administered sulforaphane at 15 μ mol/mouse/d for 5 d.⁴¹⁾ The fact that the daily dose of falcarindiol to induce GSTs and NQO1 in mice was slightly lower than that of sulforaphane suggests that among natural compounds, falcarindiol may be a potent GST and NQO1 inducer *in vivo*.

GST and NQO1 activities in liver, small intestine, kidney, and lung of mice treated with 100 mg/kg falcarindiol were significantly elevated (Table 2). It has been reported that the expression of Nrf2, an important transcription factor of GSTs and NQO1, is higher in liver, small intestine, kidney, and lung where metabolism of endogenous and exogenous xenobiotics is active.⁴²⁾ Because falcarindiol induces GST and NQO1 *via* activation of the Keap1/Nrf2/ARE pathway,⁸⁾ it is reasonable that the increase in GST and NQO1 activities in falcarindiol-treated mice was observed in the organs expressing Nrf2 at high levels.

In comparison with control mice, UGT activity was increased in small intestine and kidney of falcarindiol-treated mice, but not in liver (Table 2). The induction pattern of UGT was similar to that of GST/NQO1, except for the liver. Although mouse UGT1A6 isoform having conjugation activity toward 4-MU is known to be regulated by the Keap1/Nrf2/ARE pathway,43) Higgins et al. reported that mouse UGT1A6 mRNA was not increased in liver but was increased in small intestine with the administration of coffee containing Nrf2 activating constituents,44) indicating that the induction of UGT1A6 gene expression by Nrf2 activators in mice was tissue-selective. The fact that UGT activity toward 4-MU in falcarindiol-treated mice was enhanced in the small intestine but not in the liver may be associated with the result reported by Higgins et al.44) We need to determine UGT activity using a specific substrate for UGT1A6 rather than 4-MU known as a broad substrate for UGT isoforms.

It is noteworthy that falcarindiol did not elevate hepatic CYP1A subfamily and CYP2E1 activities (Table 2, Fig. 4). The CYP1A subfamily converts promutagens such as polycyclic aromatic hydrocarbons and heterocyclic amines to proximate reactive metabolites.^{45,46} CYP2E1 is involved in the metabolism of small molecule compounds and plays a role in creating the harmful condition such as excessive oxidative stress.⁴⁷ Induction of these CYPs leads to potentiation of carcinogenicity of benzo[*a*]pyrene and alcohol-induced oxidative stress. Hence, it is important for chemoprevention not to induce these CYPs.

Western blot analysis using a series of anti-GST antibodies revealed that some GST proteins were induced by falcarindiol in the liver, small intestine, and kidney (Fig. 1). Among these tissues, the most prominent induction of GST subunits was seen in the small intestine. The remarkable induction of intestinal GSTM1 by falcarindiol was consistent with the result that GST activity toward DCNB known as a mu-class specific substrate was observed in intestinal cytosol of falcarindiol-treated mice (Table 1, Fig. 1). According to previous reports, it seems that induced GST subunits are subtly different in response to a variety of Nrf2 activators. For instance, as to GST subunits expressed in mouse liver, Higgins et al. reported that the expression level of GSTA3 in liver of mice fed coffee was not increased.⁴⁴⁾ In contrast, hepatic GSTA3 was induced by falcarindiol in the current study. Although Chanas et al. showed that GSTA1/2 proteins in liver of mice treated with butylated hydroxyanisole known as an Nrf2 activator were induced,⁴⁸⁾ falcarindiol did not induce hepatic GSTA1/2. Our results indicate that a characteristic GST subunit induced by falcarindiol is GSTA4. GSTA4 was markedly induced in the liver, small intestine, and kidney of falcarindiol-treated mice (Fig. 1).

 CCl_4 -induced hepatotoxicity has been studied for a long time since its demonstration by Recknagel.²³⁾ The hepatotoxicity of CCl_4 has been thought to be caused by heme degradation through covalent binding of CYP2E1-generated trichloromethyl radical and caused by trichloromethyl peroxy radical-mediated lipid peroxidation. In the present study, we noted the falcarindiol-induced GSTs involved in the detoxification of membrane lipid hydroperoxides and alkenals. To investigate the protective effects of falcarindiol against CCl_4 - induced hepatotoxicity and the mechanism of its hepatoprotective effect, we performed the following experiments. Firstly, serum AST and ALT levels, which are utilized as biochemical markers of hepatic injury, were measured. Elevations of both activities by CCl₄ administration were significantly decreased in falcarindiol-pretreated mice, indicating that falcarindiol suppressed CCl₄-induced hepatotoxicity (Fig. 2A). Results of the histological analysis of hepatocytes also lend support to the hepatoprotective effect of falcarindiol (Fig. 2B). Secondly, the effect of falcarindiol on the hepatic expression level and activity of CYP2E1 was examined. Puerarin is an isoflavone glycoside, which protects against CCl₄-induced hepatotoxicity by reducing the hepatic expression level of CYP2E1.49 Because CYP2E1 generates toxic free radicals derived from CCl₄, the suppression of CYP2E1 expression by puerarin pretreatment leads to protection against CCl₄-induced hepatotoxicity. However, falcarindiol did not affect the basal expression level and enzyme activity of CYP2E1 (Fig. 4). Our data suggest that there was no meaningful relationship between falcarindiolmediated protection against the hepatotoxicity of CCl₄ and CYP2E1. Another important point in suppression of CCl₄ hepatotoxicity is the direct scavenging of free radicals. To confirm this point, we investigated whether falcarindiol exhibits radical scavenging activity. Indeed, administration of antioxidants such as vitamins C and E is reported to protect against CCl₄-induced hepatotoxicity.⁵⁰⁾ These antioxidants exert potent radical scavenging activity in vitro and in vivo. According to the results of DPPH assav and in vitro TBARS measurement in the present study, it is unlikely that free radical scavenging activity of falcarindiol plays an important role in the suppression of CCl₄-induced hepatotoxicity (Fig. 5). Finally. The ability of falcarindiol to suppress CCl₄-induced lipid peroxidation was examined. Our data indicated that fal-



Fig. 7. Proposed Mechanism of Protection against CCl₄-Induced Hepatotoxicity in Falcarindiol-Pretreated Mice

Dotted lines indicate that falcarindiol dose not have the activity. Parallel oblique line indicates the suppressive effect of falcarindiol on CCl_4 -induced hepatotoxicity.

carindiol suppressed the enhancement of the hepatic TBARS level and lipid–protein adduct formation induced by CCl_4 administration (Fig. 6). Taken together, we conclude that the suppression of lipid peroxidation by falcarindiol leads to protection against CCl_4 -induced hepatotoxicity and that the induction of GSTs may, in part, participate in the anti-lipid peroxidation activity of falcarindiol (Fig. 7). However, we should point out that the anti-lipid peroxidation activity of falcarindiol was not sufficient to completely suppress CCl_4 -induced hepatotoxicity.

In conclusion, the results of this study demonstrated that falcarindiol was absorbed and distributed extensively to various tissues after oral administration and that it can induce selectively phase 2 DMEs and NQO1 involved in the detoxification of chemical carcinogens. We found that orally administered falcarindiol mitigated CCl_4 -induced hepatotoxicity through the suppression of lipid peroxidation.

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