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Inhibition of apoptosis in rat hepatocytes treated with 'non-dioxin-like' polychlorinated biphenyls

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Polychlorinated biphenyls (PCBs) are among the most prominent persistent environmental pollutants exhibiting neurotoxic, teratogenic and tumour-promoting effects in experimental animals. 'Dioxin-like' properties have been assigned to a number of PCBs whereas other PCBs have been classified as 'non-dioxin-like'. Many of the latter congeners are inducers of cytochrome P450 (CYP) 2B1 and 2B2 similar to the liver tumour promoter phenobarbital. In contrast, 'dioxin-like' PCBs induce CYP1A isozymes, and other congeners have been classified as 'mixed-type' inducers. Inhibition of apoptosis of pre-neoplastic hepatocytes is thought to play a central role in tumour promotion in rat liver. We have used the inhibition of UV-induced apoptosis in rat hepatocytes in primary culture as an *in vitro* model for mechanistic studies on the inhibition of apoptosis. It could be shown that phenobarbital, and the 'non-dioxin-like' PCBs 28, 101 and 187 completely inhibit UV-induced apoptosis. The concentration–response curves and EC_{50} values for this effect, however, were different from those of induction of CYP2B1/2B2-catalysed 7-pentoxyresorufine *O*-dealkylase or CYP1A-catalysed 7-ethoxyresorufine *O*-deethylase activities. The PCBs and phenobarbital did not affect the spontaneous incidence of apoptotic nuclei. In conclusion, 'non-dioxin-like' PCBs are likely to promote liver carcinogenesis *via* the suppression of apoptosis. The signaling events in rat hepatocytes leading to induction of 2B1/2B2 activity by the compounds investigated are assumed to differ from those leading to inhibition of apoptosis.

Introduction

Polychlorinated biphenyls (PCBs) are among the most extensively investigated persistent environmental pollutants. Major sources of PCB emissions have been the use of technical PCB mixtures as hydraulic oils, flame retardants and lubricants in a number of technical processes (1). Furthermore, PCBs are formed during the incineration of organic materials in the presence of chlorine-containing compounds (2). Acute PCB intoxications as a result of the accidental ingestion of contaminated rice oil resulted in severe bone pain, chlorakne and malformations in newborn after exposure of pregnant women (3). Chronic exposure to increased levels of PCBs, e.g. in fish were suggested to result in delayed learning in children of women exposed during pregnancy (4).

In experimental models, PCBs lead to neurotoxicity, endocrine disturbances and tumour promotion in rodent liver (1,5,6). In a study of workers exposed to PCBs a significant increase in liver cancer was observed (7).

For practical classification, PCBs were subdivided into 'dioxin-like' and 'non-dioxin-like' congeners (6). This principle is based on the fact that a number of PCBs exert biological effects similar to those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic dioxin congener. In particular, 'dioxin-like' PCBs bind to the dioxin or aryl hydrocarbon receptor (AhR) and lead to characteristic effects on the expression of AhR-regulated genes including cytochrome P450 (CYP)1A1 (6). In contrast, a number of 'non-dioxin-like' PCBs are inactive or almost inactive as AhR-agonists but induce a battery of drug-metabolizing enzymes including CYP2B1/2B2 known as phenobarbital-inducible genes (6,8). Therefore, these PCBs are sometimes categorized as 'phenobarbital-like' inducers. It has to be kept in mind, however, that these compounds do not share many other major pharmacological or toxicological properties with the hypnotic drug phenobarbital. In addition, inducing properties concerning drug-metabolizing enzymes have not been investigated for a number of other PCBs. A variety of PCBs induce both CYP 2B1/2B2 and 1A isozymes in rat liver, and, therefore, have been categorized as 'mixed-type' inducers (8).

A common feature of TCDD, phenobarbital and a number of PCBs is their tumour-promoting potency in rat liver, when the animals have been treated previously with a genotoxic (initiating) carcinogen (5,9–11). It is assumed that the subsequent treatment with a tumour-promoting agent facilitates the clonal expansion of cells bearing a critical damage in their genome. This clonal expansion raises the risk of malignant transformation of genetically altered cells eventually resulting in the development of malignant tumours. A number of mechanisms have been proposed to explain the supportive effect of tumour promoters on the growth of preneoplastic cells (12). According to a current hypothesis, the inhibition of apoptosis, intrinsically enhanced in pre-neoplastic clones, may play a central role in the mechanism of tumour promotion (13). In fact, inhibition of apoptosis in pre-neoplastic enzyme-altered foci has been demonstrated for the liver tumour promoters phenobarbital, TCDD, and others (13–15). In the case of tumour-promoting PCBs, no data on the effect on apoptosis in pre-neoplastic rat liver are available. In rat hepatocytes in primary culture and in rat hepatoma cell lines, anti-apoptotic effects were described for phenobarbital, tumour-promoting peroxisome proliferators and TCDD (16–19).

This study was designed to investigate a possible relationship between 'phenobarbital-type' induction of CYP isozymes/activities, and the effects on UV-initiated apoptosis in rat hepatocytes after treatment with phenobarbital and certain 'non-dioxin-like' PCBs.

Abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; EROD, 7-ethoxyresorufin *O*-deethylase; DMEM, Dulbecco's modified Eagle's medium; PCBs, polychlorinated biphenyls; PROD, 7-pentoxyresorufin *O*-dealkylase; TCDD, 2,3,7,8-tetrachlordibenzo-*p*-dioxin; TEFs, toxicity equivalency factors.

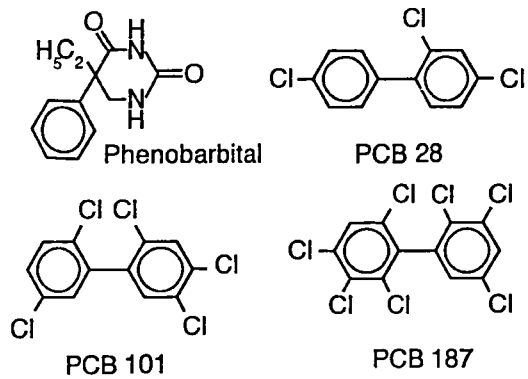

Fig. 1. Chemical structures of phenobarbital and selected 'non-dioxin-like' PCBs.

Materials and methods

Chemicals

Bovine serum albumin, collagenase type IV and phenobarbital were obtained from Sigma (Taufkirchen, Germany). Dulbecco's modified Eagles's medium (DMEM) from Seromed (Berlin, Germany), and Waymouth's medium MD 705/1 and fetal calf serum from Gibco-BRL (Heidelberg, Germany), ITS and ITS$^+$ from Becton Dickinson (Heidelberg, Germany), and the PCBs (Figure 1) IUPAC number 28 (2,4,4'-trichlorobiphenyl), 101 (2,2',4,5,5'-pentachlorobiphenyl) and 187 (2,2',3,4',5,5',6-heptachlorobiphenyl) from Promochem (Wesel, Germany). All other chemicals were purchased at the highest purity commercially available.

Hepatocytes and cell culture

Male Wistar rats were obtained from Charles River (Kisslegg, Germany) and were kept under standard conditions. Adult animals at a body weight of 150–180 g were anesthetized, and hepatocytes were isolated as described (20) using a modification of the sequential perfusion technique originally described by Seglen (21). The cells were cultured using the collagen sandwich procedure (22). For the preparation of collagen, collagen-rich fibres were isolated from eight rat tails, and were dissolved in 800 ml 3% acetic acid at 4°C. Unsoluble material was removed by centrifugation at 2300 *g* and 4°C over 90 min. Then, 1/5 volume 30% NaCl solution was added to the supernatant, and the precipitated collagen was collected by centrifugation at 2300 *g* and 4°C over 30 min. The pellet was resuspended in 175 ml 5% NaCl solution, and was centrifuged at 2300 *g* and 4°C over 30 min. The pellet was dissolved in 25 ml 0.6% acetic acid, and was filled up to a total volume of 400 ml by adding 0.6% acetic acid. After dialysis against 1 mM HCl over 48 h, the collagen solution was lyophilized. Prior to use, 1.5 mg collagen were dissolved in 1 ml 1 mM HCl to obtain a stock solution. This solution was diluted with nine volumes of 10× DMEM, the culture dishes were coated with 700 µl of the dilution, and were then kept in a tissue culture incubator for 1 h at 37°C. After hardening of the gel the cells were seeded and incubated as described (17). The cells were covered with 500 µl collagen solution in 10× DMEM 1 h before treatment.

Induction of cytochromes P450

Hepatocytes were seeded at a density of 100 000/cm^2 on collagen-coated 60 mm Petri dishes and were incubated as described (17). After 3 h the cells were covered with 700 µl of collagen dilution in 10× DMEM. Twelve hours after seeding, PCBs were added dissolved in DMSO, phenobarbital dissolved in sterile saline (0.9% NaCl). The added volume of DMSO did not exceed 0.5% of the total volume per dish. Controls were treated with DMSO or saline only. The cultures were washed, harvested and homogenized 48 h after addition of the inducers, and 7-ethoxyresorufine *O*-deethylase (EROD) and 7-pentoxyresorufine *O*-dealkylase (PROD) activities were analysed using the method of Burke and Mayer (23).

Inhibition of apoptosis

Hepatocytes were seeded at a density of 60 000/cm^2 on Quadriperm dishes (Heraeus, Frankfurt, Germany) of an area of 20 mm^2. After 12 h medium was replaced by fresh medium, and after 15 h the cells were treated with UV light as described (17). Treatment with phenobarbital or PCBs was performed 30 min after irradiation.For the counting of apoptotic nuclei, the cells were fixed, washed and air-dried 12 h after tretament with the tumour promoters as described (20), and stained with an aqueous 8 µM solution of 4',6-diamidino-2-phenylindole (DAPI) and 10 µM sulphorhodamine 101. The microscopic analysis of the encoded slides was carried out using a Zeiss (Jena, Germany) fluorescence microscope (Axioskop) equipped with a BP 450–490 excitation filter and a LP 520 emission filter. The slides were stored at 4°C protected from light. All experiments were carried out in double, and 3×1000 nuclei were examined on each slide. Condensed, half moon-shaped, and scattered nuclei were summarized as apoptotic nuclei as described earlier (17).

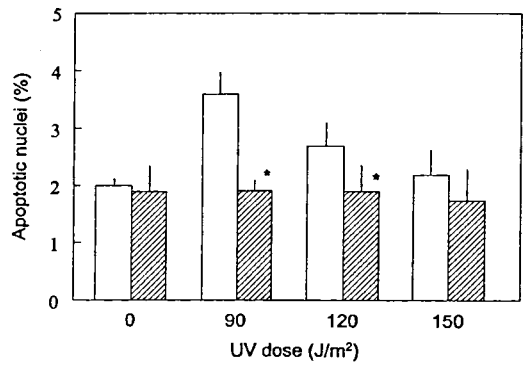

Fig. 2. Inhibition of apoptosis in rat hepatocytes in primary culture. Cultures were treated with saline (open bars) or 2 mM phenobarbital (hatched bars) 30 min after UV irradiation. Twelve hours after irradiation the number of apoptotic nuclei was determined. The bars and error bars show means and standard deviations form three independent experiments. *Significantly different from the corresponding saline-treated cultures ($P \leq 0.05$).

Statistical analysis

Means and standard deviations were calculated from independent experiments. For (multiple) comparisons of means of treated cultures with untreated controls Dunnett's test for independent samples was used.

Results

In rat hepatocytes cultured between two layers of collagen ('sandwich culture') UV pulse-irradiation at a specific intensity of 90 J/m^2 almost doubled the number of apoptotic nuclei after 12 h (Figure 2). After 6 or 18 h the numbers of apoptotic nuclei were significantly lower (data not shown). Therefore, the number of apoptoic nuclei was determined 12 h after UV treatment in all subsequent experiments. With a dose of 120 J/m^2 less apoptotic nuclei were detected, while no significant increase in apoptosis was observed 12 h after treatment with 150 J/m^2. The latter dose resulted in massive acute cell death (not shown) which probably prevented the onset of the apoptotic pathways. The liver tumour promoter phenobarbital which was reported previously to suppress UV-induced apoptosis in rat hepatocytes (17) was used as a reference compound. Addition of 2 mM phenobarbital 30 min after UV irradiation completely suppressed the increase in apoptosis (Figure 2). For further experiments with the PCBs 28, 101 and 187 standard conditions were used (single UV irradiation with 90 J/m^2, determination of apoptotic nuclei after 12 h). It was found that phenobarbital and the three PCBs tested inhibited the increase in apoptosis in a concentration-dependent manner. This effect reached a level of at least 90% (at least 90 % inhibition of additional, UV-induced apoptosis) with phenobarbital at 10^{-7} M, PCB 28 at 10^{-9} M, PCB 101 at 10^{-7} M and PCB 187 at 10^{-6} M (Figure 3A–D), i.e. the range

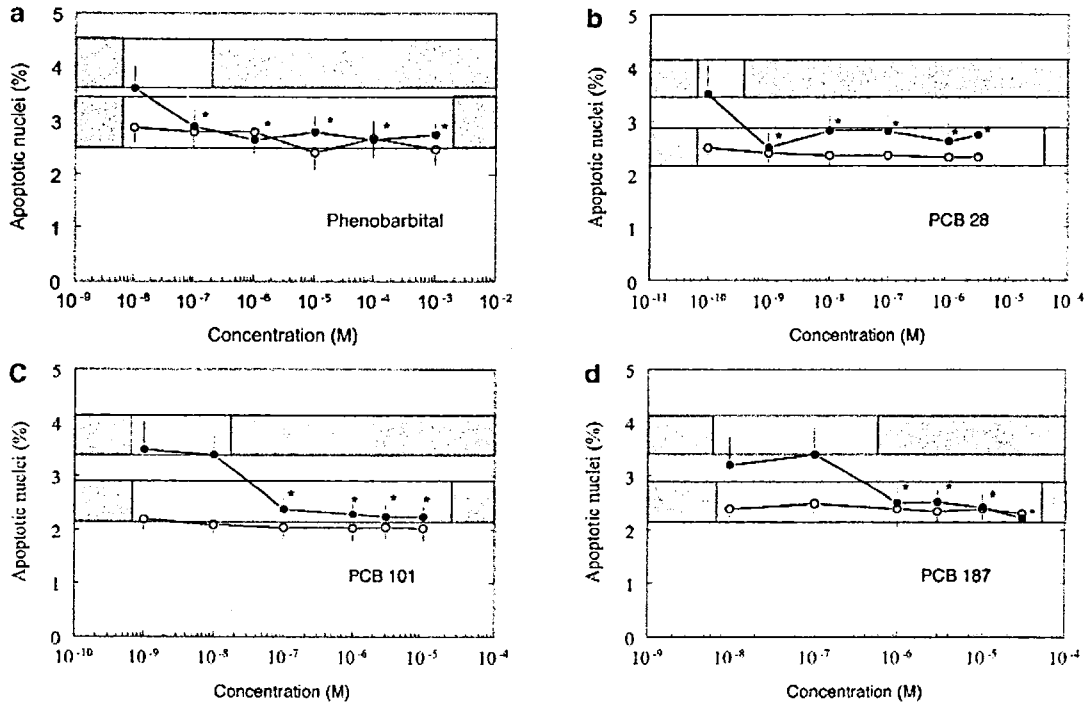

Fig. 3. Effects of phenobarbital, PCB 28, 101 or 187 on apoptosis in rat hepatocytes in primary culture 12 h after irradiation with UV-light (90 J/m^2). The compounds were added 30 min after irradiation. Symbols and error bars represent means and standard deviations from three independent experiments. *Significantly different from results obtained with corresponding DMSO-treated cultures, represented by two bands ($P \leq 0.05$). The upper bands show the standard deviation range of apoptosis in UV-treated, the lower band in DMSO-treated cultures.

of 'spontaneous' apoptosis found without UV irradiation was achieved.

All PCBs tested as well as phenobarbital also led to a concentration-dependent induction of CYP 2B1/2B2-catalysed PROD activity in cell homogenates (Figure 4A–D). Phenobarbital and the PCBs 101 and 187 exhibited a maximum efficacy in the range of 19–30 pmol/min×mg protein whereas treatment with PCB 28 resulted in a maximum level between 10 and 15 pmol/min×mg protein. With phenobarbital a complete induction curve was obtained for the CYP1A-catalysed EROD activity, whereas the PCBs were inactive as EROD inducers. Fitting of a sigmoidal dose–response function (not shown) to the experimental data using a log-probit procedure allowed the calculation of EC_{50} values and 95% confidence intervals (Table I). With respect to PROD induction, phenobarbital was ~10-fold less potent than PCB 28. The inducing potencies of the PCBs as inducers of PROD followed the rank order PCB 187 > PCB 28 > PCB 101.

Discussion

The tumour-promoting potency of PCBs and related environmental pollutants represents an important parameter in risk assessment of this group of chemicals. A variety of PCBs are members of the large and diverse group of tumour promoters which support the clonal expansion of pre-neoplastic cell clones in rat liver thus enhancing the risk of malignant transformation (12). The molecular mechanism(s) underlying tumour promotion are poorly understood. A number of hypotheses exist, however, including the notion that inhibition of apoptosis intrinsically enhanced in pre-neoplastic hepatocyte clones may play an important role (13).

The situation is complicated by the fact that PCBs can be divided into compounds with a 'dioxin-like' pattern of biochemical and toxic effects, and those with a 'non-dioxin-like' mode of action. In many cases, however, no clear borderline can be drawn between both groups. The classification of non-*ortho*-substituted PCB congeners as 'dioxin-like' is mainly based on their agonistic potency as ligands of the Ah or dioxin receptor (AhR) leading to characteristic molecular and cellular events (1,6) including the induction of CYP1A isozymes. Those 'dioxin-like' PCBs investigated so far act as tumour promoters in rat liver (5,8,9) thus resembling the most potent agonist within the family of 'dioxins', 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 10,24).

Among the 'non-dioxinlike' PCBs there are also a number of congeners acting as tumour promoters in rat liver (1,5,8,9). With another promoting agent, phenobarbital, they have in common the induction of a battery of certain drug-metabolizing enzymes including CYP 2B1/2B2 (6,8). The signalling pathway leading to 'phenobarbital-type' induction of CYP2B genes involves the constitutively active receptor (CAR) which acts as a transactivator of a distal enhancer in responsive genes called the phenobarbital-responsive enhancer module (25).

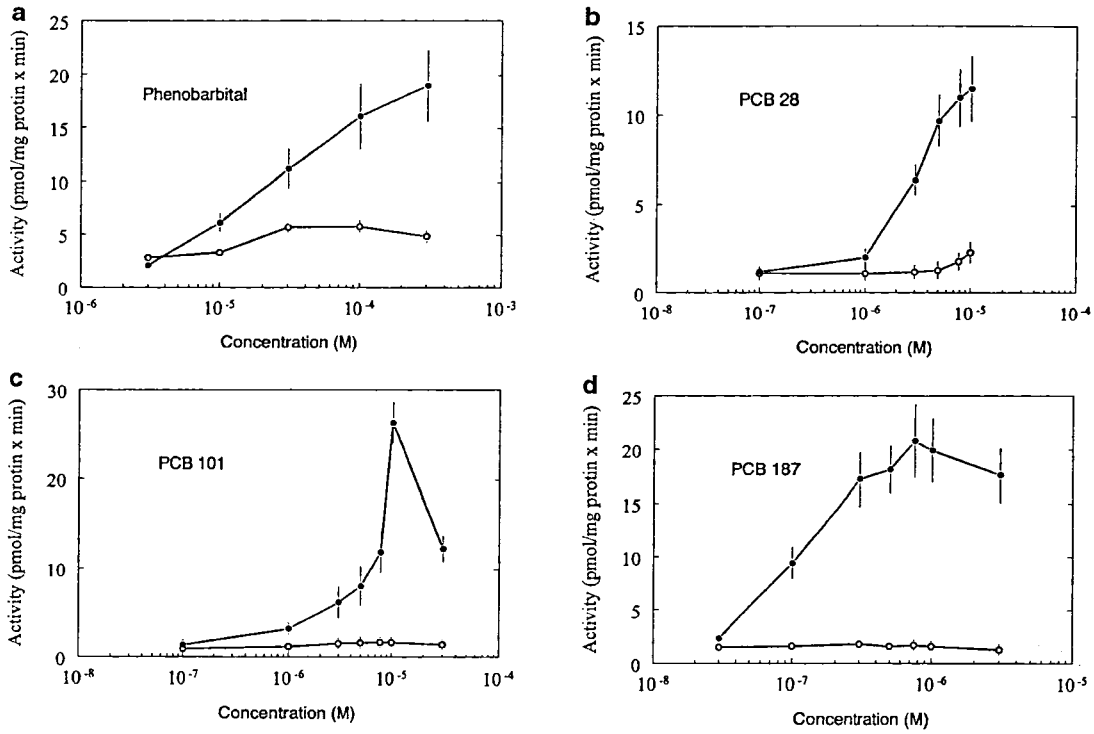

Fig. 4. Effects of phenobarbital, PCB 28, 101 or 187 on EROD activity (open circles) and PROD activity (closed circles) in rat hepatocytes after treatment with various concentrations. Forty-eight hours after addition of inducers, cells were harvested, homogenized and enzyme avctivities were determined as described under Materials and methods. Data represent means ± SD from three independent series of experiments (three different animals).

Table I. EC_{50} values for induction of PROD- and EROD-activities in homogenates from rat heptocytes in primary culture after treatment with phenobarbital, PCB 28, 101, 138 or 187

Inducer	PROD induction EC_{50} ± SD (M)	EROD induction EC_{50} ± SD (M)	Inhibition (90%) of UV-induced apoptosis (M)
Phenobarbital	$2.4\times10^{-5} \pm 0.17\times10^{-5}$	$1.8\times10^{-5} \pm 1.2\times10^{-5}$	10^{-7}
PCB 28	$3.3\times10^{-6} \pm 0.34\times10^{-6}$	–	10^{-9}
PCB 101	$5.7\times10^{-6} \pm 0.97\times10^{-6}$	–	10^{-7}
PCB 187	$1.2\times10^{-7} \pm 0.2\times10^{-7}$	–	10^{-6}

It was the major aim of the present study to investigate a possible relationship between induction of CYP1A or 2B1/2B2 activities and the inhibition of apoptosis as a possible *in vitro* surrogate for the tumour-promoting action of that class of compounds.

It was found, that UV light-induced apoptosis in rat hepatocytes was completely suppressed with phenobarbital and the three 'non-dioxinlike' PCBs 28, 101 and 187. A similar result was obtained in a previous study in rat hepatocytes using the liver tumour promoter TCDD (17). With TCDD and the PCBs used in this study, 'spontaneous' apoptosis could not be inhibited, however, arguing for two distinct types of apoptosis. One type is elicited by UV-irradiation probably initiated by DNA damage or another type of UV-induced cellular stress. Previous studies showed, that irradiation of hepatocytes resulted in induction of p53 as a characteristic response to this type of damage (26). Interestingly, both TCDD and phenobarbital could almost completely suppress the UV-induced rise in p53. The other type of apoptosis, occuring 'spontaneously' in culture is probably more abundant *in vitro* than in the liver. This may be due to various types of cellular stress during the procedures of isolation, plating, and culture of the cells.

Analysis of PROD activity as a functional parameter for CYP2B1/2B2 indicates that all PCBs tested are relatively potent inducers of this enzyme(s). In contrast, AhR-regulated

CYP1A activity (EROD) was almost unchanged with the exception of phenobarbital.

From *in vivo* experiments in rats (8) the following rank order of potency for PROD induction in the liver, probably modified by toxicokinetic influences, was reported: PCB 101 ≈ PCB187 > PCB28 which is slightly different from our *in vitro* findings where PCB 187 is found to be about 2-fold more potent than PCB 101. Comparison of EC_{50} values of PROD induction with 90% values of inhibition of apoptosis revealed no quantitative relationship. This result is of interest in several respects. First, it suggests that measurement of CYP 2B1/2B2 induction as a parameter for an anti-apoptotic potency of PCBs may be misleading for quantitative comparisons. Second, it can be speculated that the signalling events leading to induction of CYP2B1/2B2 are not directly related to those operative in inhibition of apoptosis.

Systematic investigations on possible links between the induction of CYP isozymes and liver tumour promotion are rare. With the AhR-agonist TCDD tumour promotion was observed in the livers of female but not of male Sprague–Dawley rats in spite of the fact that efficient induction of CYP1A isozymes/activities was evident in both sexes (27). On the other hand, there are indications of a relationship between the relative tumour promoting and CYP1A-inducing potencies for the class of 2,3,7,8-substituted PCDDs in the liver of female rats (2,10). Thus, it appears probable that AhR agonists may act as liver tumour promoters in a certain experimental model such as the female rat according to their affinity to the receptor. For the more diverse familiy of PCBs and other tumour promoters in rodent liver, there are some correlations between the induction of drug-metabolizing enzymes and their promoting potency (5,28). These correlations are incomplete, however, and may reflect the existence of several mechanisms in tumour promotion by phenobarbital-like inducers (29). Alternatively, a common pathway of induction and tumour promotion may diverge at a certain level, and both branches may be subject to strong modulating effects, e.g., by adaptation. An example is the enhanced formation of peroxides after phenobarbital treatment, leading to an adaptive increase in catalase activity (18).

In summary, our results show for the first time that 'non-dioxin-like' PCBs suppress apoptosis in rat hepatocytes. Furthermore, it is shown that UV-initiated but not constitutive apoptosis is inhibited and that both inhibition of apoptosis and 'phenobarbital-type' induction of cytochrome P450 activity can exhibit distinct concentration-response relationships. Further experiments are required to identify the mechanisms of action of 'non-dioxin-like' PCBs critical for tumour promotion and their links to the inhibition of apoptosis.

Acknowledgements

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