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Ascorbic Acid Deficiency Decreases Specific Forms of Cytochrome P-450 in Liver Microsomes of Guinea Pigs

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SUMMARY

Ascorbic acid (VC) deficiency resulted in a decrease in the activities of aminopyrine *N*-demethylase, aniline hydroxylase, and *p*-nitroanisole *O*-demethylase and in the content of cytochrome P-450, as spectrally determined, whereas it caused an increase in the activities of 6β-hydroxylases for testosterone and progesterone in liver microsomes of guinea pigs. Western blot analysis of liver microsomes with antibodies to rat P-448-H (P-450IA2), P-450j (P-450IIE), P-450 PB-1 (P-450IIIA), and P-450b (P-450IIB1) showed that VC deficiency decreased the amount of cytochrome P-450 immunochemically related to P-450IA2 and P-450IIE but did not change the amount of the form that was cross-reactive with antibodies to P-450IIB1 and tended to slightly increase (not statistically significantly) the amount of the form of the cytochrome immunochemically related to P-450IIIA. The larger decrease by VC deficiency in the amount of cytochrome P-450 that was cross-reactive to the rat P-450IA2 resulted in a lower capacity of liver microsomes to activate promutagens, such as 2-amino-3-methyl-imidazo(4,5-*f*)quinoline and aflatoxin B1. These results indicate that VC deficiency in guinea pigs differentially affects the content of individual forms of cytochrome P-450.

It is well established that cytochrome P-450-dependent monooxygenase is responsible for the metabolism of endogenous and exogenous compounds, including steroids, drugs, and chemical carcinogens (1, 2). The terminal oxidase, cytochrome P-450, exists in multiple forms, and the relative proportions are affected by the nutritional status of animals, as well as several other factors such as age, sex, strain, species, and environmental exposure to inducers and inhibitors (3–5). As one of the nutritional factors, VC is also known to decrease, in general, the activities of drug-metabolizing enzymes and the content of cytochrome P-450 in liver microsomes (6–9). The changes in the activities of drug-activating and -detoxifying enzymes may lead to changes in drug pharmocokinetics and chemical carcinogenesis. Rikans *et al.* (10) have demonstrated that multiple forms of cytochrome P-450 exist in liver microsomes of guinea pigs, and they suggested that VC deficiency selectively affects certain forms of the cytochrome. It remains, however, still to be clarified whether all forms of cytochrome P-450 are uniformly affected by the VC deficiency.

Experimental Procedures

Materials. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH, and $NADP^+$ were purchased from Oriental Yeast Co. (Tokyo, Japan). Protein A-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Emulgen 911 and the metabolites of testosterone that were used as standards were kindly provided by Kao-Atlas (Tokyo, Japan) and Dr. Kirk, Queen Mary College, University of London (London, UK), respectively. *Salmonella typhimurium* TA1535/pSK1002 was provided by Dr. T. Shimada, Osaka Prefectural Institute of Public Health (Tokyo, Japan). The VC-deficient diet and a normal diet consisted of basically the same components, except that the VC-deficient diet did not contain VC. The normal diet contained 20.7% protein, 4.6% corn oil, 8.8% cellulose powder, 50.7% starch and sucrose (2:1), 6.85% mineral mixture (Ca, 1.82; P, 1.08; Mg, 0.62; K, 2.20; Na, 0.60; Fe, 0.02; Cu, 0.002; Co, 0.50; Mn, 0.005; and Zn, 0.0048 g/100 g of diet), 0.35% vitamin mixture (vitamin A, 950 IU; VC, 100 mg; vitamin D3, 200 IU; vitamin E, 5 mg; vitamin K3, 0.5 mg; vitamin B1, 2 mg; vitamin B2, 2 mg; vitamin B6, 1 mg; vitamin B12, 0.0005 mg; nicotinic acid, 5 mg; Ca-pantothenate, 3 mg; choline, 150 mg; folate, 0.3 mg; biotin, 0.15 mg; and inositol, 80 mg/100 g of diet), and 8.0% water. The general energy of the normal and VC-deficient diets was 4.07 and 4.09 kcal/g of diet, respectively. Other reagents were of the highest purity commercially available.

Preparation of liver microsomes. Male albino guinea pigs (250–300 g) were maintained on a VC-free diet, for 21 days, or a normal chow diet. Animals were decapitated and exsanguinated, and their livers were immediately removed. Liver microsomes were prepared as described previously (11) and were stored at −80° until use.

Assay for the activities of cytochrome P-450-dependent monooxygenase. A typical reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating

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ABBREVIATIONS: VC, ascorbic acid (vitamin C); IQ, 2-amino-3-methyl-imidazo(4,5-f)quinoline.

system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, and 5 mM $MgCl_2$), liver microsomes (approximately 1 mg of protein), and substrate, in a final volume of 1.0 ml, except that the final volume of reaction mixture for the assay of hydroxylase activities for testosterone and progesterone was 0.5 ml. The concentration of aniline, aminopyrine, and *p*-nitroanisole was 5 mM each, and the concentration of progesterone and testosterone added as substrates was 0.6 mM. The activity of aniline hydroxylase was estimated by determination of *p*-aminophenol according to the method of Imai *et al.* (12). The activities of aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase were estimated by determination of formaldehyde and *p*-nitrophenol by the methods of Nash (13) and Kamataki *et al.* (14), respectively. The activities of testosterone hydroxylase and progesterone hydroxylase were measured as described previously (15, 16). The mutation assay was carried out as described previously (17, 18). The induction of *umu* gene expression by metabolic activation of promutagens was measured using the tester strain *S. typhimurium* TA1535/pSK1002 and was monitored by measurement of the level of β-galactosidase. The activity of β-galactosidase expressed was measured spectrophotometrically, using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The units of enzyme activity were calculated according to the method of Miller (19).

Other methods. The content of cytochrome P-450 and the activity of NADPH-cytochrome *c* reductase were estimated by the methods of Omura and Sato (20) and Phillips and Langdon (21), respectively. One unit of reductase activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of cytochrome *c*/min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot-peroxidase antiperoxidase staining were carried out essentially as described by Laemmli (22) and Guengerich *et al.* (23), respectively. Peroxidase activity was detected with 4-chloro-1-naphthol instead of diaminobenzidine. Protein concentration was measured according to the method of Lowry *et al.* (24). Purification of four forms of cytochrome P-450 from rat liver microsomes was carried out as described previously (25–28). Antibodies to the purified cytochrome P-450 were raised in rabbits, as reported (29).

Results

Table 1 shows the effects of VC deficiency on the activities of drug-metabolizing enzymes and NADPH-cytochrome *c* reductase and the content of cytochrome P-450 in liver microsomes of guinea pigs. Animals maintained on the deficient diet showed liver VC concentrations of 1.16 ± 0.28 mg/100 g of liver, which were about 4% of control concentrations. The specific content of cytochrome P-450 and the activity of NADPH-cytochrome *c* reductase were decreased by VC deficiency by about 30% and 50%, respectively. It is noteworthy that the activities of aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase were decreased by VC deficiency but to different extents. The decrease in the activity of aminopyrine *N*-demethylase apparently paralleled the decrease in the amount of cytochrome P-450, as spectrally determined, whereas the decreases in the activities of aniline hydroxylase and *p*-nitroanisole *O*-demethylase were greater than that in the amount of cytochrome P-450. These results are in agreement with observations by other laboratories (9). The effects of VC deficiency on the activities of testosterone and progesterone hydroxylases in liver microsomes were also studied. As shown in Fig. 1, the activities of 7α- and 2β-hydroxylases for testosterone and 21-hydroxylase for progesterone were virtually unchanged by VC deficiency, whereas the activities of 6β-hydroxylases for testosterone and progesterone were increased significantly by VC deficiency, as compared with normal guinea pigs. The activity of NADPH-cytochrome *c* (P-450) reductase, as well as the total content of cytochrome P-450, was decreased by the deficiency. Therefore, it is also possible that NADPH-cytochrome *c* (P-450) reductase became a rate-limiting component for exertion of the full activities in liver microsomes of the deficient guinea pigs, as in the case of rat liver microsomes (30, 31). However, the activities of cytochrome P-450-dependent monooxygenase in liver microsomes of the deficient animals were not restored to the level of control animals by externally added NADPH-cytochrome *c* (P-450) reductase (data not shown). Supporting this, the rates of cumene hydroperoxide-dependent drug oxidations were also decreased by the VC deficiency, as was seen with NADPH-dependent oxidations. Thus, to examine the hypothesis that VC deficiency differentially affects the content of individual forms of cytochrome P-450, the amounts of each form of cytochrome P-450 were quantitated by Western blot analysis, using antibodies to rat P-448-H (P-450IA2), P-450 PB-1 (P-450IIIA), P-450b (P-450IIB1), and P-450j (P-450IIE1). All of the antibodies used in this study recognized a single microsomal protein, except that two microsomal proteins were recognized by anti-P-450IA2 antibodies (Fig. 2), suggesting that the antibodies recognized cytochrome P-450 in liver microsomes of

TABLE 1

Effects of VC deficiency on the components of the electron transport system and the activities of drug-metabolizing enzymes in liver microsomes from guinea pigs

Each value represents the mean ± standard error of five to seven animals.

Measurement	Control	VC-deficient	% of control
VC (mg/100 g of liver)	29.30 ± 5.64	1.16 ± 0.28[a]	4
Cytochrome P-450 (nmol/mg of protein)	0.95 ± 0.15	0.66 ± 0.11[a]	69
NADPH-cytochrome *c* reductase (unit/mg of protein)	0.15 ± 0.02	0.07 ± 0.01[a]	47
Aniline hydroxylation (nmol/min/mg of protein)	1.1 ± 0.1	0.6 ± 0.1[a]	55
Aminopyrine *N*-demethylation (nmol/min/mg of protein)	8.1 ± 0.5	5.7 ± 1.0[a]	70
p-Nitroanisole *O*-demethylation (nmol/min/mg of protein)	2.7 ± 0.2	1.4 ± 0.1[a]	52

[a] $p < 0.01$.

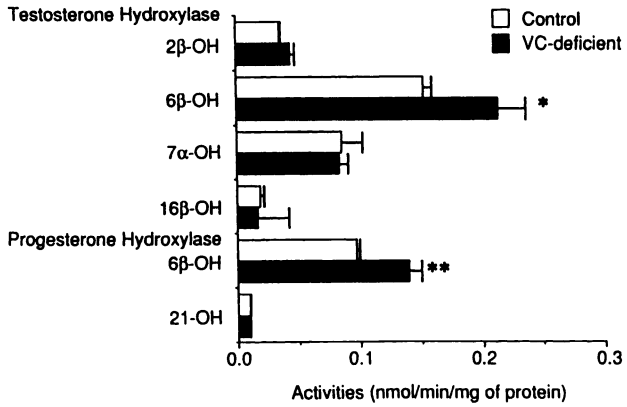

Fig. 1. Effects of VC deficiency on the activities of testosterone and progesterone hydroxylases in liver microsomes of guinea pigs. The activities of testosterone and progesterone hydroxylases were measured as described in Experimental Procedures and were expressed as nmol of hydroxylated metabolites/min/mg of microsomal protein. Each value represents the mean ± standard deviation of four animals. *, $p < 0.05$; **, $p < 0.01$.

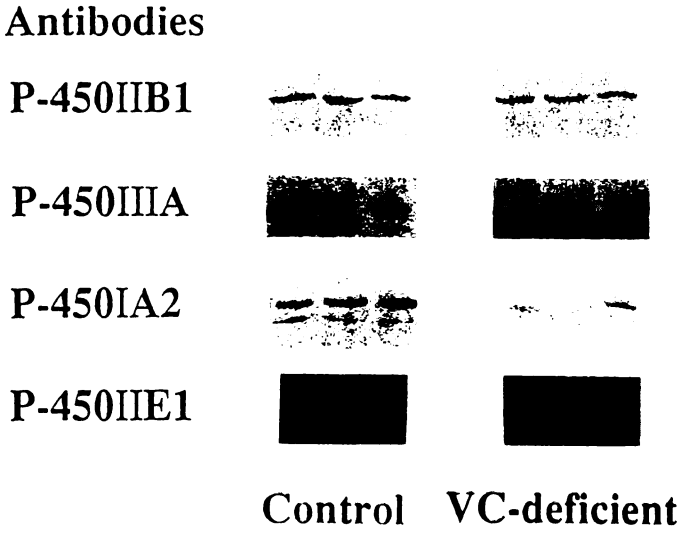

Fig. 2. Western blot analysis of liver microsomes with antibodies to rat P-450IIB1, P-450IIIA, P-450IA2, and P-450IIE1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting of liver microsomes obtained from three different guinea pigs in each group were carried out as described in Experimental Procedures. Each well contained 50 μg of microsomal protein.

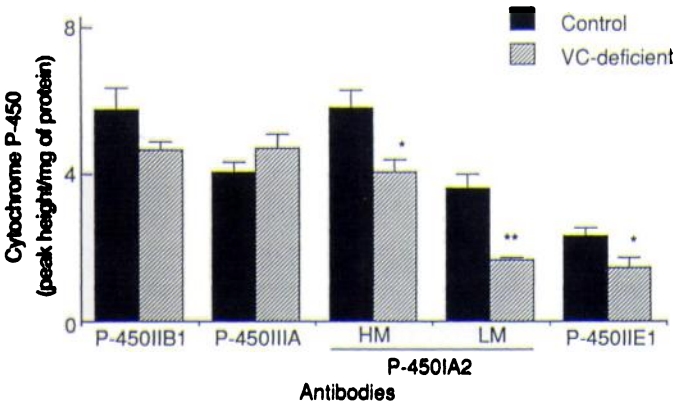

Fig. 3. Effects of VC deficiency on the forms of cytochrome P-450 immunochemically related to rat P-450IIB1, P-450IIIA, P-450IA2, and P-450IIE1. Intensities of bands stained with 4-chloro-1-naphthol were measured by means of a densitometer (Quick Scan R&D; Helena Laboratories). The forms of cytochrome P-450 that were cross-reactive with anti-P-450IA2 antibodies (*HM*, high molecular weight; *LM*, low molecular weight) were separately estimated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting of liver microsomes from normal and VC-deficient animals were conducted as described in Experimental Procedures.

guinea pigs. The intensities of stained bands, calculated per mg of microsomal protein, are shown in Fig. 3. Two forms of cytochrome P-450, which are cross-reactive with antibodies to P-450IA2, were apparently decreased by VC deficiency. The two forms of cytochrome P-450 are differentiated in Fig. 3 on the basis of their molecular weights. The amounts of cytochrome P-450 immunochemically related to P-450IIE1 were also decreased. In contrast, cytochrome P-450 that was cross-reactive with antibodies to P-450IIB1 was unaffected by VC deficiency. As expected from the results showing that the activity of testosterone 6β-hydroxylase was increased by VC deficiency, cytochrome P-450 related to P-450IIIA was slightly increased by VC deficiency.

Because the results of Western blot analysis showed that VC deficiency decreased the amount of cytochrome P-450 immunochemically related to P-450IA2 and appeared to increase the amount of cytochrome P-450 related to P-450IIIA, the effects of VC deficiency on the mutagenic activation of IQ and aflatoxin B1 were investigated (Table 2). As expected, the mutagenic activation of IQ in liver microsomes was significantly decreased by VC deficiency. The capacity of liver microsomes to activate aflatoxin B1 was also decreased by VC deficiency, suggesting that forms of cytochrome P-450 other than the cytochrome P-450 immunochemically related to P-450IIIA are involved in the mutagenic activation of aflatoxin B1 in guinea pigs. Thus, the effects of antibodies to rat P-450IA2 and P-450IIIA on the mutagenic activation of IQ and aflatoxin B1 were examined. As shown in Fig. 4, antibodies to P-450IA2 inhibited mutagen production from both IQ and aflatoxin B1 by liver microsomes of guinea pigs. Antibodies to P-450IIIA also inhibited production, but to much lesser extents.

Discussion

Multiple forms of cytochrome P-450 are present in liver microsomes of guinea pigs, as has been confirmed in other animal species including human (10, 32, 33). It was of interest that the total amount of cytochrome P-450, as determined spectrally, never decreased below 40% of the normal level, even in a severely VC-deficient status, and cytochrome P-450-dependent monooxygenase activities were affected to different extents by VC deficiency (9, 34). These findings probably suggest differential effects of VC deficiency on the forms of cytochrome P-450, as has been proposed by Zannoni and coworkers (10, 35). Thus, in the present study, the effects of VC deficiency on forms of cytochrome P-450 were investigated. The activities of aniline hydroxylase and *p*-nitroanisole *O*-demethylase in deficient animals were less than 50% of the activities in guinea pigs maintained on a normal diet, whereas the activities of testosterone 7α- and 2β-hydroxylases and progesterone 21-hydroxylase were virtually unaffected by the VC deficiency. It is worth noting that the activities of 6β-hydroxylases for testosterone and progesterone were increased by the VC deficiency.

Because the availability of heme for cytochrome P-450 synthesis has been shown to be unaltered by VC deficiency (36, 37), the results of Western blot analysis can be evaluated as a reflection of the amount of holo-cytochrome P-450. Thus, the decreased levels of activities of drug and steroid hydroxylases in the deficient animals seem to be attributable to decreases in the amounts of specific forms of cytochrome P-450. The amount of cytochrome P-450 that is cross-reactive with antibodies to P-450IIIA, which may be involved in testosterone 6β-hydroxylation in liver microsomes of guinea pigs (38), tended to increase in the deficient animals. Accordingly, the activity

TABLE 2

Decrease in the mutagen-producing activities for aflatoxin B1 and IQ in liver microsomes of VC-deficient guinea pigs

Each value represents the mean ± standard error of five animals.

Promutagen	*umu* gene expression		Decrease
	Control	VC-deficient	
	β-galactosidase units/min/nmol of P-450		%
IQ	131.4 ± 4.1	82.0 ± 5.6*	38
Aflatoxin B1	218.7 ± 14.3	83.1 ± 10.5*	62

*$p < 0.01$.

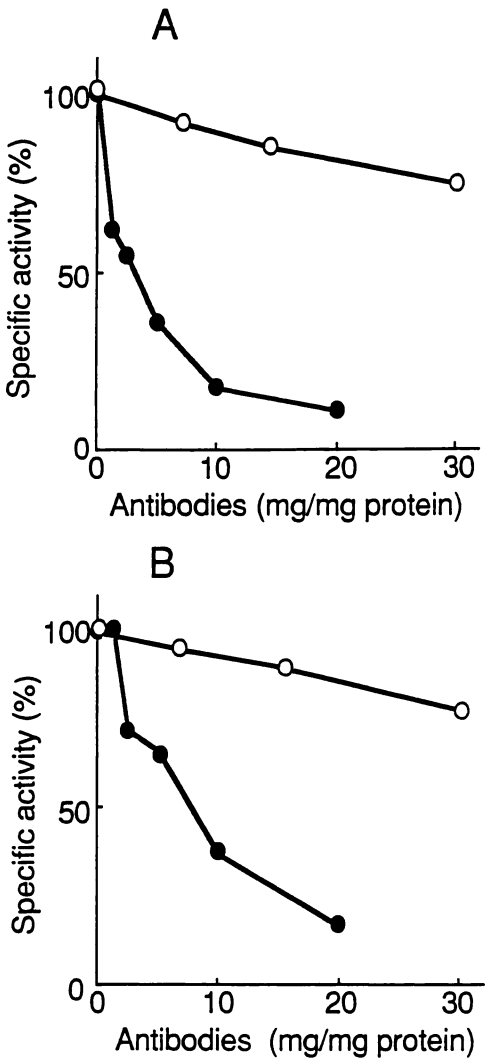

Fig. 4. Effects of anti-rat P-450IA2 IgG and anti-rat P-450IIIA IgG on activities to produce mutagens from IQ and aflatoxin B1 in liver microsomes of guinea pigs. The mutagen-producing activities for IQ (A) and aflatoxin B1 (B) were measured in the presence of various concentrations of antibodies to P-450IA2 (●) and P-450IIIA (○). The concentrations of antibodies employed are specified in the figure. To avoid the effect of turbidity on the assay for mutagenic activation, anti-P-450IA2 IgG and anti-P-450IIIA IgG were diluted with preimmune IgG to give the same final concentration of protein in each reaction mixture. Each *line* was drawn from separate experiments. Thus, the mutagen-producing activities for IQ in the absence of anti-rat P-450IA2 IgG and anti-human P-450IIIA4 IgG were 95.3 and 174.0 units/min/nmol of P-450, and the activities for aflatoxin B1 were 371.8 and 325.6 units/min/nmol of P-450, respectively.

of testosterone 6β-hydroxylase was increased significantly by the deficiency. It is well known that the forms of cytochrome P-450 that are cross-reactive with anti-P-450IA2 antibodies are generally highly active for the activation of promutagens such as 2-amino-6-methyldipyrido(1,2-*a*:3′,2′-*d*)imidazole and IQ in animal species including human (39–42). In addition, the forms belonging to the P-450IIIA gene family have been shown to be the major forms of cytochrome P-450 responsible for the mutagenic activation of aflatoxin B1 (18, 43–45). Together with these facts, we expected that the capacity of liver microsomes to produce mutagen(s) from aflatoxin B1 would be increased by the VC deficiency. However, the mutagen-producing activity for aflatoxin B1 was decreased rather than increased by VC deficiency, although cytochrome P-450 immunochemically related to P-450-PB1 (P-450IIIA) was not decreased in VC-deficient animals. The results of immunoinhibition of the mutagenic activation by antibodies to P-450IA2 and P-450IIIA indicated that cytochrome P-450 immunochemically related to P-450IA2 played a major role in the mutagenic activation of both IQ and aflatoxin B1 in liver microsomes of guinea pigs. These results suggest that the cytochrome P-450 with primary responsibility for mutagenic activation of aflatoxin B1 is different in guinea pigs than in rats and humans.

Although it is strongly suggested that the changes in the amount of cytochrome P-450 in VC-deficient guinea pigs are form specific, it is unclear at present whether VC affects the amounts of cytochrome P-450 transcriptionally or translationally. The mechanism(s) by which the specific forms of cytochrome P-450 are decreased or increased by VC deficiency remains to be elucidated.

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