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CERIUM-INDUCED STRAIN-DEPENDENT INCREASE IN Cyp2a-4/5 (CYTOCHROME P4502a-4/5) EXPRESSION IN THE LIVER AND KIDNEYS OF INBRED MICE

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Abstract—The murine Cyp2a-4 and Cyp2a-5 genes encode P450 isoforms catalysing testosterone 15α-hydroxylase and coumarin 7-hydroxylase (COH) activities, respectively. Two days after the administration of a hepatotoxic dose of cerium chloride (2 mg/kg i.v.), COH activity was increased 3.2-fold in the liver of DBA/2 mice. Three and 4 days after the cerium treatment, coinciding with the occurrence of overt liver damage, there was a dramatic decrease in COH activity. The activities of testosterone 15α-hydroxylase and the Cyp1a-1-mediated 7-ethoxyresorufin *O*-deethylase (EROD) were decreased in response to cerium. Much less pronounced changes in the enzyme activities occurred in the C57BL/6 mouse liver. Northern blot analysis showed a 21-fold increase in the hepatic Cyp2a-4/5 mRNA in the DBA/2 mice at day 2, whereas no increase occurred in the C57BL/6 mice. Also in the kidneys the increase in COH activity and in Cyp2a-4/5 mRNA was marked only in the DBA/2 mice. A polymerase chain reaction-mediated analysis method utilizing a unique *Pst*I restriction site in the Cyp2a-5 cDNA was used to differentiate between the highly homologous Cyp2a-4 and Cyp2a-5 mRNAs. Cerium was found to increase the amount of hepatic and renal Cyp2a-4 and Cyp2a-5 mRNA only in the DBA/2 mice. These data indicate that the Cyp2a-4/5 complex is regulated in a different way in DBA/2 and C57BL/6 mice and that some association exists between the development of liver damage and COH induction.

Cerium, a member of the light lanthanoids, causes profound liver damage when administered to rodents [1]. The characteristic alteration is the formation of fatty liver which is accompanied by a decrease in the P450-mediated‡ oxidative xenobiotic metabolism [1].

Coumarin 7-hydroxylase (COH§) is an activity catalysed by the Cyp2a-5 gene product (P450Coh) in mice [3]. A corresponding coumarin-metabolizing P450 isoform (designated CYP2A6) is expressed also in human liver [4], but not in rat liver [5]. Cyp2a-4, a gene with very high homology with Cyp2a-5, mediates the 15α-hydroxylation of testosterone and some other steroids in the mouse liver and kidney [6]. Cyp2a-5 is predominantly expressed in the liver of untreated mice, whereas Cyp2a-4 is the main component in the kidney [6].

Mouse hepatic Cyp2a-5 expression is often increased in conditions in which other P450 isoforms are repressed. This is especially true after treatment with hepatotoxic agents, e.g. pyrazole and heavy metals [7–10]. There is a strain-dependent difference in the hepatotoxicity of cerium with the DBA/2 (D2) strain being much more susceptible than the C57BL/6 (B6) strain [11]. Concurrently, the hepatic COH activity is increased by cerium in the D2 mice but not in the B6 mice [11]. The aim of this study was to investigate the effects of liver-damaging doses of cerium chloride on the expression of Cyp2a-4 and 2a-5 in the liver and kidney which differ in the regulation of the Cyp2a-4/5 gene complex.

MATERIALS AND METHODS

Materials. Coumarin was obtained from Serva (Heidelberg, Germany) and ethoxyresorufin from Pierce Chemical Co. (Rockford, IL, U.S.A.). 7-Hydroxycoumarin and cerium chloride ($CeCl_3 \times 7H_2O$) were purchased from Aldrich-Europe (Beerse, Belgium). Nucleic acid grade agarose, DNA and RNA molecular mass markers were from Pharmacia (Uppsala, Sweden). Guanidine thiocyanate was from Fluka (Buchs, Switzerland). ^{32}P-Labelled nucleotides and [4-^{14}C]testosterone was obtained from Amersham (Amersham, U.K.). The restriction enzymes were purchased from Boehringer Mannheim (Germany). All other reagents were of the highest commercial purity.

Treatment of mice. Adult male D2 and B6 mice (about 25 g) were used. Cerium was injected at a dose of 2 mg/kg to the tail vein of the mice. The control mice received saline only. This dose has been shown to cause severe disintegration of the liver tissue in the D2 mice while causing little effect in the B6 mice [11]. The mice were killed 6 hr to 1 week after the treatment.

Enzyme activity assays. A part of the liver and one kidney from each mouse were used to prepare

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‡ The latest P450 nomenclature [2] will be used in this article.

§ Abbreviations: COH, coumarin 7-hydroxylase; EROD, 7-ethoxyresorufin *O*-deethylase; PCR, polymerase chain reaction.

9000 *g* supernatants for enzyme activity assays. For each time-point the tissue samples from four to six mice were pooled. The Bradford [12] method was used for protein content determinations. COH activity was measured by the method of Aitio [13] using 100 μM coumarin as substrate. 7-Ethoxyresorufin O-deethylase (EROD) activity was assayed with the end-point method of Burke *et al.* [14] using 1 μM ethoxyresorufin. Testosterone 15α-hydroxylase activity was determined according to Waxman *et al.* [15].

Northern blots. The remaining part of the liver and the other kidney from the mice within each time-point were pooled and homogenized in guanidine thiocyanate and total cellular RNA was prepared by the cesium chloride centrifugation method [16]. RNA (20–30 μg) was separated on a 1% agarose gel, transferred to Hybond-N nylon membrane (Amersham). The RNA was fixed by baking at 80° for 2 hr and the membrane was hybridized with a ^{32}P-labelled *Cla*I fragment of Cyp2a-5 cDNA [17]. An end-labelled 24-mer oligonucleotide probe hybridizing to 18S ribosomal RNA [18] was used as the loading control. The autoradiography films were developed after an exposure for 2 days to 1 week at −70°. The band intensities were determined with a densitometer and the values were corrected against the corresponding 18S band intensities.

Differentiation between Cyp2a-4 and Cyp2a-5 mRNAs. A polymerase chain reaction (PCR)-based analysis method was used to differentiate between the highly homologous Cyp2a-4 and Cyp2a-5 messages. Total cellular RNA (0.5 μg) was used as a template for single-stranded cDNA synthesis (Pharmacia cDNA Synthesis Kit). Fifty pmoles of 12-mer primers (5'primer: GAGCGCATCCAA; 3'primer: CACGAGTCTAGG) constructed to bind to areas just outside the *Cla*I sites in Cyp2a-4/5 were added to the cDNA reaction tubes together with 2.5 U of AmpliTaq polymerase (Cetus). Amplification was done with a Perkin–Elmer Cetus thermocycler in 25 cycles each at: 94° 1 min, 37° 2 min and 72° 3 min. The short 12-mer primers together with low annealing temperature were found to give better results than conventional 20-mer primers. The amplification product (1 kb) was digested with *Pst*I which cuts only in Cyp2a-5 cDNA generating 710 and 290 bp fragments. The digested material was electrophoresed in a 1% agarose gel and blotted onto a nylon membrane which was hybridized with a ^{32}P-labelled *Cla*I fragment of Cyp2a-5 cDNA. The films were developed after an exposure for 60 min to overnight at room temperature.

RESULTS

Enzyme activities

The changes in the activities of hepatic COH, EROD and testosterone 15α-hydroxylase in response to cerium are shown in Fig. 1. In D2 mice COH activity was increased 3.2-fold 2 days after cerium treatment after which there was a dramatic decrease to about 10% of the control value in the activity at 3 and 4 days after the treatment. Testosterone 15α-hydroxylase activity was steadily decreased by cerium, and also the Cyp1a-1-mediated EROD activity was substantially decreased after a slight initial increase. The sharp decreases in the enzyme activities coincided with the development of overt liver damage in the D2 mice [11]. In the B6 mice the changes in the activities were much less pronounced with only a 1.7-fold increase occurring in COH activity 2 days after the treatment. No sharp decreases in the enzyme activities comparable to those in the D2 mice took place in the B6 mice.

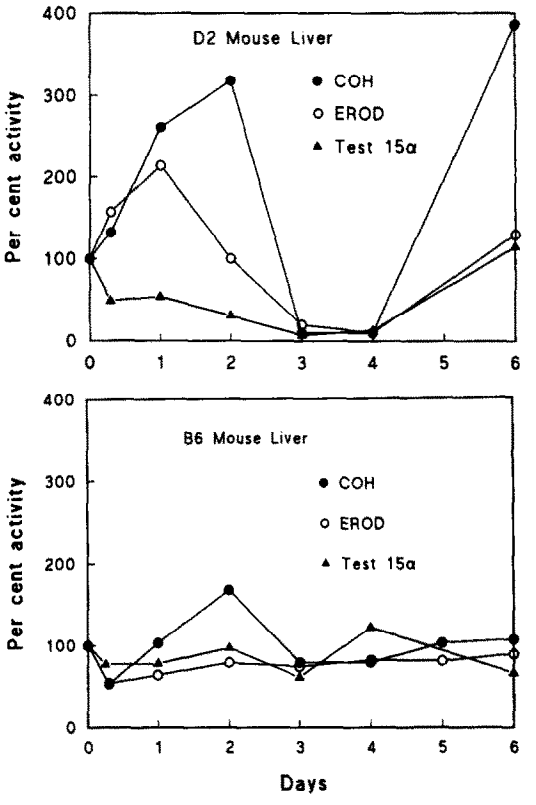

Fig. 1. Time-course of liver enzyme activities in D2 and B6 mice. COH, EROD and testosterone 15α-hydroxylase (test 15α) activities were determined. The control specific activities in the D2 mice were 10, 70 and 420 pmol/mg protein/min for COH, testosterone 15α-hydroxylase and EROD, respectively. In the B6 control mice, the corresponding activities were 7, 100 and 800 pmol/mg protein/min. As the enzyme source, the 9000 *g* supernatants of livers pooled from 4–6 mice was used. Cerium chloride was given as a single intravenous dose of 2 mg/mg.

Figure 2 shows the corresponding activities in the kidney tissue. In D2 mouse kidney, COH activity peaked to 4.4-fold over the control value at 4 days after the treatment after which the activity returned to the control level. The activities of EROD and testosterone 15α-hydroxylase were unaffected during the first 2 days, and they showed a slight reduction coinciding with the increase in COH activity at days 3 and 4 after the treatment. The activities showed a different pattern in the B6 mice. The increase in COH activity was very modest with less than 2-fold

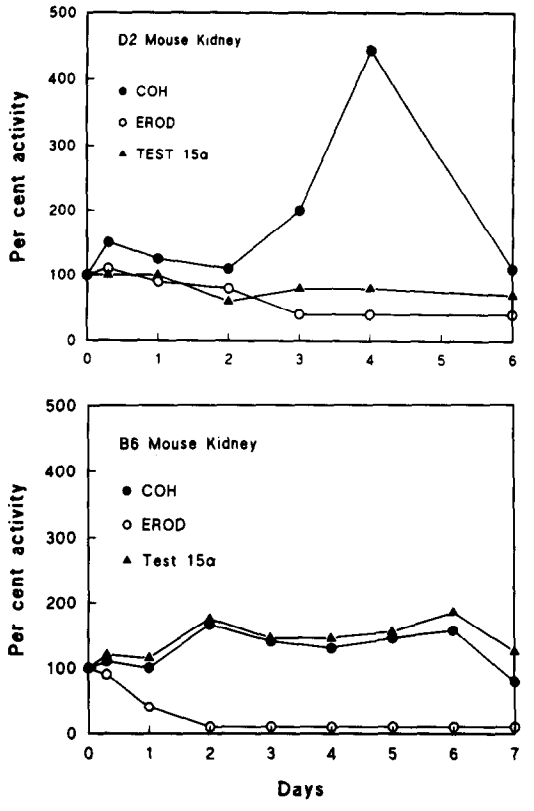

Fig. 2. Time-course of kidney enzyme activities in D2 and B6 mice. The control specific activities in the D2 mice were 3, 21 and 4 pmol/mg protein/min for COH, testosterone 15α-hydroxylase and EROD, respectively. The corresponding values in the B6 were 7, 17 and 8 pmol/mg protein/min. See the legend to Fig. 1 for the treatment of the mice.

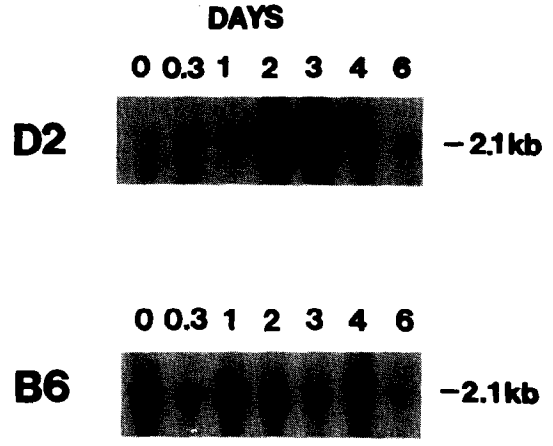

Fig. 3. Northern blot analysis of hepatic RNA from cerium-treated D2 and B2 mice. For each time-point, 20 μg of total RNA was resolved on a 1% agarose gel and transferred to a nylon membrane. The filter was hybridized with a ^{32}P-labelled *Cla*I-fragment of Cyp2a-5 cDNA. The autoradiography exposure times were 3 days for the D2 blot and 1 week for the B6 blot. Densitometric analysis of the autoradiograph, corrected against the 18S oligo signals and the control value normalized to 1, gave the following values: D2 mice; 1 (control), 1.5 (6 hr), 4.2 (1 day), 20.6 (2 days), 16.6 (3 days), 5.8 (4 days) and 3.7 (6 days) and B6 mice; 1 (control), 0.3 (6 hr), 1.2 (1 day), 0.9 (2 days), 0.6 (3 days), 0.8 (4 days) and 0.3 (6 days).

increases at the peaks (2 and 6 days after the treatment). Testosterone 15α-hydroxylase activity followed the COH activity pattern and the reduction in EROD activity was more substantial than in the D2 mice.

Northern blots. A marked increase in the amount of the 2.1 kb Cyp2a-4/5 mRNA was evident at 2 and 3 days after cerium administration in the livers of D2 mice (Fig. 3). Densitometric analysis showed that this increase is about 20-fold relative to the control value. As shown in Fig. 4, the increase in the level of Cyp2a-4/5 mRNA was also pronounced in the kidneys of D2 mice at 6 hr and 1 day after cerium injection (7- and 6-fold increases, respectively). In B6 mice no increases occurred in the mRNA levels in either the liver or kidney tissue (Figs 3 and 4).

Differentiation between Cyp2a-4 and Cyp2a-5

The relative contribution of the two genes to the mRNA signal in northern blots was determined by a PCR-enhanced diagnosis method in which the two isoforms are visualized as a 1 kb band (Cyp2a-4) or as 710 and 290 bp bands (Cyp2a-5) upon hybridization with the Cyp2a-5 probe. The method was first

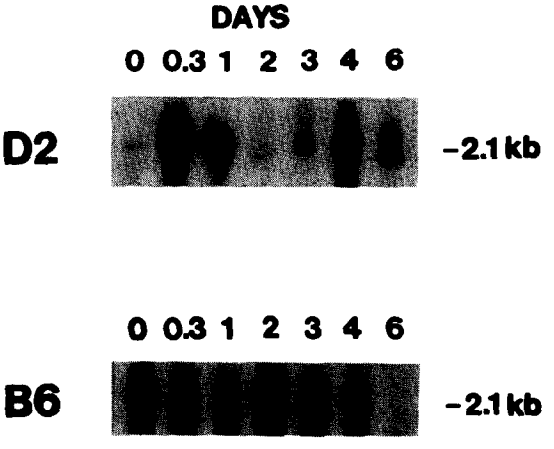

Fig. 4. Northern analysis of kidney RNA from cerium-treated D2 and B2 mice. The blot was prepared and probed like the one shown in Fig. 3. Densitometric analysis of the autoradiographs showed 7.0 (6 hr), 5.7 (1 day), 0.7 (2 days), 1.5 (3 days), 4.2 (4 days) and 2.0-fold (6 days) changes in the mRNA level in D2 mice after treatment with cerium and 0.6, 0.5, 1.2, 0.5, 0.5 and 0.2-fold changes at the corresponding time-points in B6 mice.

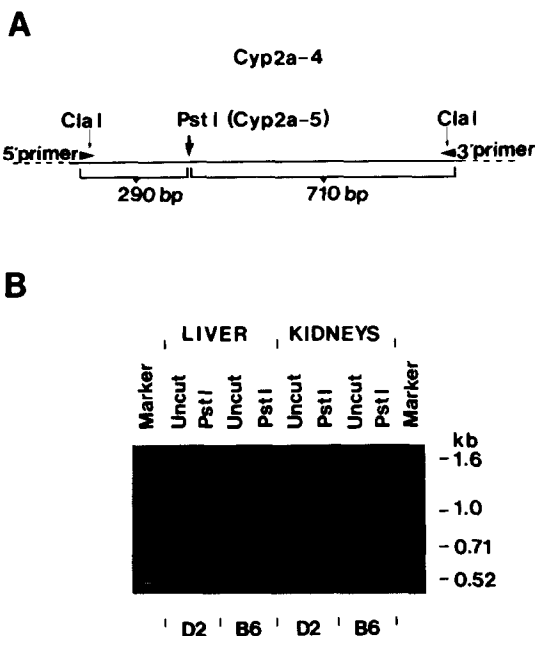

Fig. 5. Panel A: the binding sites of the primers in Cyp2a-4/5 cDNA. The 5′ primer binds to nucleotides 439–450 and the 3′ primer to nucleotides 1243–1434 in the Cyp2a-4/5 open reading frame (total length 1485). *Pst*I cuts only in Cyp2a-5 cDNA. Panel B: an ethidium bromide-stained agarose gel of uncut and *Pst*I-cut amplified DNA from control D2 and B6 mouse liver and kidney. Panel C: an autoradiogram of *Pst*I-digested DNA after hybridization with ^{32}P-labelled *Cla*I-fragment of Cyp2a-5. The D2 and B6 mice have been treated with saline (C, control), pyrazole (Pyr) or phenobarbital (PB).

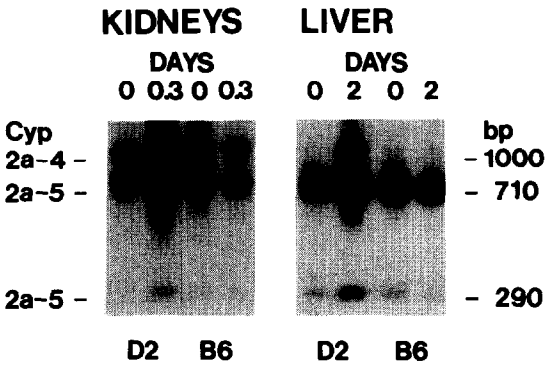

Fig. 6. An autoradiogram of *Pst*I-digested PCR-amplified DNA from the liver and kidney tissue of D2 and B6 mice treated with cerium chloride.

validated by using known inducers of Cyp2a-4/5 expression, i.e. pyrazole and phenobarbital [3, 6]. Figure 5, panel A illustrates the binding sites of the primers in the Cyp2a-4/5 cDNA. In untreated mice, Cyp2a-4 is the main component in the kidney and Cyp2a-5 is predominantly expressed in the liver [6]. As shown in the ethidium bromide-stained agarose gel (Fig. 5, panel B), the amplified 1 kb fragment was completely digested by *Pst*I in the liver samples, whereas in the kidneys the 1 kb band was predominant. Densitometric analysis of the autoradiograph (Fig. 5, panel C) showed that more than 90% of the Cyp2a-4/5 mRNA is Cyp2a-5 in control mice. The inducers appeared to affect both mRNAs to a similar extent.

For the analysis, the time-points after cerium treatment were selected to coincide with the peaks in northern blots. As shown in Fig. 6, cerium treatment increased the amount of both hepatic Cyp2a-4 and Cyp2a-5 mRNA in the D2 mice, whereas in the B6 mice no increases could be seen. The amounts of renal Cyp2a-4 and Cyp2a-5 are increased to a similar extent by cerium in the D2 mice and no increase occurred in the B6 mice (Fig. 6).

DISCUSSION

The present study shows that administration of a liver-damaging dose of cerium causes an increase in the expression of Cyp2a-4 and 2a-5 in the liver and kidneys of the cerium-susceptible D2 mice but not in the resistant B6 mice.

When administered at the dose of 2 mg/kg, cerium produces a severe liver damage in the D2 mice 72 hr after the treatment. The changes consist of diffuse panlobular necrosis, disintegration of nuclei, accumulation of fat droplets and sinusoid congestion. These changes do not occur in the B6 mice at the same dose level [11]. The differences are not due to unequal tissue distribution of cerium in the two mouse strains [11].

It is remarkable that in both the tissues (liver and kidney) where Cyp2a-4 and Cyp2a-5 are expressed, the cerium-caused increase in the expression was much more pronounced in the susceptible D2 strain. The PCR-mediated diagnosis method allowed us to dissect the relative contribution of Cyp2a-4 and 2a-5 in the Cyp2a-5-hybridizable mRNA. Taken together with previous reports [6, 19] it is evident that in the liver Cyp2a-5 is the predominant component and also the one more affected by inducing agents. In the kidney, Cyp2a-4 and 2a-5

are about equally present in the mRNA and they also appear to be similarly affected by at least cerium.

The cerium-induced increase in Cyp2a-5 expression occurs at the early stages of the developing liver damage in the D2 mice. The liver damage reaches a maximum at 3 days after cerium treatment, and at this point also a drastic reduction in P450-mediated activities takes place. There is, however, a high level of steady-state Cyp2a-5 mRNA present in the liver tissue at this time-point. This indicates that there is on-going transcription of the gene or stabilization of the message, but some block develops at the level of translation or enzyme function. Two other hepatotoxic agents, pyrazole and cobalt affect Cyp2a-5 expression by posttranscriptional mechanisms [19, 20], and this may be true for cerium also. In the kidneys the increases in the enzyme activities and mRNA levels coincide in the D2 mice. In agreement with previous studies in rats [2], the effects of cerium subsided within 1 week after the treatment.

The mechanisms behind the increased Cyp2a-5 expression in liver damage are unknown presently. For example the role of the aryl hydrocarbon (*Ah*) receptor in the toxicity has not been explored. It is also impossible to say whether COH induction is the cause or effect in the overt liver damage, since the physiological function of P450Coh is unknown. Previous studies have identified a structurally disparate group of compounds having in common the P450Coh-inducing capacity. These compounds include the heavy metals cobalt [9, 21] and indium [10], the heterocyclic nitrogen-containing agents pyrazole and its derivatives [22, 23], the classical P450 inducer phenobarbital, the mouse-specific inducer 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) [24], and some well-established hepatotoxins, such as carbon tetrachloride, hexachlorobutadiene and cocaine (Pellinen *et al.*, unpublished). Most of these agents are known to cause liver damage in rodents, but phenobarbital and TCPOBOP, for example, are not hepatotoxic. In addition, some potent liver-damaging compounds, such as dimethylnitrosamine and allyl alcohol do not affect COH activity (Pellinen *et al.*, unpublished). Thus, a linear relationship between liver damage and COH induction may not exist.

The common theme in the action of the various hepatotoxic agents is that they preferentially affect COH activity in the D2 strain in which COH is also most inducible by phenobarbital [25]. They thus obey the presently unidentified mechanism which dictates the selective inducibility in the D2 mice. The picture is made complex by both transcriptional and posttranscriptional events being involved in the increased COH activity [21, 22]. Thus, clarification of the selectivity issue requires additional work.

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