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Ascorbate-dependent elevation of mRNA levels for cytochrome P450s induced by polychlorinated biphenyls

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Abstract—Trichlorobiphenyl induced only CYP1A2 mRNA, while pentachlorobiphenyl induced both CYP1A2 and CYP2B1 mRNAs in rat liver. The mRNA levels for these P450s were elevated when ascorbic acid-deficient ODS rats (mutant rats with a hereditary osteogenic disorder) were fed a diet supplemented with ascorbic acid. The amount of CYP2B1 mRNA increased rapidly and reached a maximum level of approximately double within 24 hr of injection of pentachlorobiphenyl. Thereafter, the amount of its mRNA decreased to a steady level. This pattern was roughly paralleled by changes in the amount of CYP1A2 mRNA.

Studies of the multiplicity and substrate specificity of P450 enzymes have contributed to our current understanding of dietary effects on xenobiotic metabolism. Polychlorinated biphenyl (PCB*) isomers increase the hepatic contents of the major P450 enzymes induced by both phenobarbital and 3-methylcholanthrene [1–3]. The administration of PCB isomers causes marked increases in the tissue concentration of ascorbic acid as well as urinary excretion of ascorbic acid in the rat [4]. Guinea pigs fed an ascorbic acid-deficient diet have lower concentrations of P450. A trend has been observed toward a higher P450 content with increasing dietary ascorbic acid, suggesting that ascorbic acid influences drug metabolism [5]. The importance of ascorbic acid in many cellular reactions and processes has been known for many years. Ascorbic acid is required for the hydroxylation of proline and lysine residues to form stable triple helical procollagen [6], and enhances not only the rate of procollagen gene transcription but also the stability of its mRNA [7]. Furthermore, ascorbic acid has been shown to induce elevated levels of alkaline phosphatases in cultures prepared from hypertrophic condrocytes of chick tibiae [8]. The mechanisms of action of ascorbic acid on the transcription and/or translation of specific genes are, however, largely unknown.

In this study, a mutant rat strain with a hereditary osteogenic disorder (ODS-*od*/*od*), i.e. a lack of terminal enzyme for L-ascorbic acid synthesis, L-gulono-γ-lactone oxidase [9], was utilized to examine the effect of ascorbic acid on the mRNA levels for P450s induced by PCB isomers. The transcription of CYP1A2 and 2B1 genes was markedly enhanced when ascorbic acid-deficient ODS rats were fed a diet supplemented with ascorbic acid.

Materials and Methods

Treatment with ascorbic acid and isolation of RNAs. The ascorbic acid-free (basal) and supplemented diets were as reported by Horio *et al.* [4]. Male ODS-*od*/*od* rats (ODS rats) were purchased from Nippon Clea Co. Ltd (Tokyo, Japan). Five-week-old ODS rats were fed a basal diet supplemented with 300 mg ascorbic acid/kg diet for a week, with a 12 hr light/dark cycle in wire-bottomed cages with free access to feed and water. Ascorbic acid deficiency was induced by feeding the basal diet for 10 days. The ascorbic acid content of liver decreased to a minimal level of 13 μg/g liver, i.e. almost one-twentieth the content in ascorbic acid-supplemented rats. Furthermore, the gain in body weight began to decrease at this stage. Therefore, after 10 days of ascorbic acid depletion, a PCB isomer, trichlorobiphenyl or pentachlorobiphenyl, dissolved in corn oil was administered i.p. in a single dose (100 mg/kg body weight) to ODS rats (day 0). The ODS rats were divided into two groups; one group was fed the basal diet for 4 or 7 days and the other the ascorbic acid-supplemented diet for 7 days. At an appropriate time after injection, two ODS rats of each group were killed by decapitation and the livers were pooled. Then, total RNAs were isolated from their livers as described by Chomczynski and Sacchi [10]. Poly $(A)^+$RNA was obtained by chromatography on oligo(dT)-cellulose [11]. The concentrations of ascorbic acid in the livers were measured by the method of Roe *et al.* [12].

RNA blot analysis. Total RNAs (20 μg each) were denatured and then electrophoresed on formaldehyde-agarose gels [13]. The RNAs were blotted onto Biodyne A membranes (PALL Ultrafine Filtration Corp.) and then the filters were prehybridized, hybridized and washed according to standard procedures [14], prior to either autoradiography or quantitation with a beta-scanner (AMBIS Radioanalytic Imaging System; AMBIS Systems Inc.). RNAs for CYP1A2 and 2B1 were hybridized with random primed probes; probe D (cDNA fragment for CYP1A2; 2.15 kb *Pst*I fragment on pcP450mc3, [15]) and probe B (cDNA fragment for CYP2B1; 1.67 kb *Pst*I fragment on pcP450pb4 [16]), respectively. Mitochondrial (mt) rRNA, used as an internal standard, was rehybridized with random primed probe R (1.45 kb *Xba*I-*Kpn*I fragment encoding human mt rRNA). The radioactivity of each RNA was quantitated with a beta-scanner. The relative values for CYP1A2 and 2B1 mRNAs were normalized as to that for mt rRNA.

Other methods. Double-stranded cDNA was synthesized using poly $(A)^+$RNA and a cDNA synthesis system plus (Amersham, U.K.) and a cDNA library constructed in λ gt10 was screened with ^{32}P-labeled probe B. Nucleotide sequences were determined as described by Sanger *et al.* [17].

Results and Discussion

PCB isomers have been shown to induce many isomers of P450s responsible for hepatic drug metabolism [1–3]. To examine the ability of ascorbic acid to induce changes in gene expression for P450s, ascorbic acid-deficient ODS rats were treated with PCB isomers and fed a basal diet or ascorbic acid-supplemented diet.

The mRNAs specific for CYP1A2 and CYP2B1 were identified and quantitated by northern blot analysis. Representative blots and curves for P450 mRNAs are shown in Figs 1 and 2. Probes B and D hybridized with mRNAs for CYP2B1 and CYP1A2, respectively, of around 18S, which is consistent with the sizes estimated from their respective cDNAs [15, 16]. The low homology (less than

* Abbreviations: PCB, polychlorinated biphenyl; ODS, a mutant rat strain with a hereditary osteogenic disorder; mt, mitochondrial.

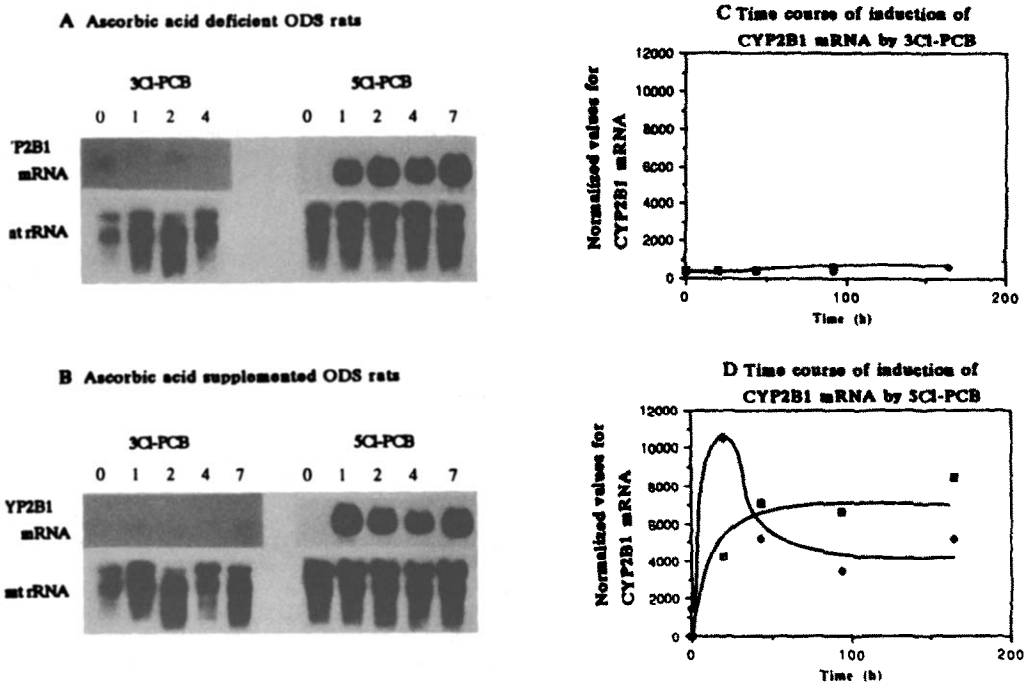

Fig.1. Ascorbate effect on the induction of CYP2B1 mRNA by PCB isomers in ODS rat liver. Ascorbic acid-deficient ODS rats were injected with trichlorobiphenyl (3Cl-PCB) or pentachlorobiphenyl (5Cl-PCB) on day 0. Total RNAs were isolated from the animals fed a basal diet (A) or an ascorbic acid-supplemented diet (B) for various periods up to 7 days. Twenty micrograms of each RNA was analysed on northern blots hybridized with either probe B or probe R, as described under Materials and Methods. The relative values for CYP2B1 mRNA were normalized as to the amounts of the corresponding mt rRNA, as described under Materials and Methods (C and D). Open and closed symbols indicate RNAs isolated from rats fed the ascorbic acid-deficient and -supplemented diet, respectively.

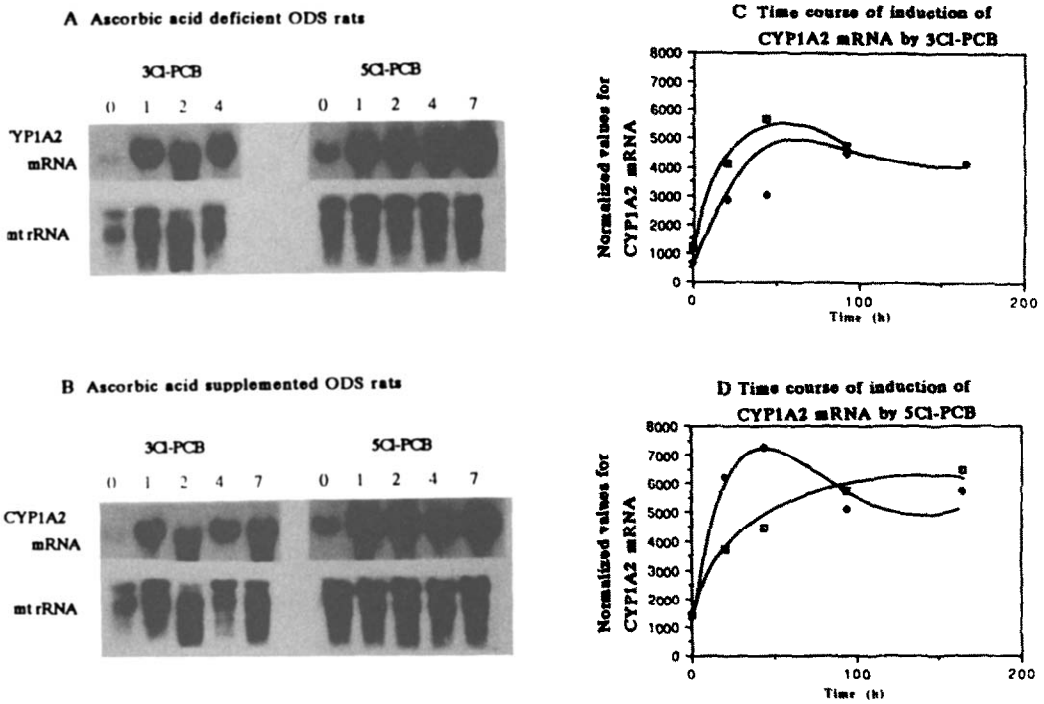

Fig. 2. Ascorbate effect on the induction of CYP1A2 mRNA by PCB isomers in ODS rat liver. The RNAs, abbreviations and symbols are the same as those in Fig. 1. The amounts of CYP1A2 mRNA were determined with probe D and normalized as described under Materials and Methods.

30%) between these DNAs made it possible to quantitate the amount of mRNA for each form without interference by the other.

The kinetics of induction of CYP1A2 and CYP2B1 mRNAs were quite different in rat livers treated with trichlorobiphenyl (Figs 1C and 2C). Trichlorobiphenyl effectively induced only mRNA for CYP1A2. The time course of induction of CYP1A2 mRNA was essentially the same in the ODS rats fed the basal diet and those fed the diet containing ascorbic acid (Fig. 2C). Ascorbic acid appeared to have no effect on the amount of CYP1A2 mRNA on induction with trichlorobiphenyl.

Both probes B and D hybridized with mRNAs of around 18S prepared from rat livers treated with pentachlorobiphenyl (Figs 1A, B and 2A, B). In contrast to trichlorobiphenyl, pentachlorobiphenyl induced mRNAs for both CYP1A2 and CYP2B1, suggesting these two forms of P450 are not under coordinate regulatory control. This may be due to differences in metabolism of the PCB isomers, since most trichlorobiphenyls are metabolized whereas most pentachlorobiphenyls are not. The amount of CYP2B1 mRNA increased rapidly and reached a maximal level of approximately two times as much as that in ascorbic acid-deficient ODS rats within 24 hr of the start of the treatment. Thereafter, the amount of the mRNA decreased to a steady level, i.e. almost one-third the maximal level (Fig. 1D). No ascorbic acid-specific effect was observed on the steady-state level of CYP2B1 mRNA, suggesting that the stability of this mRNA remains the same irrespective of the presence or absence of ascorbic acid in the diet. The level of CYP1A2 mRNA increased promptly and reached a peak after 48 hr. Then, the level of the mRNA decreased gradually to the same level as that observed in ascorbic acid-deficient ODS rats (Fig. 2D).

CYP2B1 and CYP2B2 induced in rat liver comprise 491 amino acids with highly homologous sequences (97% homology) and the respective mRNAs were difficult to determine separately under the hybridization conditions used. To examine whether or not CYP2B2 was simultaneously induced by PCB isomers, a cDNA library constructed in λgt10 from mRNA induced by pentachlorobiphenyl for 24 hr in the presence of ascorbic acid was screened with probe B. From approximately 10^6 bacteriophages screened, 16 positive clones were obtained. Although no clones carrying cDNA fragments for CYP2B2 were isolated, 15 clones contained cDNA fragments identical to that of CYP2B1. One clone, designated as λ L1, contained a cDNA fragment bearing 53% similarity with those of CYP2B1 and 2B2 and 99% similarity with that of CYP2D2, which might catalyse debrisoquine and bufuralol oxidation [18]. Furthermore, mRNA for the L1 gene was detected in kidney at a level considerably less than that in liver, but was undetectable in brain, lung, heart, adrenal glands, spleen and testis under the same conditions. A cDNA fragment bearing virtually the same sequence as that of P450L1 or CYP2D2 was, however, isolated by screening a guinea pig brain cDNA library, suggesting that CYP2D2 is expressed at a low level even in brain. These cloning data indicate that the mRNA identified as that for CYP2B1 comprises only mRNA for CYP2B1. It thus appears that ascorbic acid may be involved in the process of enhancement of the transcription rate of the mRNA for CYP2B1.

The induction of P450s by 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin or 3-methylcholanthrene is mediated by the receptor, probably a product of the *Ah* locus, for these chemicals. The receptor associated with the inducer activates the transcription of P450 genes, such as rat CYP1A1 gene, through interaction with the *cis*-acting regulatory sequence, xenobiotic responsive element or XRE [19]. The molecular mechanisms underlying the ascorbic acid-specific elevation of the CYP2B1 mRNA level could be elucidated by studying the *in vitro* transcriptional activity, using the promoter region of the CYP2B1 gene.

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REFERENCES

1. Denomme MA, Bandiera S, Lambert I, Copp L, Safe L and Safe S, Polychlorinated biphenyls as phenobarbitone-type inducers of microsomal enzymes. *Biochem Pharmacol* **32**: 2955–2963, 1983.
2. Parkinson A, Safe SH, Robertson LW, Thomas PE, Ryan DE, Reike LM and Levin W, Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats, a study of structure–activity relationships. *J Biol Chem* **258**: 5967–5976, 1983.
3. Schuetz EG, Wrighton SA, Safe SH and Guzelian PS, Regulation of cytochrome P450 by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in primary monolayer culture and *in vivo*. *Biochemistry* **25**: 1124–1133, 1986.
4. Horio F, Ozaki K, Kohmura M, Yoshida A, Makino S and Hayashi Y, Ascorbic acid requirement for the induction of microsomal drug-metabolizing enzymes in a rat mutant unable to synthesize ascorbic acid. *J Nutr* **116**: 2278–2289, 1986.
5. Peterson FJ, Holloway DE, Duquette PH and Rivers JM, Dietary ascorbic acid and hepatic mixed function oxidase activity in the guinea pig. *Biochem Pharmacol* **32**: 91–96, 1983.
6. Peterkofsky B, Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *Am J Clin Nutr* **54**: 1135S–1140S, 1991.
7. Lyons BL and Schwarz RI, Ascorbate stimulation of PAT cells causes an increase in transcription rates and a decrease in degradation of procollagen mRNA. *Nucleic Acids Res* **12**: 2569–2579, 1984.
8. Leboy PS, Vaias L, Uschmamn B, Golub E, Adams SL and Pacifici M, Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. *J Biol Chem* **264**: 17281–17286, 1989.
9. Nishikimi M, Koshizaka T, Mochizuki H, Iwata H, Makino S, Hayashi Y, Ozawa T and Yagi K, L-Gulono-*r*-lactone oxidase deficiency in rats with osteogenic disorder: enzymological and immunochemical studies. *Biochem Int* **16**: 615–621, 1988.
10. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159, 1987.
11. Aviv H and Leder P, Purification of biologically active globin messenger RNA by chromotography on oligothymidylic acid-cellulose. *Proc Natl Acad Sci USA* **80**: 1194–1198, 1972.

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12. Roe JH, Mills MB, Oesterling MJ and Damron CM, The determination of diketo-l-gulonic acid, dehydro-l-ascorbic acid and l-ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method. *J Biol Chem* **174**: 201–208, 1948.
13. Lehrach H, Diamond D, Wozney JM and Boedtker H, RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**: 4743–4751, 1977.
14. Alwine JC, Kemp DJ and Stark GR, Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci USA* **74**: 5350–5354, 1977.
15. Kawajiri K, Gotoh O, Sogawa K, Tagashira Y, Muramatsu M and Fujii-Kuriyama Y, Coding nucleotide sequence of 3-methylcholanthrene-inducible cytochrome P450d cDNA from rat liver. *Proc Natl Acad Sci USA* **81**: 1649–1653, 1984.
16. Fujii-Kuriyama Y, Mizukami Y, Kawajiri K, Sogawa K and Muramatsu M, Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. *Proc Natl Acad Sci USA* **79**: 2793–2797, 1982.
17. Sanger F, Nicklen S and Coulson AR, DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467, 1977.
18. Matsunaga E, Zanger UM, Hardwick JP, Geiboin HV, Meyer UA and Gonzalez HV, The CYP2D gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry* **28**: 7349–7355, 1989.
19. Gotoh O and Fujii-Kuriyama Y, Evolution, structure and gene regulation of cytochrome P-450. In: *Frontiers in Biotransformation* (Eds. Ruckpaul K and Rein H), pp. 195–243. Akademie-Verlag, Berlin, 1989.