MODULATING EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN ON SKIN CARCINOGENESIS INITIATED BY THE WEAK INDUCER 7,12-DIMETHYLBENZ(A)ANTHRACENE

Pierre Lesca, Nathalie Perrot and Bogumila Peryt

Laboratoire de Pharmacologie et Toxicologie, Institut National de la Recherche Agronomique, BP 3, 31931 Toulouse Cedex, France

ABSTRACT

The effects of topical pretreatment of CF1-Swiss mice with TCDD on the carcinogenesis induced by DMBA were studied. We also determined the intrinsic features of DMBA as an aryl hydrocarbon hydroxylase (AHH) inducer through either its binding ability to *Ah* receptor or its inducing effects on benzo(*a*)pyrene (BP) hydroxylase or DMBA hydroxylase. DMBA is a poor ligand of the *Ah* receptor (26-fold and 4.3-fold weaker than 3-methylcholanthrene and BP respectively) and a very weak AHH inducer (ten million-fold weaker than TCDD). Nevertheless, DMBA induces a specific isozyme of cytochrome P-450 1A1 since, for an equal dose administered to C57BL/6 mice (200 mg/kg body weight), the DMBA-hydroxylase activity was 1.72-fold increased by DMBA while it remained unchanged after BP treatment. In contrast, the BP-hydroxylase activity was 1.91-fold increased by BP and only 1.47-fold by DMBA. A dose-dependent relationship exists between the increasing dose of TCDD (from 0.001 to 1 μg per mouse) applied to mouse skin and the induction of AHH activity of skin microsomes (from 1 to 60-fold increase). For carcinogenesis experiments, mice were either untreated or pretreated with single different doses of TCDD and, after 24h, DMBA (10 or 25 μg per mouse) was applied to the skin. The average number of papillomas per mouse was dependent on 1) the dose of DMBA and 2) the metabolic capacity of the skin. For 10 μg DMBA, the TCDD only exerts an anticarcinogenic effect (from 5.5 to 0.6 tumor per mouse) whereas for 25 μg DMBA, TCDD exerts a dual effect: first, a cocarcinogenic effect (from 6.2 to 9 and 11.5 tumors per mouse for 0.001 and 0.01 μg TCDD respectively) then an anticarcinogenic effect (2.3 and 1.5 tumors per mouse for 0.1 and 1 μg

TCDD respectively). The discussion underlines the decisive importance of two factors: 1) the effective dose of the ultimate carcinogen in contact with cellular targets during a sensitive step of the cell cycle and 2) the time-persistence of a high steady state level of the carcinogen.

KEY WORDS

induction, cytochrome P-450, dioxin, carcinogenesis

INTRODUCTION

Carcinogenesis is a multistep process involving a lot of factors such as metabolizing enzyme activity, electrophilic scavengers, DNA repair efficiency, promotion, nutritional and hormonal factors, immunological competence, age, sex, time and strain- and species-differences /1/. The skin carcinogenesis model is one of the most investigated for the understanding of the carcinogenic effects of PAHs, and DMBA was currently used in these studies.

At the initiation level, several enzymes, such as cytochrome P-450-dependent monooxygenases and epoxide hydrolase, are involved in the biotransformation of procarcinogens to their reactive metabolites which bind to critical targets of the cells, in particular DNA, leading to structural damage and then genomic mutations /2-4/.

In order to appraise the influence of the induction of metabolizing enzymes for the carcinogenicity of PAHs and especially the cyt P-450 1A1 which is specifically involved in the biotransformation of PAHs into reactive metabolites /4/, it was of prime importance to control some main factors which are 1) the intrinsic inducing ability of PAHs, 2) the uninduced metabolizing activity of the tissues studied and 3) the responsiveness of animals to the inducing effect of non-carcinogenic compounds which could be used to modulate, on a large scale, the biotransformation and the activation of pre-cancerogens. In

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Abbreviations:
PAHs, Polycyclic aromatic hydrocarbons; DMBA, 7,12-Dimethylbenz(*a*) anthracene; BP, Benzo(*a*)pyrene; TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; TPA, 12-O-tetradecanoylphorbol-13-acetate; TCPOBOP, 1,4-Bis[2-(3,5-dichloro-pyridyloxy)]benzene; BPDE, Benzo(*a*)pyrene-7,8-diol-9,10-epoxide; AHH, Aryl hydrocarbon hydroxylase.

accordance with these requirements, we first studied the capacity of DMBA to bind the *Ah* receptor /5/ which, after a temperature-dependent "transformation" step /6/ leads to the induction of cytochrome P-450 1A1 through the association of the ligand-receptor complex with specific regulatory regions of DNA /7/. We also measured the inducing power of DMBA on AHH activity using either BP or DMBA as substrates.

In the second part of this work, we studied the effects of the induction of PAH-metabolizing enzymes by TCDD on the carcinogenicity of DMBA on mouse skin. Our data suggest that the rate of DMBA metabolic activation and consequently the time of persistence of the carcinogen in contact with the sensitive cells, are key parameters in chemical carcinogenesis. These results also underline the indirect influence of non-carcinogenic drugs and pollutants on this process.

MATERIALS AND METHODS

Chemicals

[^{3}H]DMBA (50 Ci/mmol), radiochemical purity 91.1% and [^{3}H]BP (31 Ci/mmol), radiochemical purity 96.1% were purchased from Amersham (Bucks, England). Unlabeled TCDD as well as [^{3}H]TCDD (50 Ci/mmol) were purchased from KOR isotopes (Cambridge, MA, USA); the radiochemical purity of the tritiated compound, >98%, was checked after storage and, if necessary, the removal of radiolysed [^{3}H]TCDD was carried out by high performance liquid chromatography according to the procedure of Gaziewicz and Neal /9/. Non-labeled DMBA and BP, salmon sperm DNA (type III, sodium salt), glucose 6-phosphate dehydrogenase (baker's yeast, type VII), DNase I (from beef pancreas), snake venom phosphodiesterase I (type II), alkaline phosphatase (*Escherichia coli*, type III), NADPH, glucose 6-phosphate, sodium dodecyl sulfate, HEPES, dextran (Mr 150,000), activated charcoal, dithiothreitol were obtained from Sigma (St Louis, MO, USA). Sodium phenobarbital and dimethyl sulfoxide were purchased from Merck A.G. (Darmstadt, Germany). 1,4-Bis[2-(3,5-dichloropyridyloxy)]-benzene, a phenobarbital-like inducer, was a gift from Dr. A. Poland (Madison, WI, USA). Sephadex LH20 was obtained from Pharmacia (Uppsala, Sweden). TPA was purchased from CCR (Eden Prairie, MN, USA).

Benzo(*a*)pyrene metabolite standards were obtained from the National Cancer Institute, Chemical Repository, NIH (Bethesda, MD, USA). All other chemicals were purchased from commercial sources.

Animals

CF1 Swiss mice (20 g), from Caworth farm, USA, were bred by Iffa-Credo (Les Oncins, France). C57 BL/6N Cr BR mice (20 g) were obtained from Charles River, Cléon, France. When the animals were pretreated by TCPOBOP, in order to increase their hepatic *Ah* receptor level /10/, they received 3 mg/kg of compound i.p. dissolved in sunflower oil. The mice were sacrificed 72 h after treatment. Sprague-Dawley rats (100-150 g) were purchased from Janvier, Le Genest, France. When the rats were pretreated, the phenobarbital (80 mg/kg), dissolved in 0.9% NaCl, was injected intraperitoneally for 3 consecutive days before the animals were killed on day 4 for removal of the liver.

For induction experiments, TCDD (from 0.001 to 1 μg/mouse) dissolved in 0.2 ml acetone was applied to the shaved back of CF1 Swiss mice. Control animals were pretreated with 0.2 ml acetone. BP (from 25 to 200 mg/kg) or DMBA (from 50 to 400 mg/kg) dissolved in sunflower oil were administered intraperitoneally to C57 BL/6 mice, control mice receiving only the solvent (0.1 ml per 10 g body weight).

Buffers

The standard buffer used for the preparation of hepatic cytosol and microsomes and for binding experiments was HEDG: 25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol (v/v), pH 7.6. Skin microsomes were prepared in TMS buffer: 50 mM Tris, 3 mM $MgCl_2$, 200 mM sucrose, pH 7.4.

Preparation of cytosol and microsomes

C57 BL/6 mice were killed by cervical fracture and Sprague Dawley rats were anesthetized by ether. The liver was perfused *in situ* with cold 0.9% NaCl solution via a needle inserted into the heart, then via the inferior vena cava with HEDG buffer. After extensive perfusion, the liver was removed, rinsed with 0.9% NaCl solution and HEDG buffer, minced and homogenized in HEDG buffer (3 ml/g of

liver) with a teflon-glass homogenizer. All procedures were performed at 4°C. The homogenate was centrifuged at 9000 *g* for 20 min, and the resultant supernatant was centrifuged at 105,000 *g* for 1 h. Cytosol was carefully drawn off without disturbing the surface lipid layer or the microsomal pellet. The latter was washed in TMS buffer and centrifuged again at 105,000 *g* for 30 min. Microsomes were resuspended in TMS and stored at -70°C. The cytosolic fraction, pooled from several animals (from 3 to 15) of the same strain, was either used immediately or stored at -70°C, for periods of up to 6 months before use in the binding experiments or *Ah* receptor and 4 S protein separation procedure; no loss of activity occurred under these conditions. *Ah* receptor and 4 S protein were separated by a sucrose density gradient method as described by Lesca *et al.* /10/. Skin microsomes were prepared from untreated or TCDD-treated CF1 Swiss mice. The mice were sacrificed 24 h after treatment, by cervical dislocation, and the skin of the shaved back was removed and placed in cold TMS buffer. Skin homogenates were obtained according to the procedure described by Kinoshita and Gelboin /11/, by scraping the epidermis of three mice with a surgical scalpel. The scraped material was homogenized in 1.5 ml TMS buffer, in a tissue grinder (Kontes Glass Company) and the microsomes were sedimented by centrifugation at 105,000 *g* for 1 h. Protein concentrations were determined by the method of Lowry *et al.* /12/ using bovine serum albumin as the standard. The microsomal cytochrome P-450 content was determined according to Omura and Sato /13/.

BP and DMBA metabolism studies

The benzo(*a*)pyrene hydroxylase activity of skin microsomes was determined by the fluorometric method described by Nebert and Gelboin /14/. The incubation mixture contained 1 ml TMS buffer, 1 mg NADPH, 0.025 mg BP (dissolved in acetone) and 0.2 ml skin microsomes containing 2 mg protein. The mixture was incubated with gentle shaking at 37°C for 20 min and the reaction stopped by addition of 25% acetone in hexane. The subsequent determination of enzyme activity was carried out as described previously /14/. The metabolism of [^{3}H]BP and [^{3}H]DMBA by untreated or pretreated C57 BL/6 mouse liver microsomes was studied by the radiometric method of Van Cantfort *et al.* /15/ with 80 μM tritiated polycyclic aromatic hydrocarbons as a substrate. For the analysis of BP and DMBA

metabolites by high performance liquid chromatography, the PAHs and their metabolites were extracted twice with 2 ml ethyl acetate. After evaporation, residues were dissolved in methanol and injected in a Waters chromatograph equipped with a pre-column phase C_{18} and an ODS microsphere column. A linear gradient from 60 to 90% methanol separated BP metabolites within 30 min; after application of a methanol-water mixture (50/50, v/v) for 10 min, a linear gradient from 50 to 100% methanol separated DMBA metabolites within 50 min. 0.4 ml fractions were collected and radioactivity was determined by liquid scintillation counting (Aquasol 2 from New England Nuclear).

[^{3}H]BP covalent binding to DNA

The procedure described by Pelkonen *et al.* was used to measure covalent binding of [^{3}H]BP reactive metabolites to DNA /16/. 20 mg of deproteinized salmon sperm DNA were incubated in a 10 ml reaction mixture including 18 mg of skin microsomal protein, 25 μmol $MgCl_2$, 1 μmol EDTA, 7 μmol glucose 6-phosphate, 1.4 unit glucose 6-phosphate dehydrogenase, 1 mmol potassium phosphate buffer pH 7.5 and 60 nmol [^{3}H]BP (1.25 mCi) added in 250 μl acetone. The reaction mixture was incubated in a shaking water bath at 37°C for 30 min in subdued light. The reaction was stopped by placing the flask on ice and the microsomes were discarded after centrifugation of the mixture at 105,000 *g* for 60 min. The isolation, purification and hydrolysis of DNA as well as the chromatographic separation of metabolite-deoxyribonucleoside complexes on a Sephadex LH20 column were exactly as described by Lesca *et al.* /17/.

Binding to *Ah* receptor and 4 S protein

The binding experiments were carried out by incubating 1 ml of cytosol or separated *Ah* receptor or 4 S protein samples with 10 nM [^{3}H]DMBA, 10 nM [^{3}H]BP or 10 nM [^{3}H]TCDD for 1 h at 0-4°C. Three to five mg protein/ml were used in all experiments. The radioligands were added to samples in 10 μl of dimethyl sulfoxide; dimethyl sulfoxide was also used as the solvent for radioactive compounds in competition experiments. Adequate amounts (5 to 50 μl) of solvent were added to the control samples. After incubation, unbound and loosely bound radioligands were removed by adding

cytosol samples to a dextran-charcoal pellet (10 mg of charcoal/mg of dextran, pelleted from HEDG buffer). Dextran-charcoal was resuspended in the sample on a vortex mixer, incubated for 15 min at 0-4°C, and then removed by centrifugation at 4000 *g* for 15 min. Aliquots (300 µl) of samples were layered onto linear sucrose density gradients (5-20%) prepared in HEDG buffer. Gradients (4.8 ml) were centrifuged at 4°C for 2 h (cytosol samples) or 1 h 30 min (*Ah* receptor or 4 S protein samples) in a vertical tube rotor (Beckman VTi-65, g_{av}=372,000) at 63,000 rpm. After centrifugation, fractions (8 drops/fraction) were collected from each gradient with a Beckman recovery system. Radioactivity in each fraction was determined by liquid scintillation counting.

Tumorigenesis experiments

Male CF1 Swiss mice, 5 weeks old, weighing 20 g, were shaved with electric clippers 2 days before treatment and only those in the resting phase of the hair growth were used. Each experimental group contained 20 preshaved mice and all chemicals were applied to the back of the mice in 0.2 ml acetone. Animals pretreated with the inducer received from 0.001 to 1 µg of TCDD 24 h before the carcinogen DMBA. Untreated mice received 200 µl acetone. Then, all the mice were treated once with either 10 or 25 µg DMBA. One week after initiation by DMBA, mice received twice-weekly application of 10 µg of 12-O-tetradecanoylphorbol-13 acetate, and promotion was continued for 20 weeks. The incidence of papillomas was recorded and the tumor response was quantified as the average number of papillomas per mouse.

RESULTS

Binding affinity of DMBA for the *Ah* receptor and 4 S protein

The ability of DMBA to bind the *Ah* receptor and 4 S protein has been examined in various experimental conditions. When [^{3}H]DMBA (10 nM) was incubated with whole hepatic cytosols from S.D. rats (Figure 1A) and responsive C57 BL/6 mice (Figure 1B) or administered *in vivo* to the same strain of mouse (20 nmole = 1 mCi) (Figure 1C), it appears that this compound elicited a preferential affinity for 4 S protein and lipoproteins rather than for the *Ah*

receptor, which sedimented in the 9 S region of the sucrose gradient (fraction 15). The low level of radioactivity bound to the *Ah* receptor suggests that this compound displays an intrinsic low affinity for this protein (Figure 1B). In order to assess this particular feature in conditions excluding the competitive effects of other binding components, a comparative binding experiment was carried out by incubating 10 nM [^{3}H]TCDD, 10 nM [^{3}H]benzo(*a*)pyrene or 10 nM [^{3}H]DMBA with a separated *Ah* receptor sample obtained from C57 BL/6 mouse hepatic cytosol, by sucrose gradient sedimentation /10/. It can be seen in Figure 2 that [^{3}H]TCDD, as expected, is a stronger ligand than [^{3}H]BP, whereas [^{3}H]DMBA bound to the *Ah* receptor to a lower extent, representing 12.5% of the [^{3}H]TCDD binding. However, since this compound is able to interact somewhat with the *Ah* receptor, it cannot be excluded that high concentrations of this PAH would be able both to compete with stronger ligands *in vitro* and to induce AHH *in vivo*. It may be helpful to mention here that high doses of TCDD do induce cytochrome P-450 1A1 in non-responsive strains of mice in spite of its very weak affinity for their defective *Ah* receptors /18,19/. In order to assess this assumption, we examined the competitive effects of DMBA, as well as those of other compounds, to the *Ah* receptor of rat hepatic cytosol incubated with 10 nM [^{3}H]- TCDD. The IC_{50} values shown in Table 1 indicate that DMBA displays a competitive potency similar to that of ellipticine, a weak AHH inducer /20/. By comparison with other carcinogenic PAHs, the ability of DMBA to compete with [^{3}H]TCDD for binding to the *Ah* receptor appears as 26-fold and 4.3-fold weaker than those of 3-MC and BP respectively. In contrast, DMBA is a good ligand of 4 S protein, better than 3-MC and β-naphthoflavone but weaker than benzo(*a*)pyrene and ellipticine (Table 1).

Comparative inducing potencies of BP and DMBA for BP and DMBA hydroxylases

One of the most discussed questions in the field of cytochrome P-450 induction concerns the ability of each compound to induce a specific isoenzyme endowed with an optimal activity for its own metabolic transformation /21/. We studied such specific isoenzymes induced by BP and DMBA. For this purpose, responsive B6 mice were treated, intraperitoneally, with various doses (from 25 to 400 mg/kg body weight) of each of these PAHs, then the cytochrome P-450 content as well as the BP- and DMBA-hydroxylase activities of

Fig. 1: Velocity sedimentation analysis of Sprague-Dawley rat and C57 BL/6 mouse (B6) hepatic cytosols after *in vitro* or *in vivo* treatment with [^{3}H]DMBA. A and B, Phenobarbital-treated rat or TCPOBOP-treated B6 mouse hepatic cytosols (5 mg of protein in 1 ml) were treated with 10 nM [^{3}H]DMBA for 1 h at 4°C. C, TCPOBOP-treated B6 mice received 20 nmoles [^{3}H]DMBA then the animals were sacrificed 4 h later and the hepatic cytosol was prepared. Following dextran-coated charcoal treatment, the samples were examined on sucrose density gradient, as detailed in Materials and Methods

receptor, which sedimented in the 9 S region of the sucrose gradient (fraction 15). The low level of radioactivity bound to the *Ah* receptor suggests that this compound displays an intrinsic low affinity for this protein (Figure 1B). In order to assess this particular feature in conditions excluding the competitive effects of other binding components, a comparative binding experiment was carried out by incubating 10 nM [^{3}H]TCDD, 10 nM [^{3}H]benzo(*a*)pyrene or 10 nM [^{3}H]DMBA with a separated *Ah* receptor sample obtained from C57 BL/6 mouse hepatic cytosol, by sucrose gradient sedimentation /10/. It can be seen in Figure 2 that [^{3}H]TCDD, as expected, is a stronger ligand than [^{3}H]BP, whereas [^{3}H]DMBA bound to the *Ah* receptor to a lower extent, representing 12.5% of the [^{3}H]TCDD binding. However, since this compound is able to interact somewhat with the *Ah* receptor, it cannot be excluded that high concentrations of this PAH would be able both to compete with stronger ligands *in vitro* and to induce AHH *in vivo*. It may be helpful to mention here that high doses of TCDD do induce cytochrome P-450 1A1 in non-responsive strains of mice in spite of its very weak affinity for their defective *Ah* receptors /18,19/. In order to assess this assumption, we examined the competitive effects of DMBA, as well as those of other compounds, to the *Ah* receptor of rat hepatic cytosol incubated with 10 nM [^{3}H]-TCDD. The IC_{50} values shown in Table 1 indicate that DMBA displays a competitive potency similar to that of ellipticine, a weak AHH inducer /20/. By comparison with other carcinogenic PAHs, the ability of DMBA to compete with [^{3}H]TCDD for binding to the *Ah* receptor appears as 26-fold and 4.3-fold weaker than those of 3-MC and BP respectively. In contrast, DMBA is a good ligand of 4 S protein, better than 3-MC and β-naphthoflavone but weaker than benzo(*a*)pyrene and ellipticine (Table 1).

Comparative inducing potencies of BP and DMBA for BP and DMBA hydroxylases

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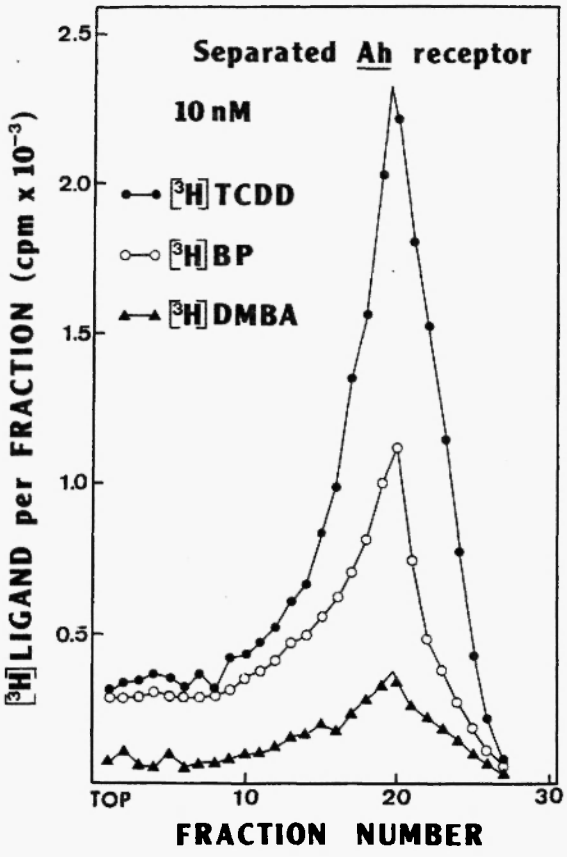

Fig. 2: Comparative binding of [^{3}H]TCDD, [^{3}H]BP and [^{3}H]DMBA to separated C57 BL/6 mouse *Ah* receptor. One ml-samples of separated *Ah* receptor (3 mg protein) were incubated for 1 h at 4°C with either 10 nM [^{3}H]TCDD, 10 nM [^{3}H]BP or 10 nM [^{3}H]DMBA. Following dextran-coated charcoal treatment, the samples were examined on sucrose density gradients as detailed in Materials and Methods.

the hepatic microsomes were measured. From the set of data shown in Tables 2 and 3, it is obvious that BP-induced cytochrome P-450 displays a higher BP-hydroxylase specific activity than the P-450 form induced by DMBA. Conversely, the DMBA-induced cytochrome P-450 metabolizes DMBA with a better specific activity than that displayed by the BP-induced cytochrome P-450. For an

TABLE 1

Competitive potency for *Ah* receptor or 4S protein of DMBA and various other chemicals[a]

Competitor	Competitive potency (IC_{50}) [b]	
	Ah receptor	4S protein
	nM	nM
DMBA	260	620
3-MC	10	1000
BP	60	100
TCDF	14	-
β-NF	-	3000
Ellipticine	270	80

[a]$[^3H]$TCDD (10 nM) or $[^3H]$BP (10 nM) and competing ligands (from 1 nM to 100 μM) were incubated with either separated *Ah* receptor or separated 4S protein from phenobarbital-treated rat liver as described in Materials and Methods. The ability of DMBA as well as other chemicals to compete for either *Ah* receptor or 4S protein was assessed by determining the effective concentrations of competitor necessary to reduce specific binding of $[^3H]$TCDD or $[^3H]$benzo(*a*)pyrene respectively to 50% of the maximal value obtained in the absence of competitor in the 9S or in the 4S region of sucrose density gradients.

[b]The IC_{50} value (concentration giving 50% inhibition of $[^3H]$TCDD or $[^3H]$BP binding to *Ah* receptor or 4S protein respectively) for each competitor was determined by interpolation from the dose-response curves and was the mean of at least two individual experiments (<10% variations).

All values are the average of three independent experiments (≤ 10% variations).

equal dose of inducer administered to mice (200 mg/kg body weight) the BP-hydroxylase specific activity was increased 1.91-fold by BP and only 1.47-fold by DMBA. In the same way, the DMBA-hydroxylase activity was increased 1.72-fold by DMBA whereas it remained practically unchanged after BP treatment.

TABLE 2

Comparative inducing potencies of BP and DMBA for BP-hydroxylase

Dose of inducer	BP - treated mice (a)						DMBA - treated mice (a)					
	Cyt-P-450		BP-hydroxylase activity (b)				Cyt-P-450		BP-hydroxylase activity (b)			
mg x kg^{-1}	nmol x mg prot^{-1}		nmol x min^{-1} x mg prot^{-1}		nmol x min^{-1} x nmol P450^{-1} (Specific activity)		nmol x mg prot^{-1}		nmol x min^{-1} x mg prot^{-1}		nmol x min^{-1} x nmol P450^{-1} (Specific activity)	
		%		%		%		%		%		%
Control	0.694	(100)	0.236	(100)	0.340	(100)	0.694	(100)	0.236	(100)	0.340	(100)
25	0.610	(88)	0.382	(162)	0.626	(184)	-		-		-	
50	1.038	(150)	0.573	(243)	0.552	(162)	0.585	(84)	0.217	(92)	0.370	(109)
100	1.110	(160)	0.694	(294)	0.625	(184)	-		-			
200	1.436	(207)	0.934	(396)	0.650	(191)	0.909	(131)	0.455	(193)	0.500	(147)
400	-		-				1.160	(167)	0.587	(249)	0.506	(149)

(a)The liver microsomes as enzyme source were obtained from groups of three C57BL/6 mice treated intraperitoneally with DMBA or BP dissolved in sunflower oil. Control mice received only the solvent (0.1 ml per 10 g body weight).
(b)The microsomal BP-hydroxylase activity was determined according to the method of Van Cantfort /15/ with 0.4 mg protein/ml. All values are the average of three independent experiments (≤ 10% variation).

TABLE 3

Comparative inducing potencies of DMBA and BP on DMBA-hydroxylase

Dose of inducer	DMBA - treated mice (a)					BP - treated mice (a)				
	Cyt-P-450		DMBA-hydroxylase activity (b)			Cyt-P-450		DMBA-hydroxyase activity (b)		
mg x kg^{-1}	nmol x mg prot^{-1}	%	rmol x min^{-1} x mg prot^{-1}	%	nmol x min^{-1} x nmol P450^{-1} (Specific activity)	nmol x mg prot^{-1}	%	rmol x min^{-1} x mg prot^{-1}	%	nmol x min^{-1} x nmol P450^{-1} (Specific activity)
Control	0.694	(100)	0.585	(100)	0.842	(100)	0.694	(100)	0.585	(100)	0.842	(100)
50	0.585	(84)	0.614	(105)	1.094	(124)	1.038	(150)	0.725	(124)	0.698	(83)
100	0.729	(105)	1.006	(172)	1.379	(164)	1.110	(160)	1.175	(201)	1.058	(125)
200	0.909	(131)	1.316	(225)	1.447	(172)	1.436	(207)	1.199	(205)	0.834	(99)
400	1.160	(167)	1.684	(288)	1.451	(172)	-		-		-	

(a)The liver microsomes as enzyme source were obtained from groups of three C57 BL/6 mice treated intraperitoneally with DMBA or BP dissolved in sunflower oil. Control mice received only the solvent (0.1 ml per 10 g body weight).
(b)The microsomal DMBA-hydroxylase activity was determined according to the method of Van Cantfort /15/ with 0.4 mg protein/ml. All values are the average of three independent experiments (≤ 10% variation).

Enhancing effects of TCDD on skin microsomal AHH activity and on BPDE-binding to DNA

The influence of the PAH activation rate on the induction of cancer *in vivo* has been studied in CF1-Swiss mice. This strain of mice exhibits a weak PAH metabolizing capacity, which allows the precise determination of the increase in activity of the induced enzymes. For this purpose, we applied different doses of TCDD to the shaved back of three CF1 Swiss mice (0.001 or 0.01 or 0.1 or 1 μg/mouse), control animals receiving only the solvent. Table 4 shows that a dose-dependent relationship exists between the increasing dose of TCDD applied to mouse skin and the induction of AHH activity of skin microsomes as well as the *in vitro* binding of the ultimate

TABLE 4

Modulation by TCDD of skin microsomal AHH activity and DNA-binding of BP-7,8-diol-9,10-epoxide, (BPDE), *in vitro*

Treatment [a] μg/mouse	AHH activity pmol 3-OHBP/min/mg prot	Ratio to untreated	BPDE-binding [b] pmol/mg DNA
no TCDD	0.88	1	0.9
TCDD 0.001	9.30	10.6	5.5
TCDD 0.01	20.00	22.8	6.8
TCDD 0.1	53.00	60.0	17.0
TCDD 1	54.00	61.0	20.0
DMBA 1.000	1.12	1.3	-

[a]Skin microsomes as enzyme source were obtained from groups of three CF_1 Swiss mice, the shaved backs of which were treated, for 24 hours, with 0.2 ml acetone (no TCDD) or with 0.2 ml acetone solution containing either TCDD or DMBA at the doses indicated.
[b]BPDE-binding to DNA was measured according to the method of Pelkonen *et al.* /16/. The details of the enzyme assay and BPDE-binding to DNA are described in Materials and Methods.

carcinogenic metabolite BP-7,8-diol-9,10-epoxide (BPDE) to DNA. It is noteworthy that 1 mg of DMBA applied to the skin of CF1 mice induces AHH activity about 10-fold less than 0.001 μg TCDD, this last compound appearing to be 10 million times as potent as DMBA for skin AHH induction.

Modulating effects of TCDD on skin carcinogenesis by DMBA

After 24 h induction by different doses of TCDD, as described above, DMBA (10 or 25 μg) was applied once to the skin of 20 untreated or TCDD-treated CF1 mice. One week later, promotion was carried out by a twice weekly treatment of 12-O-tetradecanoyl-phorbol-13-acetate (10 μg) for 20 weeks. As shown in Figure 3, the

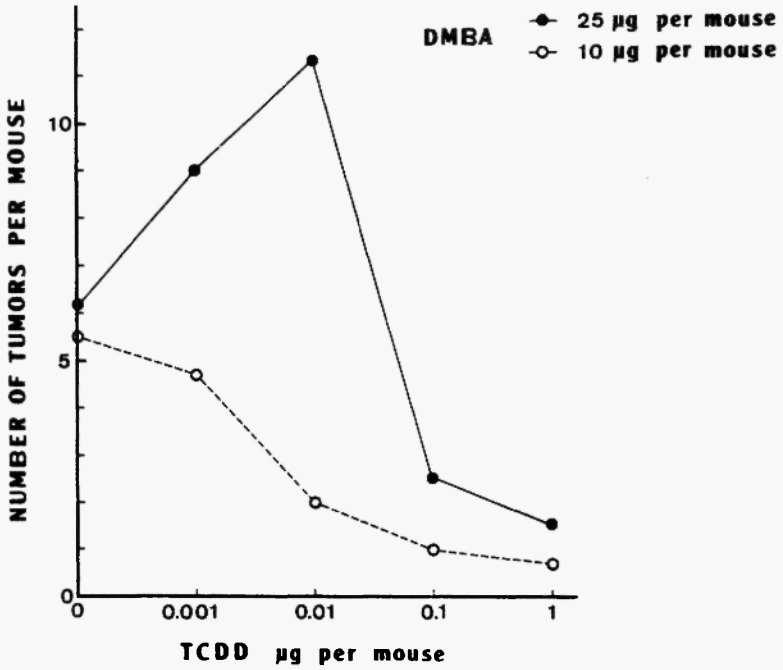

Fig. 3: Modulating effects of TCDD on skin carcinogenesis by DMBA. Twenty male CF1 Swiss mice were used per experimental group. The shaved backs of mice were either not pretreated or pretreated with TCDD (from 0.001 to 1 μg per mouse) for 24 h then the animals were initiated with either 10 μg (O) or 25 μg (●) DMBA per mouse. One week after initiation, mice received 10 μg TPA twice weekly (promotion).

average number of papillomas per mouse was dependent on 1) the dose of DMBA applied to mice and 2) the metabolic capacity of the skin. The increased metabolic activity due to the induction of enzymes by TCDD led to a decrease of tumorigenicity of DMBA when the weaker dose (10 μg) of this carcinogen was applied. In contrast, for a higher dose of DMBA (25 μg), a dual effect of TCDD was observed: first, a cocarcinogenic effect of the lower doses of TCDD corresponding to a moderate induction of metabolism, then an anticarcinogenic effect of the highest doses of the inducer. So, it appears that when the mice were treated with 0.01 μg TCDD, an optimal rate of DMBA activation was reached which corresponded to the highest number of tumors.

DISCUSSION

The goal of this work was to assess the influence of the induction of metabolizing enzymes on the initiation of skin tumorigenesis by DMBA.

Our results demonstrate that DMBA is a poor ligand of the *Ah* receptor and a very weak inducer (ten million-fold weaker than TCDD) of metabolizing enzymes.

The contrasting effects of AHH induction by TCDD on DMBA skin tumorigenesis underlines the decisive importance of two factors: 1) the effective dose of the ultimate carcinogen which is in contact with the critical macromolecules of cells in a particular sensitive step of their cycle, and 2) the persistence in the tissue of a high steady state level of the carcinogen. However, in the case of the weaker dose of DMBA (10μg per mouse), the induction of metabolism by TCDD exerted an anti-carcinogenic effect, by the rapid elimination of the carcinogen, in spite of the increase in ultimate carcinogen level. The fact that, in these conditions, no increase of tumorigenesis was observed seems to indicate that the second factor is of prime importance. Indeed, with a higher dose of DMBA, the carcinogen can remain for a longer time in contact with the sensitive cells and produce a higher concentration of ultimate carcinogen. Increased further by a moderate induction, this higher and higher concentration of ultimate carcinogen leads to a lot of damage and to a great number of tumors. When the metabolism is still more strongly increased by the highest doses of the inducer (above 0.01 μg TCDD per mouse),

the detoxifying effect of carcinogen elimination again prevails. It cannot be excluded that the various DNA repair mechanisms also have some important influence on the final effect. Indeed, for moderate damage, the fidelity and the efficiency of the DNA-excision repair system leads to a decrease of mutations whereas with a higher percentage of DNA damage, the adaptive SOS-repair system becomes error-prone and mutagenic /22/.

Our observations are in good agreement with previous investigations. For example, DiGiovanni *et al.* /23/ reported that the modifier TCDD inhibited DMBA initiation in Sencar mice with a ED_{50} (dose of TCDD producing half-maximal inhibition) equal to 1.8 x 10^{-3} μg/mouse when 2.5 μg DMBA per mouse was applied to the skin. According to the values shown in Figure 3, the ED_{50} for our experiment is 7 x 10^{-3} μg/mouse for 10 μg DMBA per mouse. In another report from the same group /24/, the application of a high dose of TCDD (1 μg per mouse) 3 days before that of various carcinogens (DMBA, BP or 3-MC), led, in all cases, to anticarcinogenic effects either for 2.5 or for 25 μg PAH per mouse. But there was no effect when TCDD was administered 5 min before or 1 day after the application of DMBA. On the other hand, we believe that the dual and contrasting effects of TCDD observed by Kouri *et al.* /25/ with C57 BL/6 and DBA/2 mice support the hypothesis of a close relationship between the PAH-metabolism rate and the initiation of tumorigenesis. When the PAH-poor metabolizer (DBA/2 mouse) was pretreated subcutaneously with TCDD (100 μg/kg), the 3-MC carcinogenic index was increased 13-fold. In contrast, the same treatment applied to C57 BL/6 mice (PAH-high metabolizer) did not change the carcinogenic index. We have also observed such apparently conflicting results by using the AHH inducer β-naphthoflavone which diminished the DMBA tumorigenesis in NMRI Swiss mice (PAH-high metabolizers) and strongly increased the number of tumors in CF1 Swiss mice (PAH-poor metabolizers) /8/. The rate of PAH metabolism directly influences the steady state level of the carcinogen in the skin and the length of time within which the reactive metabolites act in the cells, as recently demonstrated by several authors /26-28/. For example, Iversen /28/ has shown that a single dose of 51.2 μg DMBA gave 45 tumors per 32 mice, when the same dose divided into 50 applications gave 250 tumors per 32 animals. Kaufmann *et al.* /29/ studied the pattern of cell cycle-dependent variation in hepatocyte sensitivity to

initiation of carcinogenesis by the BP ultimate carcinogen (±) 7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (BPDE) and they concluded that the cells in transit from the G_1 to the S phase displayed the greatest incidence and yield of hepatocellular neoplasms. Taken together, it seems to be true that only a short critical time exists during the cell cycle for the deleterious effects of the ultimate carcinogen. So, one can consider that, at a given time, only a small percentage of cells are sensitive and can be damaged and the final number of transformed cells will be dependent on the duration of the exposure to the ultimate carcinogen.

The results shown in this work together with other data /17,23-25,30/ indicate that non-carcinogenic compounds can strongly modify the metabolism and the mutagenicity of precarcinogens by their enzyme inducing capacity exerting either co-carcinogenic or anti-carcinogenic effects.

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