Reappraisal of the liver benzpyrene hydroxylase synthesized de novo after treatment of rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene

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The dynamics of the inductive effects of MC and TCDD upon rat liver microsomal benzpyrene hydroxylase and the main properties of the de novo synthesized hemoproteins have been compared. The inadequacy of expression of the enzyme activity per total cytochrome P-448 content has been established. It was concluded that TCDD microsomes have a relatively low content of benzpyrene hydroxylase with a higher molecular activity than the enzyme from MC microsomes.

Benzpyrene hydroxylase *Enzyme activity* *Hemoprotein synthesis* *Cytochrome P-448* *Antibody*

1. INTRODUCTION

Among hundreds of xenobiotics, which are inducers of the microsomal monooxygenases, TCDD takes a particular place. This xenobiotic is the most potent inducer of the MC type so far known and has therefore been used widely in studies on the key steps of intracellular reception of an inducer and expression of structural genes of aromatic hydrocarbon hydroxylases in the livers of mice, rats and rabbits [1,2]. This study has been undertaken because the usage of TCDD and MC as typical inducers led to contradictory results concerning the similarity molecular mechanisms of induction and the synthesized forms of cytochrome P-450 [3,4]. The results of a reappraisal of the dynamics of induction by TCDD and MC of benzpyrene hydroxylases, and of the catalytic, spectral, immunochemical and electrophoretic properties of the de novo synthesised hemoproteins are presented here.

Abbreviations: MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Chemicals used were purchased from the following sources: MC, Serva; 3,4-benzpyrene, Fluka; NADPH, Boehringer; L-[1-^{14}C]leucine, Amersham. Reagents for SDS-PAGE were from Pharmacia. TCDD was a generous gift from Dr Daniel Nebert (NICHHD, Bethesda).

Male Wistar rats (50 g) were treated with either MC (40 mg/kg) or TCDD (25 μg/kg) 16 h before killing. The rats were also injected i.p. with L-[1-^{14}C]leucine (500 μCi) 4 and 8 h before killing. In other experiments the rats were treated with MC or TCDD for 72 h beforehand.

Microsomes were prepared from livers by conventional differential centrifugation. Cytochrome P-450 (P-448) and protein were determined as described [5,6]. Benzpyrene hydroxylase activity was determined fluorimetrically as in [7]. All spectral measurements were performed with a Hitachi

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556 spectrophotometer and MPF-4 spectrofluorimeter.

SDS-PAGE was carried out on 7,5–15% gradient gels according to Laemmli [8] with GE-2/4 slab gel apparatus (Pharmacia). Fluorography of PAGE slabs was performed as described by Chamberlain [9]. Radioactivity was determined with a Mark-III liquid scintillation spectrometer.

Cytochrome P-448 was purified from the MC microsomes [10] to a concentration of 16–18 nmol per mg protein and its homogeneity verified using SDS-PAGE. Antibodies against isolated cytochrome P-448 were obtained from the sera of rabbits as described in [7]. Rocket immunoelectrophoresis of liver microsomes for specific quantitation of benzpyrene hydroxylase was performed according to [11] with Multiphor 2117 (LKB). Metabolism of 3,4-benzpyrene in the presence of antibodies was determined as in [7].

3. RESULTS AND DISCUSSION

The main results obtained are presented in table 1 from which it can be seen that 16 h after injection of MC or TCDD into rats there was no increase in the total microsomal cytochrome content, nor shift of the $Na_2S_2O_4$ CO peak to 448 nm. Nevertheless, at the same time benzpyrene hydroxylase activity increased significantly. This fact has been acknowledged in earlier experiments with mice pretreated with MC or TCDD [12]. Table 1 shows that in MC microsomes the K_m for 3,4-benzpyrene decreased to the level characteristic of maximal induction. The specific hemoprotein form amounts to 1/3 of the total cytochrome content, as determined by rocket immunoelectrophoresis using antibodies against MC cytochrome P-448 (56 kDa). However, it is precisely this particular form of the cytochrome that catalyses metabolism of

Table 1

A comparison of inducing effects of MC and TCDD upon rat liver microsomal benzpyrene hydroxylases

Parameters determined	Time after inducer injection (h)				Control group
	16		72		
	MC	TCDD	MC	TCDD	
(A) Hemoprotein content					
1. Total content (nmol/mg protein)	0.55 ±0.02	0.59 ±0.02	1.06±0.02	0.87±0.02	0.53±0.01
2. Content of specific form (nmol/mg protein)	0.198±0.007 (36)[a]	0.0235±0.005 (4)	0.90±0.02 (85)	0.209±0.005 (24)	– (<3)
3. Position of CO peak (nm)	448.5	449	448	448	450
(B) Benzpyrene hydroxylases					
1. K_m for 3,4-benzpyrene (μM)	0.39 ±0.04	0.54 ±0.07	0.40±0.02	0.37±0.03	0.53±0.03
2. Total activity (nmol/min)					
-per mg protein	2.85 ±0.15	1.00 ±0.06	3.45±0.20	2.90±0.18	0.23±0.01
-per nmol total cytochrome	5.18 ±0.085	1.70 ±0.04	3.25±0.13	3.33±0.13	0.43±0.01
3. Specific activity (nmol/min)					
-per mg protein	2.71 ±0.14 (95)[b]	0.82 ±0.05 (82)	3.28±0.19 (95)	2.81±0.17 (97)	–
-per nmol specific form	13.69 ±0.22	34.86 ±1.38	3.64±0.13	13.46±0.52	–
(C) In vivo incorporation of [^{14}C]-leucine into microsomes (%)	450	290	n.d.	n.d.	100[c]

[a]Percent of total cytochrome content
[b]Percent of inhibition by antibodies against MC cytochrome P-448
[c]100% = 133 cpm/mg protein

Values are means ± SE from 3–5 experiments

3,4-benzpyrene in MC microsomes. This was evinced by almost complete inhibition of the reaction by antibodies against the purified MC cytochrome P-448.

SDS-PAGE of MC and TCDD microsomes showed two very faint bands in the region of migration of the hemoproteins (48–58 kDa) (fig.1). Fluorography of the gels after SDS-PAGE separation of the [^{14}C]leucine-labeled MC and TCDD microsomes revealed that these bands belong to the de novo synthesized polypeptides of 53 and 56 kDa (fig.2). These bands were lacking in the preparations of control microsomes. These two hemoproteins isolated and purified from MC microsomes were both registered spectrally as cytochrome P-448 but only the latter posessed high benzpyrene hydroxylase activity in a reconstituted system as well as cross-reactivity with homologous antibodies.

Unlike the MC microsomes, the K_m for 3,4-benzpyrene in the liver microsomes prepared 16 h after injection of TCDD did not exceed that in control microsomes. In the TCDD microsomes the rate of the benzpyrene hydroxylation reaction calculated per nmol total hemoprotein was relatively low (see table 1). However, it should be

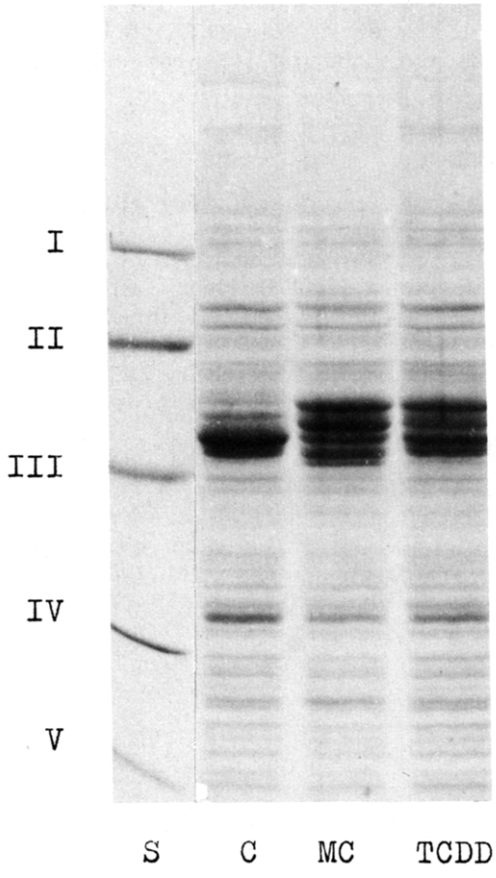

Fig.1. SDS-PAGE in gradient (7.5–15.0%) gels of microsomal protein (25 μg) from control (C), and MC- and TCDD-treated rats. Standard protein mixture (S) contained: I, phosphorylase *b* (94 kDa); II, albumin (67 kDa); III, ovalbumin (43 kDa); IV, carbonic anhydrase (30 kDa); V, α-lactalbumin (14.4 kDa).

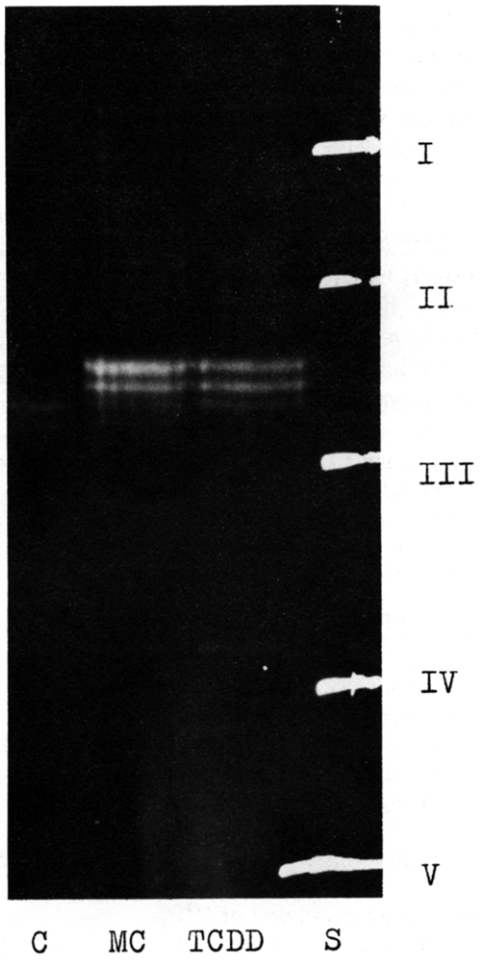

Fig.2. Fluorogram of SDS-PAGE slab after electrophoresis of [^{14}C]leucine-labeled microsomal proteins (25 μg) from control (C), and MC- and TCDD-treated rats. The mixture of standard proteins was as in fig.1.

emphasized that 16 h TCDD microsomes contain an extremely small amount of the specific hemoprotein form with benzpyrene hydroxylase activity. Therefore, calculation of the activity per nmol of this specific form of the hemoprotein has revealed a considerably higher turnover number of the enzyme in TCDD microsomes than that of cytochrome P-448 in MC microsomes. The same results were also obtained with 7-ethoxyresorufin as a substrate (not shown). The decisive contribution of this form of the cytochrome to hydroxylation of 3,4-benzpyrene was proved by inhibitory analysis using homologous antibodies.

72 h after injection of TCDD into rats the liver microsomes displayed the characteristic blue shift of the maximum CO peak and an increase in the total cytochrome P-448 content. There was also a 6-fold increase in benzpyrene hydroxylase when compared with the level at 16 h but it still comprised only 24% from the total cytochrome content. Therefore, the activities of the enzyme calculated per nmol total cytochrome content are the same in both MC and TCDD microsomes. However, when the activity is calculated per nmol specific hemoprotein form determined immunochemically, then benzpyrene hydroxylase in TCDD microsomes is 3.7-fold more active than in MC microsomes.

The molecular activity and K_m values for the substrate are similar in 72 h TCDD microsomes and 16 h MC microsomes. The supposition about the slower rate of biosynthesis of the enzyme in rats pretreated with TCDD was confirmed in the in vivo experiments with pulses of [^{14}C]leucine. It may be seen from table 1 that incorporation of the label into TCDD microsomes was 1.5-fold less effective than into MC microsomes.

Thus, only direct comparison of the inductive effects of MC and TCDD has elucidated the fact that treatment of Wistar rats with TCDD induces the synthesis of benzpyrene hydroxylase which represents only a minor part of the total microsomal cytochrome but has high molecular activity.

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