Biochemical and Molecular Action of Nutrients

Curcumin and Especially Tetrahydrocurcumin Ameliorate Oxidative Stress-Induced Renal Injury in Mice

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ABSTRACT Protective effects of curcumin (U1), one of the major yellow pigments in turmeric and its derivative, tetrahydrocurcumin (THU1), against ferric nitrilotriacetate (Fe-NTA)-induced oxidative renal damage were studied in male ddY mice. Single Fe-NTA treatment (5 mg Fe/kg body intraperitoneally) transiently causes oxidative stress, as shown by the accumulation of lipid peroxidation products and 8-hydroxy-2′-deoxyguanosine in the kidney. Mice were fed with a diet containing 0.5 g/100 g U1 or THU1 for 4 wk. THU1 significantly inhibited 2-thiobarbituric acid reactive substances and 4-hydroxy-2-nonenal-modified proteins and 8-hydroxy-2′-deoxyguanosine formation in the kidney; U1 inhibited only 4-hydroxy-2-nonenal-modified protein formation. To elucidate the mechanisms of protection by U1 and THU1, the pharmacokinetics and radical-scavenging capacities of U1 and THU1 were investigated by HPLC and electron spin resonance spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide, respectively. Induction of antioxidant enzymes was also investigated. The amounts of THU1 and its conjugates (as sulfates and glucuronides) in the liver and serum were larger in the THU1 group than in the U1 group. The amounts of U1 and its conjugates were small even in the U1 group. These results suggest that THU1 is more easily absorbed from the gastrointestinal tract than U1. Furthermore, THU1 induced antioxidant enzymes, such as glutathione peroxidase, glutathione *S*-transferase and NADPH: quinone reductase, as well as or better than U1 and scavenged Fe-NTA-induced free radicals in vitro better than U1. These results suggest that U1 is converted to THU1 in vivo and that THU1 is a more promising chemopreventive agent. J. Nutr. 131: 2090–2095, 2001.

KEY WORDS: • *curcumin* • *tetrahydrocurcumin* • *lipid peroxidation* • *oxidative stress* • *rats*

Several lines of evidence indicate that oxidative stress may play an important role in various pathological conditions, including cancer, neurodegeneration, atherosclerosis, diabetes and rheumatoid arthritis, as well as drug-associated toxicity, postischemic reoxygenation injury and aging (1). An iron chelate, ferric nitrilotriacetate (Fe-NTA),[2] induces acute renal proximal tubular necrosis, a consequence of free radical-mediated oxidative tissue damage that eventually leads to a high incidence of renal cell carcinoma in rodents (2–4). It has been shown that the amount of free radical-associated modified molecules as assessed by lipid peroxidation products, aldehyde-modified proteins and a variety of modified DNA bases, such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), reaches the maximum as early as 3 h after single Fe-NTA treatment and gradually decreases thereafter (5,6).

Curcumin is a major yellow pigment in turmeric (the ground rhizome of *Curcuma longa* Linn), which is widely used as a spice and coloring agent in several foods, such as curry, mustard and potato chips, as well as cosmetics and drugs. A wide range of biological and pharmacological activities of curcumin has been investigated (7,8). Curcumin is a potent inhibitor of mutagenesis and chemically induced carcinogenesis (9–11). It possesses many therapeutic properties including anti-inflammatory and anticancer activities (12). Curcumin is currently attracting strong attention due to its antioxidant potential as well as its relatively low toxicity to rodents. Curcuminoids also exhibited antioxidant activities in some in vitro lipid peroxidation systems (13,14) and suppressed 12-*O*-tetradecanoylphorbol 13-acetate-induced hydrogen peroxide production and oxidized DNA formation in the mouse epidermis (15). Curcumin is an inhibitor of neutrophil responses (16) and superoxide generation in macrophages (17).

Tetrahydrocurcumin (THU1; **Fig. 1**), one of the major colorless metabolites of curcumin (U1), in the form of glucuronide conjugate, had stronger antioxidant activity than curcumin in several in vitro systems (13,14). Therefore, THU1 has been hypothesized to be one of the major metabolites with greater physiological and pharmacological activities than U1 in the intestine. However, there are few data concerning the metabolism and antioxidant functions of U1 and THU1 in

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[2] Abbreviations used: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; 1- CDNB, chloro-2, 4-dinitrobenzene; DMPO, 5,5′-dimethyl-1-pyrroline-1-oxide; ESR, electron spin resonance; Fe-NTA, ferric nitrilotriacetate; GPx, glutathione peroxidase; GST, glutathione *S*-transferase; HNE, 4-hydroxy-2-nonenal; NTA, nitrilotriacetic acid; QR, quinone reductase; TBARS, 2-thiobarbituric acid reactive substances; THU1, tetrahydrocurcumin; U1, curcumin.

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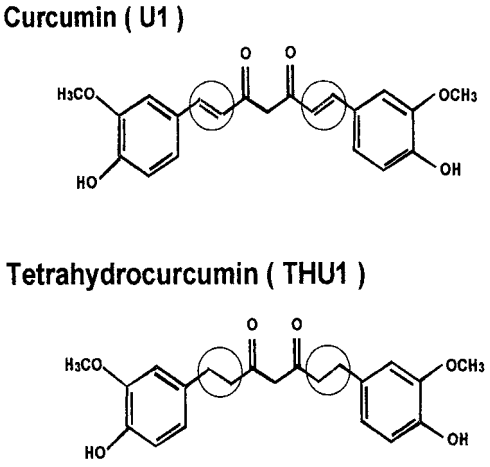

FIGURE 1 Chemical structures of U1 and THU1.

vivo. Furthermore, there is a controversy as to which molecule would be more effective as a chemopreventive agent. THU1 has recently been reported to be a less effective chemopreventive agent in the mouse skin than curcumin (18,19). However, feeding 0.5% THU1 in the diet significantly inhibited 1,2-dimethylhydrazine-induced mouse colon carcinogenesis, whereas the inhibitory effect of U1 was not significant (20).

In the present study, we fed mice diets containing 0.5% U1 or THU1 and evaluated their effects on Fe-NTA-induced oxidative renal injury, focusing on the ability of U1 and THU1 to act as antioxidants, and we studied the metabolism of these compounds.

MATERIALS AND METHODS

Animals and diet. A total of 54 male ddY mice (Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 25–35 g (6 wk old) were used. Groups were housed jointly ($n = 6$) in plastic cages at a temperature of 23 ± 2°C and an alternating 12 h/12 h light and dark cycle. All the mice were allowed free access to food and deionized water (Millipore Japan, Osaka, Japan) for 1 wk to adapt to the new environment. The mice were divided into three diet groups of 18 mice each and consumed ad libitum control or experimental diets containing 0.5% U1 or 0.5% THU1 (**Table 1**) for 1 mo. Each group was further divided into three groups of six: untreated control, killed 1 h or 3 h after Fe-NTA treatment. Mice were killed by cervical dislocation. Blood was taken from the abdominal aorta and the serum was separated. The liver and both kidneys of each mouse were immediately removed. The kidneys were homogenized with a Teflon homogenizer in 10 volumes of 50 mmol/L sodium phosphate buffer (pH 7.2). The homogenate was centrifuged at 10,000 × *g* for 10 min and the supernatant was used for the enzyme activity and thiobarbituric acid assay. The supernatant was centrifuged at 105,000 × *g* for 60 min to obtain the microsome fraction, while the supernatant was considered the cytosolic fraction.

Materials. U1 and THU1 were kind gifts of Nikken Fine Chemicals (Shizuoka, Japan), THU1 (>99% pure) was prepared from U1 (>99% pure) obtained from the rhizomes of turmeric by hydrogenating the two double bonds conjugated to the β-diketone (Fig. 1). Ferric nitrate enneahydrate and sodium carbonate were from Wako (Osaka, Japan) and nitrilotriacetic acid (NTA) disodium salt was from Nacalai Tesque (Kyoto, Japan). The protein concentration was measured using the bicinchoninic acid protein assay reagent obtained from Pierce (Rockford, IL). All the chemicals used were of analytical quality.

Preparation and injection of Fe-NTA. The Fe-NTA solution was prepared immediately before use as previously described (5). Briefly, ferric nitrate enneahydrate and the NTA disodium salt were each dissolved in deionized water to form 300- and 600-mmol/L solutions. They were mixed at the volume ratio of 1:2 (molar ratio, 1:4) and the pH was adjusted with sodium hydrocarbonate to 7.4. Each mouse was given an intraperitoneal injection of Fe-NTA at a dose of 5 mg Fe/kg body.

Quantitative analysis of curcuminoids. A JASCO MD-910 multiwavelength detector (Tokyo, Japan) was used with the HPLC instrument. A Devolosil ODS-HG-5 column (0.46 cm o.d. × 25 cm; Nomura Chemical, Aichi, Japan) was used for the analysis. The tissue homogenates or the serums with or without glucuronidase/sulfatase treatment were used for the analysis (21). The enzyme treatment was performed as follows: 200 μL of 2 volumes tissue homogenates or serums were treated with β-glucuronidase (500 U) and sulfatase (40 U) in 10 mmol/L PBS (pH 5.0 containing 20 g/L ascorbic acid, 0.1 g/L EDTA). The analysis was carried out with a mobile phase of acetonitrile/H_2O (1 g/L trifluoroacetic acid); (50:50 v/v) at a flow rate of 1.0 mL/min. The peak corresponding to U1 was detected at 430 nm after 13 min and THU1 at 280 nm after 10 min.

Measurement of antioxidative activity. In previous study, single intraperitoneal Fe-NTA treatment (5 mg Fe/kg body) caused oxidative stress, monitored by the accumulation of lipid peroxidation products and by the formation of 8-OHdG in the time course study (22). The renal 2-thiobarbituric acid reactive substances (TBARS) or 8-OHdG content has been shown to reach the highest level 3 h or 1 h after intraperitoneal injection of Fe-NTA, respectively. Hence, we subsequently assessed the formation of the 4-hydroxy-2-nonenal (HNE)-modified proteins, as one of the major oxidatively modified proteins, in the kidney of mice treated with Fe-NTA. Amounts of TBARS, HNE-modified proteins and 8-OHdG levels were measured by the assays as previously described (22).

Electron spin resonance (ESR) spectral measurement. ESR spectra were measured at room temperature with an ESR spectrometer (JES-TE2000; JEOL, Tokyo, Japan) according to the method of Kawabata et al. (23) after slight modification. 5,5′-Dimethyl-1-pyrroline-1-oxide (DMPO; 10 μL), a radical trapping agent, was added to 200 μL of each tissue homogenate ($n = 4$, 10 g protein/L). Then, Fe-NTA (final concentration 10 mmol/L as Fe) was added and mixed well for 10 min. Formation of the DMPO-trapping radical spectra was calculated as the integrated area of the signal.

Enzyme assays. Glutathione peroxidase (GPx) activity was measured by NADPH oxidation in a coupled reaction system containing t-butyl hydroperoxide and oxidized glutathione (24). Assays of NADPH:quinone reductase (QR) activity was determined by a procedure reported by Benson et al. (25) after slight modification. The glutathione *S*-transferase (GST) activity toward 1-chloro-2, 4-dinitroben-

TABLE 1

Composition of control and experimental diets

Ingredient	Control diet	0.5% U1 diet	0.5% THU1 diet
		g/100 g	
Casein	25.0	25.0	25.0
Cellulose	4.0	4.0	4.0
Corn oil	5.0	5.0	5.0
Mineral mixture[1]	3.5	3.5	3.5
Vitamin mixture[2]	1.0	1.0	1.0
Corn starch	61.5	61.0	61.0
U1	—	0.5	—
THU1	—	—	0.5

[1] Composition of mineral mixture (g/100 g mix): $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; Fe-Citrate, 0.623; $MgSO_4$, 4.8764; $ZnCl_2$, 0.02; $MnSO_4 \cdot 5H_2O$, 0.121; KI, 0.0005; $CaCO_3$, 29.29; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025; cellulose-powder, 5.1036.

[2] Composition of vitamin mixture (g/100 g mix): retinyl acetate, 0.1; cholecalciferol, 0.00025; all-rac-α-tocopheryl acetate, 0.5; menadion, 0.52; thiamin-HCl, 0.12; riboflavin, 0.4; pyridoxine-HCl, 0.08; vitamin B-12, 0.00005; vitamin C, 3.0; biotin, 0.002; folic acid, 0.2; calcium-pantothnate, 0.5; *p*-aminobenzoic acid, 0.5; nicotinic acid, 0.6; choline-chloride, 20.0; cellulose powder, 73.0577.

zene (CDNB) as a substrate was measured according to the method of Habig et al. (26), and GST activity toward 4-HNE was measured according to the method of Alin et al. (27). Catalase (28) and superoxide dismutase (29,30) activities were determined as described.

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed by means of a two-way ANOVA. All post hoc multiple comparisons were made with the Scheffé test. The statisitical significance level was set at 5% ($P < 0.05$). StatView software (StatView J-4.5; Abacus Concepts, Berkeley, CA) was used for the analysis in each case.

RESULTS

Body, liver and kidney weights. Body weight and weights of liver and kidney were not different among the three groups of mice (data not shown), suggesting that the U1 and THU1 diets were not negatively affect food consumption.

Concentrations of U1 and THU1. In liver and serum, most of the U1 and THU1 was present as conjugated glucuronides or sulfates; only a small amount of the free forms were detected (**Tables 2** and **3**). A small amount of U1 and its conjugates (as sulfates and glucuronides) were found in the serum of the U1 diet group, but it was not detected in the liver (Table 2) or kidney (data not shown). The THU1 concentrations and its conjugates were larger than those of U1 of the liver and serum of the U1 group. Thus, when U1 is fed, it is transformed into its metabolite, THU1. The concentrations of THU1 and its conjugates in the liver and serum were higher in THU1 group than in the U1 group (Table 3). U1 and THU1 were not detected in the kidney because of the limited sensitivity of HPLC analysis.

Effects of U1, THU1 on oxidative stress in kidney of Fe-NTA-treated mice. The THU1 diet significantly suppressed the increase in lipid peroxidation and oxidative modification of DNA induced by Fe-NTA (**Fig. 2**). The U1 diet significantly suppressed the HNE-modified protein concentration in kidney but did not significantly decrease TBARS or 8-OHdG concentrations relative to the Fe-NTA-treated controls. Thus, THU1 generally had stronger inhibitory effects than U1.

TBARS and ESR measurements in control kidney homogenates. **Figure 3***A* shows the TBARS levels in the renal homogenates of controls after treatment with U1 or THU1 at the concentrations indicated, followed by direct Fe-NTA administration. The TBARS contents in the control renal homogenates decreased dose dependently. The effect of THU1 was not significantly different from that of U1. Figure 3, *B* and *C* shows the ESR spectra of radical spin adducts of DMPO generated from Fe-NTA treatment in the presence of U1 and THU1. THU1 inhibited the formation of the DMPO-trapping radicals mediated by Fe-NTA treatment (Fig. 3C). U1 was not significant.

Enzyme activity in kidney of mice treated with Fe-NTA. Activities of superoxide dismutase and catalase were decreased

TABLE 2

Distribution of curcuminoids in liver and serum of mice fed U1[1]

	Liver		Serum
Curcuminoid	Free	Conjugate	Free + conjugate
	nmol/mg		*μmol/L*
U1	ND	ND	0.6 ± 0.1
THU1	ND	3.5 ± 0.4	14.4 ± 3.9

[1] Values are means ± SD, $n = 6$.
ND, not detected.

TABLE 3

Distribution of curcuminoid in mice fed with THU1[1]

	Liver		Serum
Curcuminoid	Free	Conjugate	Free + conjugate
	nmol/mg		*μmol/L*
U1	ND	ND	ND
THU1	2.5 ± 0.6	7.9 ± 1.6	43.4 ± 15.5

[1] Values are means ± SD, $n = 6$.
ND, not detected.

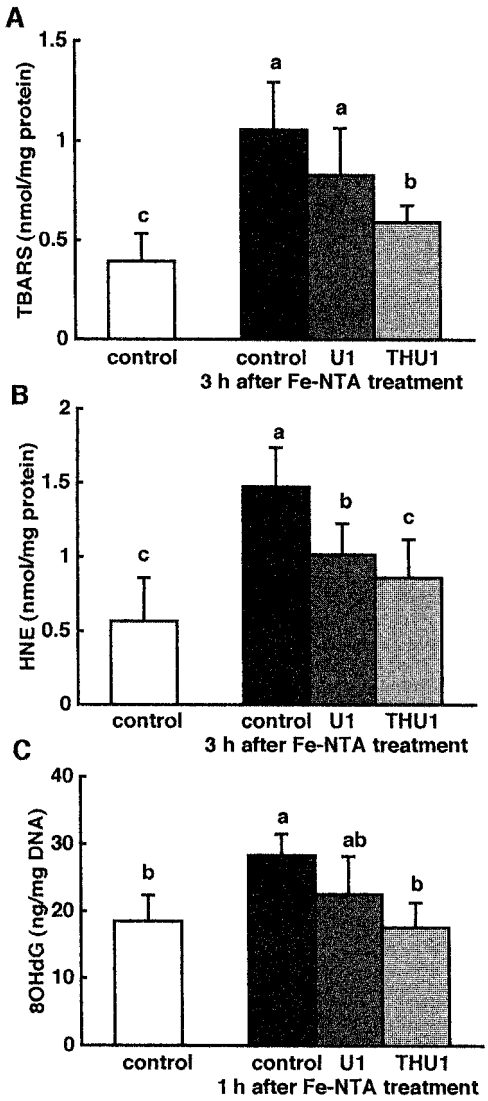

FIGURE 2 Effects of dietary U1 and THU1 on Fe-NTA-induced oxidative stress suppression in the kidney of mice. Mice were intraperitoneally treated with Fe-NTA (5 mg Fe/kg body), and oxidative stress was monitored by the formation of TBARS (*A*), HNE-modified proteins (*B*) and 8-OHdG (*C*). Data are expressed as means ± SD, $n = 6$. Means without a common letter differ, $P < 0.05$.

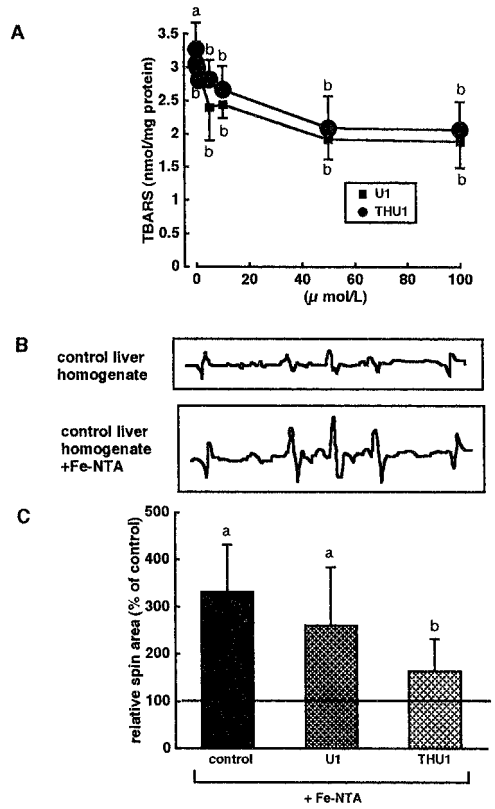

FIGURE 3 Effect of U1 and THU1 on lipid peroxidation and ESR spectra of DMPO spin adducts in the in vitro experiments with control kidney homogenates after Fe-NTA addition. *A*, Lipid peroxidation in mouse kidney samples that were incubated with various concentrations of U1 and THU1 (0–100 μmol/L), ascorbic acid (100 μmol/L) and Fe-NTA (10 μmol/L). *B*, ESR spectra of DMPO spin adducts formed. Twenty microliters of DMPO and Fe-NTA were added to; 200 μL of mouse tissue homogenate. ESR spectra were recorded 1 min after DMPO addition. *C*, Relative spin area of ESR spectra compared with control homogenates without Fe-NTA. Data are presented as percent control. Data are expressed as means ± SD, $n = 4$. Means without a common letter differ, $P < 0.05$.

by Fe-NTA treatment and did not differ between the U1 and THU1 diet groups (data not shown). However, suppression of GPx activity was less in U1 and THU1 groups than in controls (**Fig. 4***A*). Cytochrome P450 activity, the phase I detoxification enzyme, was not affected (data not shown), while suppression of the NADPH:QR and GST activities, the phase II detoxification enzymes, were inhibited in the THU1 group (Fig. 4*B–D*). U1 was not significant. In the THU1 group, the phase II enzymes generally were induced more than in the U1 group. The THU1 diet not only inhibited the decrease in whole GST activity due to Fe-NTA treatment, but also induced stronger GST activity toward HNE (Fig. 4*D*) than those of the untreated control kidney sample.

DISCUSSION

Recently, there has been an increasing interest in the protective function of dietary antioxidants, which are candidates for cancer chemoprevention and for extending lifespan. Several antioxidants, such as vitamin E, vitamin C, β-carotene, uric acid, ubiquinols and flavonoids, have been found to play important roles in the nonenzymatic protection against oxidative stress. However, it has been pointed out that one component in these antioxidants is not enough to prevent carcinogenesis. Therefore, a small number of the components of food are anticipated to have effects on prevention of carcinogenesis. In our recent studies, when given orally, U1 compounds converted to tetrahydro types that had strong antioxidant activities (13,14) in the intestinal tubes. These changes were also detected in cultured cells (31). We anticipated that THU1 has anticancer effects.

The results of our studies suggest that THU1 has better absorption properties than U1 (Tables 2 and 3). If the same amount of THU1 diet was given as that of U1, the amounts of THU1 and its conjugates (as sulfates and glucuronides) were larger in the THU1 group than in the U1 group. In contrast, the amounts of U1 and its conjugates were small in both groups. THU1 had the same level of antioxidant capacity as U1 in vitro (Fig. 3A). However, THU1 was more effective than U1. Indeed, ESR spectra signal of the radical spin adducts of DMPO mediated by Fe-NTA was more reduced in the THU1-mixed kidney homogenate than in the U1-mixed homogenate, which did not differ from the control (Fig. 3, *B* and C).

Preferential induction of phase II biotransformation enzymes such as GST and NADPH:QR (as opposed to phase I biotrans-formation enzymes of the cytochrome P450 systems)

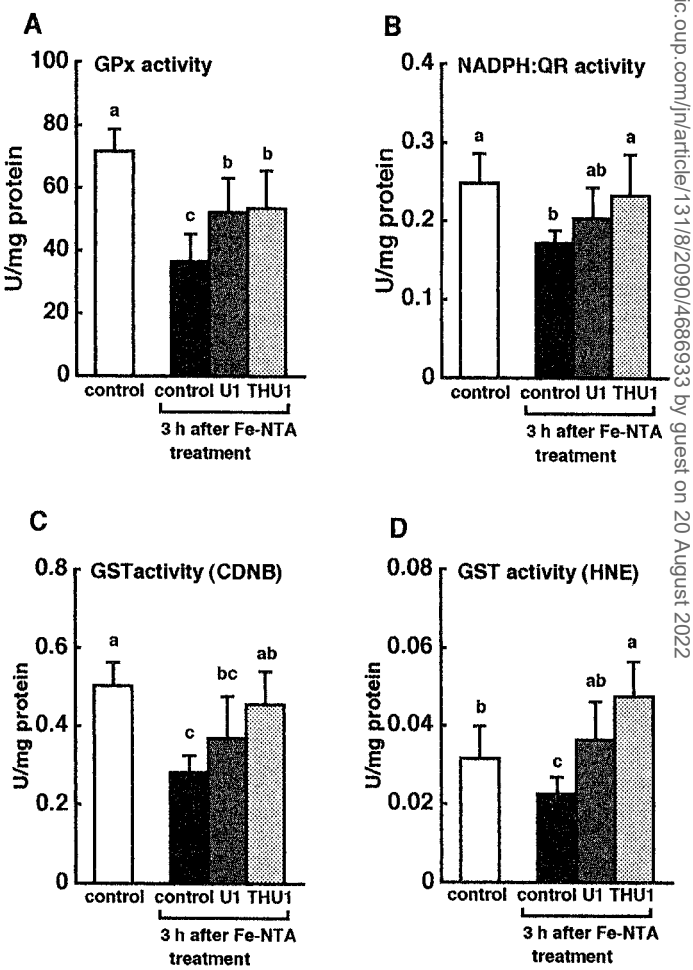

FIGURE 4 Effect of dietary U1 and THU1 on GPx (*A*), NADPH:QR (*B*) and GST (*C* and *D*) activities in the kidney of mice treated with Fe-NTA. *C*, GST activity toward CDNB. *D*, GST activity toward HNE. Data are expressed as means ± SD, $n = 6$. Means without a common letter differ, $P < 0.05$.

has been suggested to be a possible mechanism for the effects of a number of antioxidants in cancer prevention (32–34). Orally administered U1 has been shown to slightly increase GST activity toward CDNB in mouse liver even with a high dose (35). However, GST activity toward HNE is relatively high in mouse liver (36). GST consist of several catalytically distinct isozymes, each of whose expression is differentially regulated by the oxidant or antioxidant environment within the cell (37). Therefore, the present studies were designed to investigate the effects of oral U1 and THU1 administration on GSH-linked antioxidant defenses, including GPx activity and GST activities toward CDNB and toward HNE in the mouse kidney after Fe-NTA injection.

We found that the antioxidant effects of U1 generally were augmented through restoration of decreased GPx and the HNE-metabolizing GST isozymes activities by Fe-NTA (Fig. 4). The effect of THU1 was stronger than that of U1 in these antioxidant enzyme inductions. In the present study, it was also shown that there was a significant effect of oral THU1 exposure during phase II enzyme induction (Fig. 4). Namely, in the THU1 group, the GST activity toward HNE was induced more than in the untreated control group. This induction might result from THU1 exposure alone. More detailed study to clarify the molecular mechanisms concerning how these antioxidants induce enzymes is currently in progress.

Phase I enzymes inactivate a foreign substance by a redox reaction and hydrolysis. Cytochrome P450, one of the major phase I enzymes, is, thus, induced by factors, such as drugs, industrial chemical substances, food additives, tobacco, alcohol and various food components. However, a variety of chemical substances is also activated by cytochrome P450. Compounds of this kind have been classified either as bifunctional inducers, which elevate both the phase I and phase II enzymes, or monofunctional inducers, which selectively elevate the phase II enzymes. The induction of the phase I enzymes, such as cytochrome P450 isozymes, is required for the metabolic disposal of xenobiotics (38), but is also considered to be a risk factor due to the potential of activating procarcinogens (39). Therefore, the finding of U1 and THU1 as monofunctional inducers gives them biologically important merit.

In conclusion, the present study provided clear evidence for the suppression of oxidative stress-induced renal damage by dietary U1 and THU1. U1 and THU1 are probably working in two different ways: direct chelating or scavenging effects and induction of the antioxidant enzymes (monofunctional inducers). The in vivo antioxidant effects of THU1 were greater than were those of U1. THU1 may be more easily absorbed than U1 from the gastrointestinal tract. THU1 also has some advantages as a food additive because it is colorless and yet is easily prepared by the standard hydrogenation of U1. Additional studies of the effects of curcuminoids on oxidative stress, especially on their molecular mechanisms, are necessary. We believe that THU1 has the potential to be used as a chemopreventive agent in humans.

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