L-Gulono- γ -lactone Oxidase Is Not Induced in Rats by Xenobiotics Stimulating L-Ascorbic Acid Biosynthesis

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Summary L-Ascorbic acid (AsA) is synthesized from D-glucose in rats; the terminal step of this synthetic pathway is catalyzed by L-gulono- γ -lactone oxidase (GLO). In this study, we examined the effects of phenobarbital (PB) and 3-methylcholanthrene (MC), both of which are known to stimulate AsA biosynthesis in rats, on the hepatic levels of GLO activity, GLO mRNA, and AsA. Firstly, the existence of GLO mRNA was examined in the liver, kidney, lung, small intestine, spleen, testis, and prostate from a male rat; and GLO mRNA was found to be present only in the liver, in which GLO activity was also detected. The intra-peritoneal injection with PB (100 mg/day/kg body weight, once a day for 2 days) or MC (20 mg/day/kg body weight, once) significantly elevated the hepatic level of AsA and the urinary excretion of AsA in rats (5-week-old males). The hepatic levels of cytochrome P-450IIB1 mRNA and cytochrome P-450IIB2 mRNA and those of cytochrome P-450IA1 mRNA and cytochrome P-450IA2 mRNA were also elevated in the rats treated with PB and MC, respectively, indicating a normal response of these animals to these compounds. However, the level of GLO mRNA and the activity of GLO in the liver tended to be slightly decreased by the administration of PB or MC, though the differences were not significant. Thus it is clear that the treatment with PB or MC stimulates the biosynthesis of AsA by increasing the activity of some enzyme(s) participating in the synthesis prior to GLO.

Key Words L-gulono- γ -lactone oxidase, L-ascorbic acid, xenobiotics, rats

L-Ascorbic acid (AsA) is synthesized from D-glucose in the liver of phylogenetically higher animals *via* the D-glucuronic acid pathway (1). L-Gulono- γ -lactone oxidase [GLO, EC 1.1.3.8] catalyzes the terminal step of AsA biosynthesis.

Nearly all species of higher animals can synthesize AsA and do not require it as an essential nutrient, although humans and other primates (2), guinea pigs (2), and a rat mutant (ODS-*od/od* rat) (3–5) are deficient in GLO and, as a consequence, require dietary AsA to prevent scurvy.

Previous studies (6–15) demonstrated that the administration of various kinds of xenobiotics causes a marked increase in the biosynthesis of AsA in rats, resulting in the accumulation of AsA in various tissues. In our previous paper, we reported that the hepatic activities of UDPglucuronosyltransferase and UDPglucose dehydrogenase, which are involved in the D-glucuronic acid pathway, were increased in rats by the administration of xenobiotics such as polychlorinated biphenyls (15). However, no detailed study has been made on the effect of xenobiotics on the expression of the GLO gene.

In this study, therefore, we estimated the hepatic levels of GLO and GLO mRNA in rats administered phenobarbital (PB) or 3-methylcholanthrene (MC), both of which are potent stimulators of AsA biosynthesis in rats (10).

METHODS

Animals and diet. Male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu) weighing about 90 g (4 weeks old) were used and fed a synthetic diet. The composition (g/kg diet) of the diet was as follows: casein, 300; carbohydrate (sucrose–starch, 1 : 2), 563; AIN76 salt mixture, 35; AIN76A vitamin mixture (AsA free), 10; choline chloride, 2; corn oil, 50; and cellulose powder (AVICEL type FD-101, Asahi Chemical Industry Co., Tokyo), 40. The animals were kept at 24°C with a 12-h light cycle (from 0800 h to 2000 h). All rats were individually housed in wire screen-bottomed cages and were provided with food and water with free access. The rats were killed by decapitation between 1000 h and 1200 h in all experiments. Animal experiments were performed following Guidelines for Animal Experimentation of Nagoya University.

Experimental protocol. For Northern blot analysis for GLO mRNA in various tissues, the liver, kidney, lung, small intestine, spleen, testis, and prostate were dissected from a 5-week-old rat, and total RNA was extracted.

To examine the effect of xenobiotics on AsA biosynthesis, 12 rats weighing about 110 g (5 weeks old) were divided into 4 groups of 3 rats each. Two of the groups were injected intraperitoneally once a day for 2 days with PB (sodium salt; 100 mg/day/kg body weight, dissolved in saline) or saline as a control, and were killed 72 h after the second injection. In addition, the remaining 2 groups were injected intraperitoneally once with MC (20 mg/day/kg body weight, dissolved in corn oil) or corn oil as a control, and were also killed 72 h after the injection. Urine was collected in 5% metaphosphoric acid during the last 24 h. Liver was dissected and used for extraction of total RNA and for the GLO assay.

cDNA. The isolation of a rat GLO cDNA was described previously (16). An *Eco*RI fragment of the cDNA, designated 15L (17), was used as a probe for Northern

blotting. A cDNA clone for rat cytochrome P-450IA2 (330 bp) (18) was kindly provided by Dr. K. Kawajiri of Saitama Cancer Research Institute. This cDNA hybridizes with both cytochrome P-450IA2 mRNA and cytochrome P-450IA1 mRNA as separate bands in Northern blot analysis. A cDNA clone for rat cytochrome P-450IIB1 (1,600 bp) (19) was kindly provided by Dr. Y. Fujii-Kuriyama of Tohoku University. This cDNA hybridizes with both cytochrome P-450IIB1 mRNA and cytochrome P-450IIB2 mRNA as a single band in Northern blot analysis. A cDNA clone for mouse β -actin (1,100 bp) (20) was a gift from Dr. K. Hitomi of Nagoya University.

RNA extraction and Northern blot analysis. Tissues were homogenized in 25 mM sodium citrate (pH 7.0), containing 4 M guanidium thiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol; and total RNA was extracted as described (21). The RNA (20 μ g) was separated by electrophoresis on a 1% agarose gel containing 6.6% formaldehyde, and were immobilized on Hybond N+ membranes (Amersham Japan, Tokyo) in 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate). The membranes were baked at 80°C for 2 h, and then hybridized with probes (2 $\times$ 10⁶ cpm/ml ³²P-labeled cDNAs) specific for GLO, cytochrome P-450IA2, cytochrome P-450IIB1, or β -actin mRNA at 42°C overnight in a solution containing 50% formamide, 5 $\times$ Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 5 $\times$ SSC, 50 mM sodium phosphate, and 0.05% denatured salmon sperm DNA. The blots were washed twice with 0.1 $\times$ SSC containing 0.1% SDS at 55°C for 15 min. The washed blots were subjected to autoradiography, after which the radioactivity on the bands detected was quantified with a Radioanalytic Imaging System apparatus (AMBIS System Inc., San Diego, California). The probe DNAs were radiolabeled with [α -³²P]dCTP (110 TBq/mmol; Amersham Japan) by use of a multiple labeling system (Amersham Japan) according to the manufacturer's manual.

Assay of GLO activity. GLO activity was measured essentially as described by Kiuchi *et al.* (22). Liver was homogenized in 4 volumes of ice-cold 0.25 M sucrose. The homogenate was centrifuged for 15 min at 10,000 $\times g$ at 4°C, and the postmitochondrial supernatant was centrifuged for 60 min at 105,000 $\times g$ at 4°C. The microsomal pellets were suspended in 1.15% KCl containing 10 mM EDTA (pH 7.4), and used as the enzyme sample. The GLO assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 50 mM sodium citrate, 1 mM dithiothreitol, 2.5 mM L-gulono- γ -lactone, and enzyme sample. The mixture (1.0 ml) was incubated at 37°C for 15 min, and the reaction was stopped by the addition of trichloroacetic acid to 5%. After the precipitate had been removed by centrifugation, the amount of AsA in the supernatant was measured by a modification of the dinitrophenylhydrazine method (23), in which the oxidation of AsA was accomplished with 2,6-dichlorophenolindophenol.

Measurement of cytochrome P-450 and AsA. The hepatic content of cytochrome P-450 was determined by the dithionite difference method of Omura and Sato (24). For the measurement of the hepatic level of AsA, liver was

homogenized in ice-cold 5% metaphosphoric acid and centrifuged for 10 min at $1,600 \times g$. The AsA in the supernatant was measured by the same method as used in the assay of GLO activity. Urine was collected in 5% metaphosphoric acid, and used for the measurement of AsA.

Statistical analysis. Statistical significance was determined by Student's *t*-test; $p < 0.05$ was considered significant. All values were presented as means $\pm$ SE.

RESULTS

We previously isolated a cDNA for rat GLO from a rat liver cDNA library in λ gt11 (16). The 1.5-kbp *Eco*RI fragment of this cDNA, designated 15L (17), was used as a probe for Northern blot analysis of total RNA from various rat tissues. As shown in Fig. 1A, a single band of GLO mRNA (~ 2.4 kb) was observed in the liver, while no positive signal was observed in the other tissues tested (kidney, lung, small intestine, spleen, testis, and prostate). Hybridization of the same blot with a probe for β -actin showed a band of the expected size, indicating the authenticity of the RNA samples used (Fig. 1B). In parallel with this result, the activity of GLO was detected only in the liver.

Effect of the administration of MC or PB on AsA biosynthesis and hepatic level of GLO mRNA

The hepatic level of AsA and urinary excretion of AsA in each group are shown in Table 1. The administration of PB or MC caused a higher level of AsA in the liver than the level in the respective control group. In addition, the urinary

(A) L-Gulono- γ -lactone oxidase

(B) β -Actin

Fig. 1. Northern blot analysis of total RNA from various rat tissues. Total RNA (20 μ g) isolated from various tissues was separated on a 1% agarose gel containing 6.6% formaldehyde. The RNA in the gel was transferred to a Hybond N+ membrane and hybridized with 32 P-labeled rat liver L-gulono- γ -lactone oxidase cDNA (panel A) or 32 P-labeled mouse β -actin cDNA (panel B). Lane 1, liver; lane 2, kidney; lane 3, lung; lane 4, small intestine; lane 5, spleen; lane 6, testis; lane 7, prostate.

Table 1. Effects of treatment with sodium phenobarbital (PB) or 3-methylcholanthrene (MC) on growth, liver weight, hepatic level of L-ascorbic acid, urinary excretion of L-ascorbic acid, hepatic activity of L-gulono- γ -lactone oxidase, and hepatic level of cytochrome P-450.

Group	Initial body weight (g)	Final body weight (g)	Liver weight (g/100 g body weight)	Hepatic level of L-ascorbic acid (μ g/g)	Urinary excretion of L-ascorbic acid (μ g/day/100 g body weight)	Hepatic activity of L-gulono- γ -lactone oxidase (nmol/min/mg protein)	Hepatic level of cytochrome P-450 (nmol/mg protein)
Control	109 $\pm$ 1	134 $\pm$ 2	5.05 $\pm$ 0.09	199 $\pm$ 8	119 $\pm$ 22	10.1 $\pm$ 2.1	0.31 $\pm$ 0.05
PB	108 $\pm$ 3	128 $\pm$ 7	4.84 $\pm$ 0.32	265 $\pm$ 7**	521 $\pm$ 59**	8.4 $\pm$ 1.2	0.50 $\pm$ 0.04*
Control	109 $\pm$ 1	136 $\pm$ 2	5.07 $\pm$ 0.13	203 $\pm$ 3	143 $\pm$ 23	8.5 $\pm$ 0.6	0.30 $\pm$ 0.01
MC	108 $\pm$ 2	130 $\pm$ 1	5.64 $\pm$ 0.46	290 $\pm$ 16*	770 $\pm$ 190*	7.5 $\pm$ 0.5	0.52 $\pm$ 0.06*

Values are mean $\pm$ SE. A mean with an asterisk is significantly different from that of the corresponding control group (* p < 0.05; ** p < 0.01).

Fig. 2. Northern blot analysis of total RNA from liver of rats treated with sodium phenobarbital (PB) or 3-methylcholanthrene (MC). Total RNA (20 μ g) isolated from livers was separated on a 1% agarose gel containing 6.6% formaldehyde. The RNA in the gel was transferred to a Hybond N+ membrane and hybridized with 32 P-labeled rat L-gulono- γ -lactone oxidase cDNA, 32 P-labeled rat cytochrome P-450IIB1 cDNA, or 32 P-labeled rat cytochrome P-450IA2 cDNA. Panel A, total RNA from the liver of rats treated or not treated with PB. Lanes 1–3, control group (saline injected); lanes 4–6, PB group (PB injected). Panel B, total RNA from the liver of rats treated or not treated with MC. Lanes 1–3, control group (corn oil injected); lanes 4–6, MC group (MC injected).

excretion of AsA in the PB group increased 4.3-fold compared with that in the control group, and the value in the MC group increased 5.4-fold compared with that in the control group.

Figure 2A and 2B show the results of Northern blot analysis of hepatic RNA from rats treated with PB or MC. Quantification of the radioactivity of the probe hybridized (Fig. 3) showed that the administration of PB or MC tended to slightly decrease the hepatic level of GLO mRNA, though the difference was not significant. A similar tendency was observed in the hepatic activity of GLO, as shown in Table 1. However, cytochrome P-450IIB1 mRNA and cytochrome P-450IIB2 mRNA were markedly induced by the treatment with PB (Fig. 2A); and cytochrome P-450IA1 mRNA and cytochrome P-450IA2 mRNA, by the treatment with MC (Fig. 2B). In addition, the hepatic levels of cytochrome P-450 in the PB and MC groups were higher than those in the respective control groups (Table 1).

DISCUSSION

It is well documented that AsA biosynthesis occurs in the liver of most mammalian species. GLO, which catalyzes the terminal step of the AsA biosynthetic pathway, is known to be located in the microsomes of the liver. There is also a

Fig. 3. Hepatic level of L-gulono- γ -lactone oxidase mRNA in rats treated with sodium phenobarbital (PB) or 3-methylcholanthrene (MC). The value of PB group or MC group was normalized to that of each control group. The bars indicate mean values and the vertical lines above the bars indicate SE.

report stating that GLO activity was detected in the prostate (25). GLO has been usually assayed by the determination of the AsA formed in the enzymatic reaction. Since an appreciable amount of AsA is present in crude enzyme samples, even in the microsomal fraction (26), GLO assays are prone to be done erroneously when the activity is low. Thus the judgment of occurrence of GLO in certain tissues by the activity measurement is not decisive. In contrast, the measurement of the level of GLO mRNA appears to be more reliable for the assessment of the expression of GLO in a tissue.

In this study, we examined the distribution of the GLO mRNA in various organs by Northern blot analysis, and observed that the kidney, lung, small intestine, spleen, testis, and prostate gave no signal under the conditions where the liver gave a strong signal. The prostate develops with the sexual maturation in male rats, so we also tested the prostate from a 12-week-old male in addition to that from the 5-week-old male. However, no signal for the GLO mRNA was detected. In agreement with this result, the GLO activity was not detectable in the prostate from either rat.

It has already been shown that AsA biosynthesis in rats is increased by treatment of the animals with various kinds of xenobiotics (6–15). Hollmann and Touster (10) reported the enhancement of AsA biosynthesis in rats administered PB or MC. In line with their result, we observed that the treatment with PB or MC stimulated AsA biosynthesis, as evidenced by an increased hepatic level of AsA and an increased urinary excretion of AsA in rats treated with these compounds (Table 1). The administration of PB significantly increased the hepatic levels of cytochrome

P-450IIB1 mRNA and cytochrome P-450IIB2 mRNA, and the administration of MC increased the hepatic levels of cytochrome P-450IA1 mRNA and cytochrome P-450IA2 mRNA (Fig. 2). These results indicate that the rats used in this study responded normally to PB or MC administered as a stimulator of AsA biosynthesis and drug metabolism. However, the hepatic level of GLO mRNA and hepatic GLO activity were not elevated, but rather tended to be decreased by the administration of PB or MC (Table 1 and Fig. 3). In addition, the hepatic level of GLO mRNA did not change at any time, 24, 48, or 72 h, after the start of treatment with PB or MC (data not shown).

These results clearly indicate that the treatment with PB or MC increases the biosynthesis of AsA by inducing some enzyme(s) participating in the synthesis prior to GLO, and not by stimulating the expression of the GLO gene. UDPglucuronosyltransferase and UDPglucose dehydrogenase may be such enzymes, since the administration of the xenobiotics was shown to increase the hepatic activities of them (10).

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