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Proline Is Required for Transcriptional Control of the Aromatic Hydrocarbon-inducible P_1450 Gene in C57BL/6 Mouse Monolayer-cultured Hepatocytes

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Expression of aryl hydrocarbon hydroxylase (AHH) and the corresponding gene, *Cyp1A1*, or P_1450 in mice, in C57BL/6 mouse hepatocytes in primary culture was investigated after exposure to benz[*a*]-anthracene with respect to proline-related metabolic regulation. When the cells were cultivated in complete Waymouth MB752/1 (Way), prominent induction of AHH by benz[*a*]anthracene was observed, whereas the induction was inefficient in the same but proline-deficient medium [Way-(-pro)]. Constitutive AHH activities decreased with increasing culture period. P_1450 gene transcripts were slightly expressed when the medium was changed, independently of whether the cells were cultivated in either Way or Way(-pro), followed by decrease within 24 h and no apparent induction of AHH. However, treatment with Δ^1-pyrroline-5-carboxylic acid (P5C), a biosynthetic precursor for proline, dose-dependently increased basal AHH activities in the cells cultivated in Way-(-pro). Benz[*a*]anthracene induction of AHH in cells cultivated in Way(-pro) was additively increased in the presence of P5C as much as with proline. Treatment with *o*-aminobenzaldehyde, which inactivates P5C, drastically reduced the induced AHH activities in hepatocytes cultivated in either P5C-added Way(-pro) or Way medium. Benz[*a*]anthracene induced both P_1450 and P_3450 mRNAs. Neither proline nor P5C increased the induced transcripts within 12 h after the start of benz[*a*]-anthracene treatment, but the two compounds increased the amounts of P_1450 mRNA found at later time points. After treatment with actinomycin D, the half-life of the induced P_1450 mRNA was approximately 12 h, being independent of the presence of either proline or P5C. Our observations suggest that induction of AHH after treatment with polycyclic aromatic hydrocarbon is dependent on proline-related metabolism which influences the transcriptional process of P_1450 gene expression.

Key words: Aryl hydrocarbon hydroxylase — Proline — Hepatocyte culture

Most environmental chemical carcinogens require metabolic activation before they can cause damage to cellular macromolecules.[1] Microsomal P450-dependent monooxygenases play an important role in the activation process.[2,3] Purification and isolation of enzyme proteins, DNAs and cDNAs of several P450 species have allowed definition of their substrate specificities.[4-9] The *CYP1A1* gene or P450IA1 protein species is responsible for activation of carcinogenic polycyclic aromatic hydrocarbons and a representative enzyme is AHH.[1] Its activity can be induced by treatment with several kinds of aromatic hydrocarbons[10] and Nebert and his group reported after extensive studies that mouse strains can be sorted into responsive and non-responsive classes on the basis of their AHH inducibility.[11,12] The responsiveness has been concluded to rely on the binding affinity of cytosolic Ah receptor for inducers, namely, in responsive mouse strains it can bind to aromatic hydrocarbons, whereas in non-responsive strains it has poor affinity. Kouri and Nebert subsequently studied relationships between hepatic AHH induction and susceptibility to cancer in lung and skin by aromatic hydrocarbons, reaching the conclusion that these two parameters are well correlated.[13] However, the induction mechanism has not been completely elucidated. For example, further research is necessary to characterize the Ah receptor and to verify the hypothesis that an Ah receptor-inducer complex transfers into the nucleus to bind to a regulatory sequence in the 5′-flanking region of P_1450 gene.[2] Regulatory factors for AHH induction other than the Ah receptor itself are therefore important targets for investigation in *in vitro* cell culture systems. In mouse hepatoma cell lines a short-lived suppressor protein[14] and in hamster cultured kidney cells cyclic nucleotide-dependent AHH expression[15] have already been documented. Elevated induction of AHH activity in rat hepatocytes in primary culture was also reported by ourselves after treatment with specific inhibitors of poly(ADP-ribose) polymerase.[16]

Recently we found proline-dependent expression of aromatic hydrocarbon-inducible AHH in mouse hepatocytes in primary culture.[17] The present study concerns

[1] The abbreviations used are: C57BL/6; C57BL/6NCrj, AHH; aryl hydrocarbon hydroxylase, cDNA; complementary DNA, SSC; standard saline citrate, P5C; Δ^1-pyrroline-5-carboxylic acid, Way; Waymouth MB752/1, Way(-pro); proline-deficient Waymouth MB752/1.

[2] The terms P_1450 and P_3450 genes have been assigned to genes isolated from C57BL/6 mouse liver.

the regulation mechanism of AHH induction and P_1450 gene expression over time after aromatic hydrocarbon treatment of hepatocytes from C57BL/6 mice maintained in monolayer culture, the results indicating that proline-related metabolism is essential for the transcription process during AHH induction.

MATERIALS AND METHODS

Chemicals Materials for culturing hepatocytes were purchased from GIBCO Laboratories, Grand Island, NY, Collaborative Research Inc. Bedford, MA, and Kyokuto Seiyaku, Tokyo. Percoll and collagenase were products of Pharmacia, Uppsala, and Sigma Chemical Co., St. Louis, MO, respectively. P_1450 and P_3450 cDNAs cloned from mouse liver[18, 19] were generous gifts from Dr. Daniel W. Nebert, NIH, Bethesda, MD. Human prealbumin cDNA was obtained from the Japanese Cancer Research Resources Bank. 3-Hydroxybenzo[*a*]pyrene was supplied by the NCI Chemical Repository, Bethesda, MD. ^{32}P-Labeled deoxycytidine triphosphate was a product of ICN Biomedicals Inc., Costa Mesa, CA. Other compounds were purchased from Wako Pure Chemicals and Daiichi Chemicals, Tokyo.

Preparation of primary cultures of hepatocytes The livers of female C57BL/6 mice (Charles River Japan) weighing 25–30 g were subjected to collagenase perfusion and then Percoll isodensity centrifugation for isolation of viable hepatocytes using the method previously described.[17] The cells were dispersed in Leibovitz-15 medium containing bovine serum albumin (2 g/liter) and insulin (5 mg/liter) and allowed to seed in dishes coated with collagen type I at a density of $0.5–1.0\times10^6$ cells/4 ml/60 mm dish. The non-attached cells were discarded by aspiration, followed by changing the medium to Way containing bovine serum albumin (2 g/liter), insulin (5 mg/liter), transferrin (5 mg/liter), selenium (5 μg/liter) and dexamethasone (4 μg/liter) 2 h after seeding. The cultures were maintained at 37°C in a humidified CO_2 incubator. Medium was normally renewed every other day. Benz[*a*]anthracene, an inducer, was dissolved in dimethyl sulfoxide, of which the final concentration was less than 0.4%. The vehicle by itself did not induce AHH. Usually experiments were carried out after 48 h cultivation in Way medium, unless otherwise specified.

Assay of AHH The activity of AHH in hepatocytes was measured as described previously,[17] essentially by the modified method of Nebert and Gelboin.[20] One ml of reaction mixture contained hepatocytes from one dish, 1.08 μmol NADPH, 3 μmol $MgCl_2$, 100 nmol benzo[*a*]pyrene and 50 μmol Tris-HCl, pH 7.5. The reaction was stopped after 30 min by addition of cold acetone and the metabolites were then extracted with *n*-hexane, followed by extraction with 1 *N* NaOH. The emission at 522 nm with excitation at 396 nm was measured with 3-hydroxybenzo[*a*]pyrene as a standard. Enzyme activity was expressed as pmol of 3-hydroxybenzo[*a*]pyrene produced per 10^6 cells per min.

Hybridization of hepatocyte RNA with P450 probes Total RNA was prepared from the hepatocytes according to the guanidinium thiocyanate/phenol method[21] and subjected to hybridization as described by Maniatis *et al.*[22] More than 50 μg of total RNA was usually obtained from $0.8–1.0\times10^6$ cells. Northern transfer experiments were carried out after size-fractionation of the denatured RNA on formaldehyde containing 1.3% agarose gels. Hybridization was carried out at 42°C overnight in a mixture containing 50% formamide, 1× Denhardt, 5×SSC, 50 m*M* sodium-phosphate, pH 6.4, salmon testis DNA at 0.25 mg/ml, and ^{32}P-labeled probe. Washing was performed four times for 5 min with 2× SSC and 0.2% SDS at room temperature, and then twice for 15 min with 0.1×SSC and 0.2% SDS at 60°C. Exposure to Kodak X-ray film was carried out at −70°C with an intensifying screen (Du Pont). Although the species of stably expressed mRNA in hepatocytes in primary culture has not been identified yet, and in fact profound morphological alterations were observed with increasing culture period, albumin mRNA was employed for normalization of the applied RNA amounts on the gel, because albumin is a marker protein in the liver.

RESULTS

Effect of *cis*-hydroxy-L-proline or ornithine on AHH induction Proline is a major component of collagen, so that radioisotopically labeled proline has often been used as an indicator for collagen synthesis. *cis*-Hydroxy-L-proline, a potent inhibitor of collagen synthesis, was found to exert no inhibitory action on AHH induction in mouse hepatocytes (Fig. 1), although, when the cells were treated with the same experimental protocol within 2 days of cultivation after seeding, cytotoxic effects were observed at high concentrations. Ornithine, which is a precursor of polyamines and can be synthesized from proline, could not replace proline for enhancing benz[*a*]anthracene-induced AHH activity (Table I).

Enhancement of AHH activity by P5C Addition of proline to the culture medium slightly increased basal AHH activity ($P<0.05$). However, P5C, a biosynthetic intermediate for proline, significantly ($P<0.001$) elevated the enzyme activity (Fig. 2). Benz[*a*]anthracene-induced AHH activity was induced significantly ($P<0.001$) but was low when the cells were cultivated in Way(-pro). However, the induced AHH activity was enhanced by proline dose-dependently up to 0.2 m*M*. P5C also dose-dependently increased both uninduced and induced AHH activities. As shown in Fig. 3, with increas-

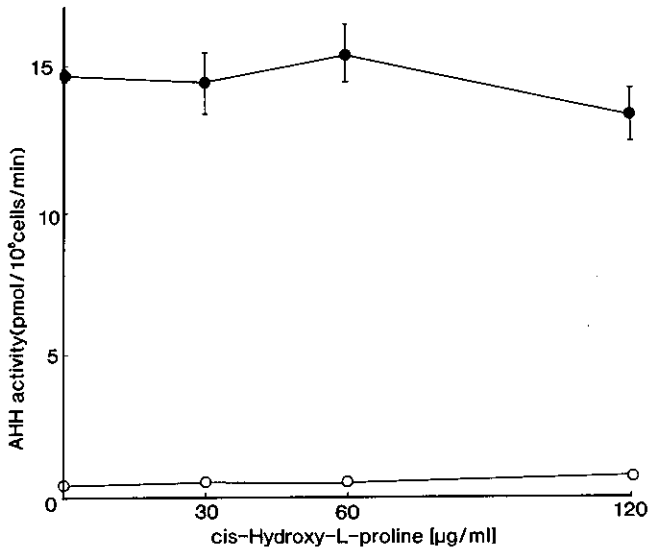

Fig. 1. Effect of *cis*-hydroxy-L-proline on AHH induction. Hepatocytes from C57BL/6 mice were treated with 13 μM benz[*a*]anthracene from 48–72 h and the indicated amounts of *cis*-hydroxy-L-proline. AHH activities at 72 h were assessed on the basis of four experiments. ○, untreated; ●, with benz[*a*]anthracene.

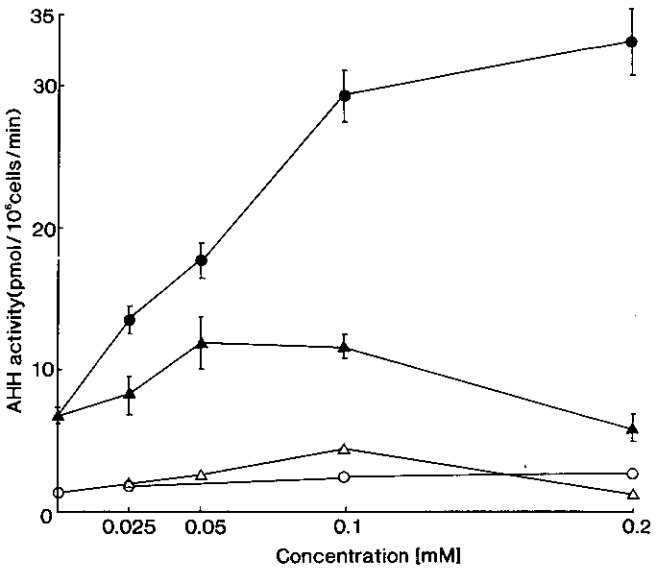

Fig. 2. Enhancement of benz[*a*]anthracene-induced AHH activity by proline or P5C. Hepatocytes were cultivated in Way medium from 0–48 h and then the medium was washed several times with Way(-pro). Proline or P5C at the indicated concentrations with or without 13 μM benz[*a*]anthracene was then added to the culture medium, and enzyme activities were determined at 72 h. Each point represents the mean±SD of data from four experiments. ○, proline; ●, proline+benz[*a*]anthracene; △, P5C; ▲, P5C+benz[*a*]anthracene.

Table I. Effect of Ornithine on AHH Activity

Treatment	AHH activity (pmol/10^6 cells/min)
Control	1.00±0.03
Proline (0.4 mM)	2.07±0.15
Benz[*a*]anthracene (13 μM)	3.72±0.19
Benz[*a*]anthracene + proline	11.16±1.27
Ornithine (0.01 mM)	0.82±0.04
(0.1 mM)	0.71±0.07
(1.0 mM)	0.70±0.08
Benz[*a*]anthracene + ornithine (0.01 mM)	3.69±0.24
(0.1 mM)	3.68±0.36
(1.0 mM)	3.65±0.38

Hepatocytes were cultivated in Way for 0–48 h, and then in Way(-pro) from 48–72 h. Addition of the indicated compounds was started at 48 h and the AHH activity was measured at 72 h. Each activity value was calculated as the mean±SD from data for four experiments.

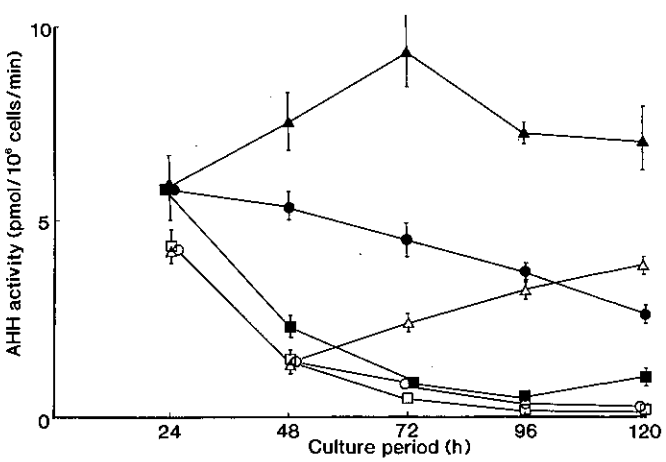

Fig. 3. Time course of AHH induction by benz[*a*]anthracene in the presence of proline or P5C. Hepatocytes cultivated in Way medium were treated from 0–24 h with 13 μM benz[*a*]anthracene and then the medium was changed to Way(-pro), Way or P5C(0.05 mM)-added Way(-pro). The medium was changed every day. Each point represents the mean±SD of data from four experiments. □, untreated; ■, with benz[*a*]anthracene; ○, proline; ●, proline+benz[*a*]anthracene; △, P5C; ▲, P5C+benz[*a*]anthracene.

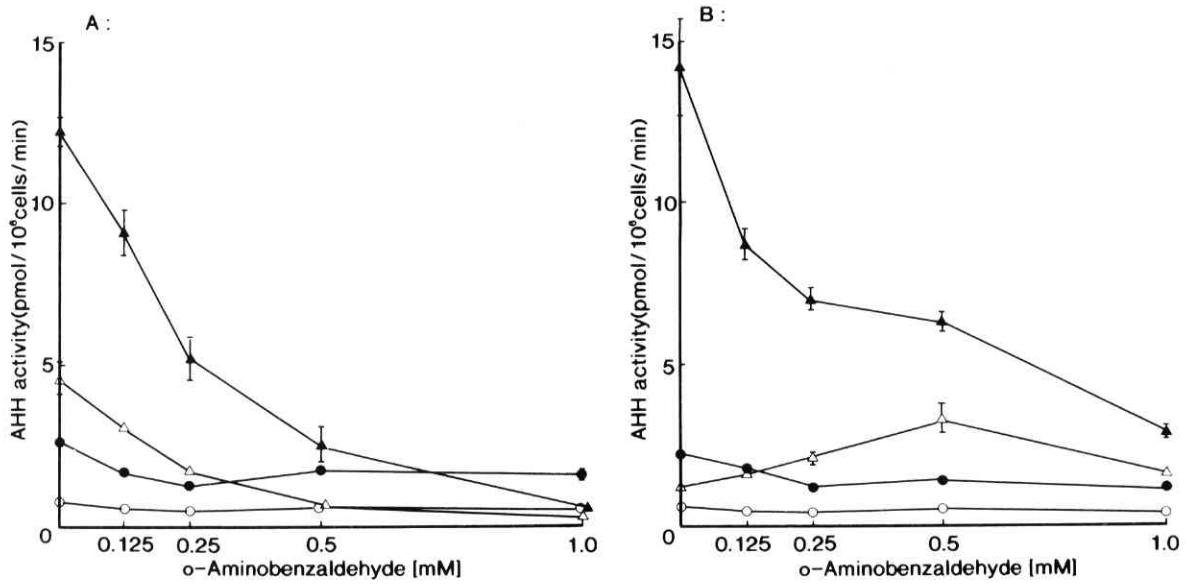

Fig. 4. Effect of *o*-aminobenzaldehyde on AHH induction by benz[*a*]anthracene in mouse hepatocytes. After cultivation of hepatocytes in Way medium for 48 h, the medium was changed to Way(-pro) after several washings with the latter. The cells were then cultivated in P5C (0.05 m*M*)-supplemented Way(-pro) (A) or in Way (B) medium. Treatment with 13 μM benz[*a*]-anthracene was started from 48 h and enzyme activities were determined at 72 h. *o*-Aminobenzaldehyde at the indicated amounts was added at 48 h and 60 h. Each point represents the mean ± SD of data from four experiments. ○, no addition; △, P5C (A) or proline (B); ●, benz[*a*]anthracene; ▲, benz[*a*]anthracene + P5C (A) or proline (B).

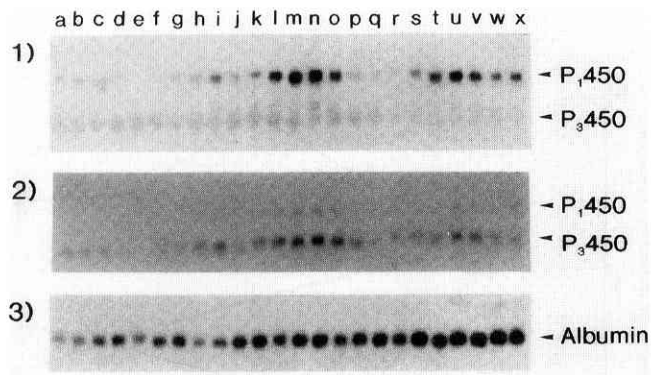

Fig. 5. Effect of *o*-aminobenzaldehyde on expression of P_1450 gene by benz[*a*]anthracene. Treatment with 13 μM benz[*a*]-anthracene (BA) and/or 0.4 m*M* *o*-aminobenzaldehyde (o-ABA) was started after 48 h cultivation of hepatocytes in Way medium. Total RNAs were subjected to Northern-blot hybridization using cDNA probes for 1) mouse P_1450, 2) mouse P_3450 and 3) human prealbumin. The same filter was used for either hybridization. Control RNAs were prepared from untreated cells after a medium change at 48 h. Treatment: lanes a–e; untreated. f–j; o-ABA-treated. k–q; BA-treated. r–x; o-ABA + BA-treated. Harvest time after medium change: a, f, k, r; 3 h. b, g, l, s; 6 h. m, t; 9 h. c, h, n, u; 12 h. d, i, o, v; 24 h. p, w; 36 h. e, j, q, x; 48 h.

ing culture period, basal AHH activity decreased when the cells were cultivated in Way(-pro) medium. In the relevant experiments the medium was changed every day and either proline or P5C was added at each changeover. The increase after addition of either compound was negligible at 48 h, but proline slightly elevated basal enzyme activity after 72 h, without preventing the gradual decrease of activity. In contrast P5C elevated the basal activity significantly (Fig. 3). After washing out of benz[*a*]anthracene at 24 h, the enzyme activity of hepatocytes cultivated in Way(-pro) medium rapidly decreased, while proline moderately restrained this decrease. However at day 5 the enzyme activity of benz-[*a*]anthracene-negative cells in P5C-added Way(-pro) medium was greater than that of benz[*a*]anthracene-treated cells in Way. Addition of P5C further increased the benz[*a*]anthracene-induced AHH activity.

Effect of *o*-aminobenzaldehyde on AHH induction

Δ^1-Pyrroline compounds, such as P5C, specifically and covalently bind with *o*-aminobenzaldehyde, resulting in loss of their activity.[23] Addition of *o*-aminobenzaldehyde to the culture medium drastically reduced the induction of AHH by benz[a]anthracene (Fig. 4). The reduction was more prominent for the cells cultivated in P5C-added Way(-pro) medium than for those in complete

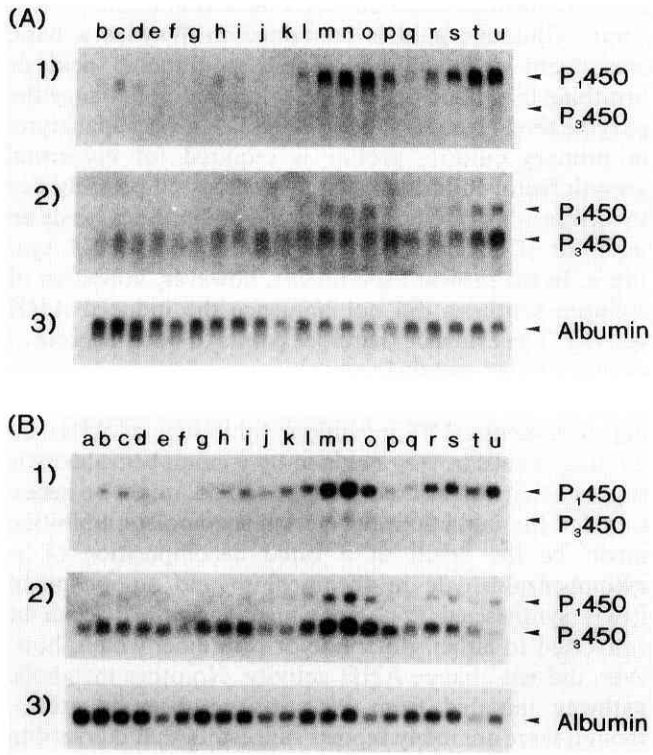

Fig. 6. Effect of proline or P5C on transcription of P450 genes in hepatocytes treated with benz[*a*]anthracene. After cultivation of hepatocytes in Way medium for 48 h the medium was changed to Way(-pro). Total RNAs were prepared for hybridization as described in the legend to Fig.5 after addition of 13 μM benz[*a*]anthracene (BA) in the presence of either proline (0.42 m*M*) or P5C (0.05 m*M*). A) Experiment in Way medium. B) In P5C-supplemented Way(-pro) medium. Treatment: lanes a–f; untreated. g–k; proline (A) or P5C (B) added. l–p; BA-treated. q–u; BA+proline (A) or P5C (B) added. Harvest time after medium change: a; 0 h. b, g, l, q; 3 h. c, h, m, r; 6 h. d, i, n, s; 9 h. e, j, o, t; 12 h. f, k, p, u; 24 h.

Way medium. The compound by itself did not cause cytotoxicity as evaluated morphologically. The hepatocytes cultivated in complete Way medium responded to the treatment with *o*-aminobenzaldehyde with slightly increased AHH activity. Total RNAs were prepared from the hepatocytes treated with either benz[*a*]anthracene or *o*-aminobenzaldehyde and then subjected to Northern-blot hybridization using P_1450, P_3450, or prealbumin cDNA as probes (Fig. 5). Sizes of the hybridized RNAs were 2.9 kb for P_1450 and 2.1 kb for P_3450 RNA, as reported previously.[18, 19] Constitutively expressed P_3450 mRNA decreased with increasing culture period as described in our previous paper for several kinds of responsive and non-responsive mouse strains.[24] After a medium change, expression of P_1450 gene was

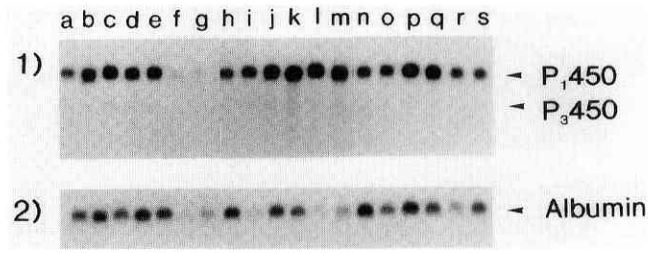

Fig. 7. Effect of proline or P5C on benz[*a*]anthracene-initiated P450 gene transcription in hepatocytes. Hepatocytes cultivated in Way medium were treated with 13 μM benz[*a*]-anthracene from 48 h after seeding. The medium was changed to Way-(-pro) 12 h later, after washing out several times. Control RNAs were prepared from cells cultivated in Way (-pro). Addition: lanes a–g; nothing. h–m; P5C. n–s; proline. Harvest time after the last medium change: a; 0 h. b, h, n; 3 h. c, i, o; 6 h. d, j, p; 9 h. e, k, q; 12 h. f, l, r; 24 h. g, m, s; 36 h.

observed at low levels, but the amounts were reduced with time, becoming negligible after 24 h. *o*-Aminobenzaldehyde slightly increased the amounts of both transcripts. Benz[*a*]anthracene induction of the transcripts was maximal from 9–12 h after the beginning of the treatment. Concomitant treatment with *o*-aminobenzaldehyde and benz[*a*]anthracene decreased the amounts of both P_1450 and P_3450 mRNAs by 24 h. Amounts of transcripts for albumin were not appreciably influenced by *o*-aminobenzaldehyde, although they decreased with incubation period.[24]

Effects of proline or P5C on P_1450 gene transcription in hepatocytes Figure 6 illustrates the effects of proline or P5C on levels of benz[*a*]anthracene-induced P_1450 mRNA. Proline by itself did not alter the P_1450 mRNA levels altered after a medium change. However, P5C treatment maintained the maximal amounts of P_1450 mRNA due to medium change. In the presence of proline, amounts of benz[*a*]anthracene-induced P_1450 mRNA increased to a maximum level around 9–12 h as did those in hepatocytes cultivated in Way(-pro) medium, but those at 24 h were higher than in the latter case. High levels of benz[*a*]anthracene-induced P_1450 transcription were also maintained at 24 h in the presence of P5C. In contrast, the amounts of benz[*a*]anthracene-induced P_3450 mRNA at 24 h were reduced in the presence of P5C, although they were not so much influenced by proline. Figure 7 also shows the effects of proline or P5C on the amounts of benz[*a*]anthracene-induced P_1450 mRNA, though with the difference in the experimental protocol that treatment with benz[*a*]anthracene was continued for only the initial 12 h and then the compound was washed out by changing the medium several times, followed by addition of proline or P5C.

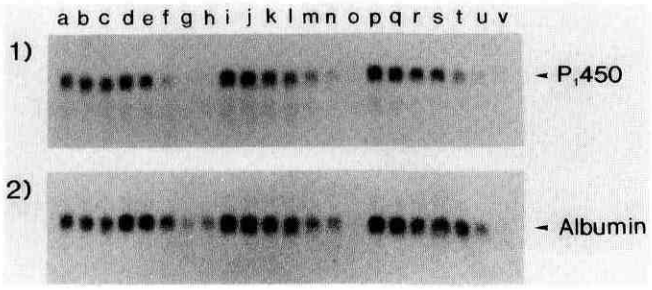

Fig. 8. Effect of proline or P5C on decrease in benz[*a*]-anthracene-induced P_1450 gene transcripts after treatment with actinomycin D. Hepatocytes cultivated in Way medium for 48 h were treated with 13 μM benz[*a*]anthracene after changing the medium to Way(-pro) and washing out several times. Actinomycin *D* at 2.5 μg/ml was added 12 h later at the same time as either proline (0.42 m*M*), or P5C (0.05 m*M*). Preparation of total RNAs was started at this time as the 0 h sample. Addition: lanes a–h; nothing. i–o; proline. p–v; P5C. Harvest time: a; 0 h. b, i, p; 3 h. c, j, q; 6 h. d, k, r; 9 h. e, l, s; 12 h. f, m, t; 24 h. g, n, u; 36 h. h, o, v; 48 h.

Amounts of P_1450 mRNA were increased about twice in controls, probably due to the medium change, but declined to a low level at 24 h. In contrast, the amounts of induced mRNA were maintained at high levels for up to 36 h in the presence of either P5C or proline. Transcription of the albumin gene was not altered by the presence of either compound. Decreasing patterns of benz[*a*]anthracene-induced P_1450 mRNA amounts after treatment with actinomycin D were almost the same whether hepatocytes were cultivated in Way(-pro), complete Way or P5C-added Way(-pro) medium (Fig. 8). Half-lives for the transcripts were calculated by densitometric measurement to be approximately 12 h in each case.

DISCUSSION

AHH induction by polycyclic aromatic hydrocarbons in mouse hepatocytes in primary culture was earlier found to be strongly dependent on proline as a medium constituent.[17] The present investigations revealed that P5C, a biosynthetic precursor for proline, exerts a similar potential for enhancing AHH induction to proline. Thus, the amounts of P_1450 mRNA after treatment with benz-[*a*]anthracene were increased in the presence of either proline or P5C.

Proline is not an essential amino acid, because there are several metabolic pathways which result in its production. For example glutamine and glutamic acid are both precursors of proline and ornithine in the urea cycle can be converted to it . However, these compounds were found to have no effect on AHH induction in the present study. Glutamic acid is contained in Way as a basic constituent and addition of either glutamic acid or ornithine to proline-deficient medium did not change the enzyme level (see ref. 17 and Table I). In rat hepatocytes in primary culture, proline is required for epidermal growth factor-initiated DNA synthesis,[25,26] possibly due to its role as a prerequisite for collagen synthesis, since an inhibitor of collagen synthesis also inhibited DNA synthesis. In the present experiments, however, inhibition of collagen synthesis did not influence the induced AHH activity. The evidence therefore points to other aspects of proline-related metabolism.

We observed that P5C could replace proline in obtaining prominent AHH induction. Inhibition of AHH induction of cells in Way medium by *o*-aminobenzaldehyde suggests that oxidation of proline to P5C might be necessary for the induction. Severe but incomplete inhibition might be the result of a rapid decomposition of *o*-aminobenzaldehyde in the medium and an escape of newly synthesized P5C from inactivation. P5C can be converted to either glutamate or ornithine, which, however, did not change AHH activity. No other metabolic pathway initiated from P5C has yet been identified, though there are many reports suggesting that conversion between proline and P5C might be an important process influencing other biological activities.[27] Interconversion between proline and P5C is not catalyzed by a single enzyme, but by proline oxidase in mitochondria and P5C reductase in the cytosol.[27] During the cycle of oxidation of proline and reduction of P5C, ATP and NAD(P) are generated. The NAD(P)/NAD(P)H redox state is important in several metabolic processes.[27,28] For example, stimulatory effects of P5C on pentose phosphate pathway activity and on levels of phosphoribosyl pyrophosphate might be accounted for by an increased availability of NAD(P).[29] In rat hepatocyte culture there is a link between NAD concentration and maintenance of P450 content.[30,31] We also observed an elevation of AHH activity by a specific inhibitor of poly(ADP-ribose) polymerase in monolayer-cultured hepatocytes from rats[16] and mice (unpublished observation), indicating that an altered concentration of NAD, a substrate for the polymerase, might be an important factor for the induced AHH activity.

There are several reports that expression of orthologous genes to mouse P_1450 is regulated primarily at the transcription level,[32] whereas that of orthologous genes to P_3450 is largely controlled at the post-transcriptional level by stabilization of P_3450 mRNA.[32-34] The present Northern-blot hybridization experiments with RNAs from mouse hepatocytes suggested that the increase in P_1450 mRNA may be due to prolonged transcription induced by proline or a P5C-based mechanism. Precise

quantitation of the transcription rate of P_1450 gene will be required to confirm this possibility. However, since the amounts of P_1450 mRNA within 12 h after the start of treatment with benz[*a*]anthracene were not so different as the AHH activities when either proline or P5C was involved in the medium, the other possibility that proline or P5C could influence translation or the P450-based electron transport system can not be excluded.

Expression of P_1450 mRNA after a medium change was not observed in hepatocytes cultivated for less than one day (unpublished observations), but became prominent thereafter. Elevation of basal AHH activity by P5C was observed in a similar way (Fig. 3), indicating that P5C might prolong transcription of medium change-induced P_1450 gene expression, and the slight increase in AHH activity of the hepatocytes in complete Way might be due to conversion of small portions of proline to P5C without affecting the P_1450 mRNA level much. The Way medium does not contain any known inducers of AHH. Insulin, transferrin and selenium were added as growth factors, but exclusion of them did not eliminate the expression and, furthermore, dexamethadone did not change the appearance pattern of P_1450 mRNA (unpublished data), despite the finding of a nucleotide sequence resembling glucocorticoid regulatory element in the 5′-flanking region of the *CYP1A1* gene.[35] These observations suggest that expression of P_1450 gene after a medium change might be some kind of adaptive response of hepatocytes to culture conditions and therefore that sporadic expression of P_1450 gene might be expected *in vivo* under certain circumstances. Kimura *et al.*[36] reported induction of P_1450 mRNA after partial hepatectomy or during embryogenesis in the absence of any known foreign inducers, and they suggested that the P_1450 gene may play an important developmental function during differentiation. Although hepatocytes *in vivo* are considered to be in G_0 or G_1 phase, they started to synthesize DNA after transfer to primary culture, and some functional characteristics change with increasing culture period.[31, 37] Expression of P_1450 gene in primary culture might be required for cellular differentiation during culture.

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