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Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21$^{WAF1/Cip1}$, cyclin D1 and Ogg1

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To evaluate the risk of exposure to so-called non-genotoxic chemicals and elucidate mechanisms underlying their promoting activity on rat liver carcinogenesis the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), cytochrome P-450 (P-450) and hydroxyl radicals induction, DNA repair and alteration to cellular proliferation and apoptosis in the rat liver were investigated during 2 weeks of phenobarbital (PB) administration at a dose of 0.05%. Significant increase of hydroxyl radical levels by day 4 of PB exposure accompanied the accumulation of 8-OHdG in the nucleus and P-450 isoenzymes CYP2B1/2 and CYP3A2 in the cytoplasm of hepatocytes. Conspicuous elevation of 8-OHdG and apoptosis in the liver tissue were associated with reduction of the proliferating cell nuclear antigen (PCNA) index after 8 days of PB application. Thereafter, 8-OHdG levels decreased with an increase in mRNA expression for the 8-OHdG repair enzyme, DNA glycosylase 1 (Ogg1). Analysis with LightCycler quantitative 2-step RT–PCR demonstrated induction of cyclin D1 (CD1) and p21$^{WAF1/Cip1}$ mRNA expression on days 4 and 6, respectively, preceding marked elevation of PCNA and apoptotic indices. These results suggest that similar to genotoxic, non-genotoxic chemicals might induce reversible alteration to nuclear 8-OHdG in the rat liver after several days of continuous application; however, by a different mechanism. Increased 8-OHdG formation is caused by developing oxidative stress or apoptotic degradation of DNA and coordinated with enhanced expression of CD1 mRNA and cell proliferation, subsequent increase of p21$^{WAF1/Cip1}$ mRNA expression, cell-cycle arrest and apoptosis, while activation of 8-OHdG repair mechanisms contributes to protection of tissue against reactive oxygen species-induced cell death.

Introduction

Non-genotoxic liver tumor promoter, phenobarbital (PB), a sedative and anti-convulsant used as an anti-epilepsy drug in humans, exerts promoting effects on hepatocarcinogenesis after suitable initiation in rats (1), and enhances the proliferation of carcinogen-exposed hepatocytes *in vitro* (2). Chronic administration of PB to rats and mice results in hepatocarcinogenicity (3,4). Depending on the time point, inhibition of cell proliferation, DNA synthesis and increased concentrations of TGF-β_1 have all been observed in the liver tissue (5–8). On the other hand, growth of pre-neoplastic lesions induced by PB might predominantly be attributable to an inhibition of programmed cell death/apoptosis or to induction of reactive oxygen species (ROS), oxidative DNA damage and genetic alterations by spontaneous errors in DNA replication and repair (9–11). Understanding the mechanism of PB-promoted tumor formation in rodents is an integral step toward more accurately assessing the potential risk of exposure to the non-genotoxic chemicals for human health.

ROS are involved in intracellular regulation of numerous factors, such as transcription factors kappa B, NFκB and AP-1, partly through protein kinase C (PKC) activation (12–15). Oxygen radicals attack DNA bases and deoxyribose residues, producing damaged bases and single strand breaks or oxidize lipid and protein molecules, generating intermediates, which can react with DNA and form adducts. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is known as a marker of oxidative DNA damage, potentially involved in carcinogenesis in various experimental models (16). This modified base causes mutations, predominantly G to T transversions (17). The time- and dose-dependent generation of 8-OHdG in rat hepatic DNA have been demonstrated after single i.p. administration of genotoxic carcinogens including *N*-nitrosodiethylamine and aflatoxin B1 (16,18). In addition, 8-OHdG levels assessed after 1 week of dietary 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline application were also dose-dependently increased, although the effect was not observed at the end of the treatment period (19). These effects are suggested to be involved in initiation of hepatocarcinogenesis by genotoxic chemicals. On the contrary, it has been reported that only long-term high dose treatment with non-genotoxic liver tumor promoter PB results in the accumulation of 8-OHdG (20). As 8-OHdG in DNA is attributed to the production of oxygen radicals, especially hydroxyl radicals (21) and can be rapidly repaired (22), the actual level in the tissue is determined by changes in the rates of these processes. How PB treatment impacts on these remains unclear.

Recently, DNA damage has been shown to influence several genes involved in the cell-cycle checkpoint responses, inducing *p53* (23), *p21*$^{WAF1/Cip1}$ (24) and *ATM* (mutated in ataxia telangiectasia) (25). Although regulation of the cell cycle in response to DNA damage induced by genotoxic carcinogens has been extensively studied, there have been relatively few studies of this kind concerning the effects of non-genotoxic chemicals. Furthermore, links among oxidative stress and formation of DNA base modifications, cell-cycle regulation and DNA repair are not clearly understood.

Abbreviations: CYP2B1/2 and CYP3A2, 2B1/2 and 3A2 isoenzymes of cytochrome P-450, respectively; CD1, cyclin D1; CDK, cyclin-dependent kinase; DAB, 3,3′-diaminobenzidine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMPO-OH, spin adduct of DMPO and hydroxyl radicals; ESR, electron spin resonance; GAPDH, glyceraldehyde-3-P dehydrogenase; HPLC, high-performance liquid chromatography; LC-RT–PCR, LightCycler RT–PCR; MnO, manganese oxide marker; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; Ogg1, 8-oxoguanine DNA glycosylase 1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; P-450, cytochrome P-450; PB, phenobarbital; ROS, reactive oxygen species; ssDNA, single-strand DNA.

To elucidate the risk of exposure to PB and investigate mechanisms underlying its promoting activity on rat liver carcinogenesis, in this study, our attention was focused on interrelations between the induction of oxidative stress, cytochrome P-450 (P-450) and hydroxyl radicals, formation of 8-OHdG and its repair and alterations to cellular proliferation and apoptosis in the livers of rats during continuous administration of the chemical. First, to analyze the formation of oxidative stress, the generation of hydroxyl radicals in the liver microsomal fraction was measured by electron spin resonance (ESR). Secondly, the cytochrome P-450 total content, activity and protein levels of isoenzymes CYP2B1/2 and CYP3A2 were examined. To evaluate the effect of oxidative stress on DNA damage and cell growth regulation, the levels of 8-OHdG, PCNA and apoptosis were determined by immunohistochemistry. In addition, the mRNA levels for the 8-OHdG repair enzyme, DNA glycosylase (Ogg1), were examined by semi-quantitative RT–PCR. To investigate whether this non-genotoxic chemical exerts its carcinogenic effect in part by altering the cell-cycle regulation and cellular response to DNA damage, real-time LightCycler (LC) 2-step RT–PCR was used for the quantitative determination of cyclin D1 (CD1) and $p21^{WAF1/Cip1}$ mRNA expression.

Materials and methods

Chemicals

PB sodium salt (CAS no. 57-30-7) (purity ≥98%) was purchased from Wako Pure Chemicals Industries (Osaka, Japan) and other reagents from Wako Pure Chemicals Industries or Sigma (St Louis, MO). The spin-trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan).

Animals

Five-week-old male Fisher 344 rats (Charles River, Japan, Hino, Shiga, Japan) were quarantined for 1 week before the start of the experiment. Animals were housed in an animal facility maintained on a 12 h (08:00–20:00) light/dark cycle, at a constant temperature of 25 ± 1°C and relative humidity of 44 ± 5% and were given free access to tap water and food (Oriental MF powder diet, Oriental yeast Co., Tokyo, Japan).

Experimental design

Forty-eight male 6-week-old F344 rats were randomized into two groups, each including control and experimental subgroups. PB (0.05%) was administered in MF powder diet to rats for 1, 2, 4, 6, 8, 10, 12 and 14 days. At each time point three animals each in the experimental and control groups were killed. The numbers were selected in order to be able to complete the killing in 1 h. Animals were anesthetized with diethyl ether. The peritoneal cavity was then opened, and the portal vein was cannulated using an Insyte-W™ Vialon E I.V. catheter (Becton Dickinson Infusion Therapy Systems, Sandy, UT). The vena cave inferior was then cut and perfusion was begun *in situ* at 2 ml/min with ice-cold 1.15% KCl buffer (1.15% KCl, 1 mM EDTA, 0.25 mM PMSF) at room temperature. This was continued for 8 min, following which the liver was immediately removed. Separate portions were fixed in Bouin's solution and 10% buffered formalin. In addition, samples were frozen in liquid nitrogen and stored at –80°C for molecular analysis. The remaining liver tissue was immediately processed for microsome isolation, as described previously (26).

P-450 examination

The rat liver microsomal fraction was used for the examination of P-450 content (27), and activity determination in terms of P-450-mediated hydroxylation of testosterone, by high-performance liquid chromatography (HPLC) (28) and western blotting (29).

ESR

DMPO was employed as a spin-trapping agent with ESR signals measured using a quartz flat cell (inner size, 60×10×0.31 mm) and a JES-TE200 ESR Spectrometer (Japan Electronics Datum Co., Osaka, Japan). The reaction was started by addition of 10 mM nicotinamide adenine dinucleotide phosphate reduced form (NADPH) to liver microsomes (100 μg) in 0.1 M potassium phosphate buffer (pH 7.4) with 100 mM of the superoxide dismutase inhibitor diethyldithiocarbamate, followed by incubation at 37°C for 5 min. ESR spectra were recorded 30 s after addition of DMPO to the DMPO-free pre-incubation mixture with a reactive sample volume of 300 μl. As negative controls, several samples were run without addition of NADPH to the reaction mixture. After recording, the signal intensity of the spin adduct of DMPO and OH· (DMPO–OH) was evaluated from the peak height of the third signal of the quartet and normalized relative to the standard signal intensity of the manganese oxide marker (MnO).

Localization of 8-OHdG

Liver sections fixed in Bouin's solution were used for immunohistochemical assessment of 8-OHdG. All sections were prepared 1 day before the immunohistochemical examination, which was performed in quintuplicate. After deparaffinization, sections were sequentially pre-treated with 0.3% H_2O_2 in distilled water for 30 min, 0.05 M NaOH in 40% ethanol for 12 min and 250 μg/ml RNAse A for 60 min at 37°C. Then they were exposed to avidin, then biotin, for 20 min each step to block non-specific binding of Avidin/Biotin System reagents (Vector Blocking Kit, cat. SP-2001, Vector Lab., Burlingame, CA). The sections were subsequently incubated with 10% horse blocking serum for 20 min at 37°C to prevent background staining before incubation with diluted anti-8-OHdG mouse monoclonal antibody (IgG1, 20 μg/ml) 1:500 overnight. Immunoreactivity was detected by the ABC Method (Vectastain Elite ABC kit, Vector Lab.) with color development using 3,3′-diaminobenzidine (DAB). Mayer's hematoxylin was then added as a counterstain for 1 min. Negative controls were immunostained as above, but with primary serum instead of the anti-8-OHdG antibody. To check the possibility of the immunohistochemical detection of cells that might be susceptible to oxidation by H_2O_2, liver sections were pre-treated with different concentrations of H_2O_2 and staining intensity has been compared. Furthermore, control detection of liver DNA 8-OHdG levels has been performed by HPLC method (30). Total hepatocyte numbers in areas of liver sections and all positive nuclei were counted with the aid of a color image analyzer (IPAP, Sumika Technos, Osaka, Japan) and results were expressed as the number of positive nuclei per 1000 cells.

Double immunohistochemistry for 8-OHdG and P-450

Double staining for 8-OHdG and P-450 isoenzymes was performed using anti-8-OHdG mouse monoclonal antibody (1:500) and polyclonal rabbit anti-CYP2B1/2 (1:1500) and anti-CYP3A2 (1:1000) (IgG, 100 μg/ml) antibodies. After deparaffinization sections were sequentially pre-treated with 0.3% H_2O_2 in distilled water and goat serum to block the background staining, they were exposed to polyclonal rabbit anti-CYP antibodies overnight (4°C). The sites of peroxidase binding were demonstrated with alkaline phosphatase [Vectastain ABC-AP kit, Vector Red (SK-5100)] solution. Thereafter, sections were sequentially treated with 0.2 M glycine, pH 2.2, for 2 h to remove immune complexes. Immunohistochemistry for 8-OHdG was performed as described above, except with the 0.3% H_2O_2 treatment. The red color cytoplasm of hepatocytes reflected binding of rabbit polyclonal primary antibody to cytochrome P-450, while brown or black staining of nuclei showed a positive immunoreaction of monoclonal primary antibody with 8-OHdG.

Detection of apoptosis

Formation of single-stranded DNA (ssDNA) in the liver of F344 rats treated with 0.05% PB, which occurs during programmed cell death/apoptosis, was detected in Bouin-fixed sections with polyclonal rabbit anti-ssDNA antibody (IgG, 100 μg/ml, Dako Japan Co., Kyoto, Japan) (31). After deparaffinization, sections were sequentially pre-treated with 0.3% H_2O_2, washed with Tris buffer solution (TBS) and incubated with horse blocking serum to prevent background staining. Exposure to anti-ssDNA antibody was at 1:400 dilution for 60 min at room temperature. Immunoreactivity for the primary antibody was detected with goat anti-rabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer in Tris–HCl buffer containing a carrier protein and an anti-microbial agent (Dako EnVision+™ Peroxidase, Rabbit kit, Dako). Color was developed using DAB. The apoptotic index was estimated as for 8-OHdG, as the number of positive nuclei per 1000 cells.

Double immunohistochemistry for 8-OHdG and apoptosis

Double staining for 8-OHdG and apoptosis has been performed as described above using alkaline phosphatase (Vectastain ABC-AP kit, Vector Blue) solution for the immunohistochemical detection of apoptosis, and ABC method with color development by DAB for the determination of 8-OHdG. Brown- and blue-stained nuclei reflected binding of primary antibody against 8-OHdG and ssDNA, respectively, while deep black staining showed positive immunoreaction with both 8-OHdG and ssDNA.

PCNA

Formalin-fixed sections were incubated with monoclonal anti-PCNA antibody (PC-10, IgG2a; Dako) at 1:500 dilution, followed by ABC-peroxidase procedures. The substrate was hydrogen peroxide with the coloring agent DAB and the PCNA index was assessed as for 8-OHdG and apoptotic indices.

Table I. Nucleotide sequence for PCR primers, hybridization probes and conditions for amplification and sequence-specific detection of cDNA with the LightCycler System by real-time quantitative 2-step RT-PCR

Target genes	Nucleotide sequence	Orientation/HybProbe	GC content, %	Length (bp)
Primers				
p21$^{WAF1/Cip1}$	5′-TGG CCT TGT CGC TGT CTT-3′	sense	55.6	18-mer
	5′-CTA AGG CAG AAG ATG GGG AA-3′	antisense	50.0	22-mer
cyclin D1	5′-TGG AGC CCC TGA AGA AGA G-3′	sense	57.9	19-mer
	5′-AAG TGC GTT GTG CGG TAG C-3′	antisense	57.9	19-mer
GAPDH	5′-CTG GGA TGG AAT TGT GAG GGA G-3′	sense	57.1	21-mer
	5′-CTC TGG AAA GCT GTG GCG TGA-3′	antisense	54.5	22-mer
HybProbes				
p21$^{WAF1/Cip1}$	5′-GAC CGG GAC ATC TCA GGG C-3′	3′LCFluorescein	68.4	19-mer
	5′-GAA AAC GGA GGC AGA CCA GC-3′	5′LCRed640	57.9	20-mer
cyclin D1	5′-CGA ACA CTT CCT CTC CAA AAT GC-3′	3′LCFluorescein	60.0	23-mer
	5′-AGA GGC GGA TGA GAA CAA GCA-3′	5′LCRed640	52.4	21-mer
GAPDH	5′-GGG TGA GAA CAC GGA AGG CC-3′	3′LCFluorescein	65.0	20-mer
	5′-TGC CAG TGA GCT TCC CGT TC-3′	5′LCRed640	54.5	20-mer

RNA preparation

Total RNA was isolated from rat liver (pieces <5 mm in diameter) using Isogen (Nippon Gene, Toyama, Japan) (32). RNA was isopropanol precipitated, dissolved in DEPC-treated distilled water and kept at –80°C until use. RNA concentrations were determined with a spectrophotometer (Ultraspec 3000, UV/Visible Spectrophotometer; Pharmacia Biotech, Tokyo, Japan). Reverse transcription of 3 μg of total RNA was performed with Oligo-dT primer, and cDNA samples were stored at –20°C until assayed.

Analysis of Ogg1 mRNA induction

The samples prepared for PCR analyses were amplified in a 35 cycle-PCR reaction with DNA glycosylase (Ogg1) mRNA-specific primers as described by Tsurudome *et al.* (33). To perform RT–PCR semi-quantitatively, we confirmed that PCR products were increased linearly with the input of cDNA. Glyceraldehyde-3-P dehydrogenase (GAPDH) mRNA expression was used as an internal standard. PCR products were separated on 3% NuSieve agarose gels, and analyzed using a FMBIO II Multi-View Image Analyzer Scanning Unit (Hitachi, Japan).

Standards for RT–PCR

Using the RNA extracted from the livers of rats without any chemical treatment as a template, *CD1*, *p21*$^{WAF1/Cip1}$ and *GAPDH* were amplified by RT–PCR and subcloned in pT-AdV vector plasmid using an AdvanTAge™ PCR cloning Kit (Clontech Laboratories, CA, USA). Finally, amplified plasmids were isolated with QIAGEN Plasmid Mini Kit (Funakoski Co., Tokyo, Japan), measured in a spectrophotometer, and molecule concentrations were calculated. Sequence analysis was performed to check the obtained plasmid quality. Serial dilutions ranging from 10^9 to 10^2 molecules were then prepared.

Real Time quantitative LightCycler (LC) PCR

PCR for *CD1*, *p21*$^{WAF1/Cip1}$ and internal control, *GAPDH*, was performed using single pairs of primers and fluorescent probes (Table I) and a LightCycler–FastStart DNA Master HybProbes Kit (Roche Molecular Biochemicals, Germany). Probes were designed to hybridize to the antisense strands of transcripts and were labeled with 6-carboxy-fluorescein phosphoramidite at the 5′ end, and, as a quencher, 5-carboxy-tetramethyl-rhodamine (Nihon Gene Research Lab., Sendai, , Japan). The 20 μl PCR reaction mix contained 2 μl 10 PCR buffer, 8 mM (*CD1*) or 2.5 mM (*p21*$^{WAF1/Cip1}$) $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer and 0.2 μM probe, 2 U of FastStart *Taq* DNA polymerase and 100 ng of sample cDNA. PCR amplification began with a 10 min pre-incubation step at 95°C, followed by 45 cycles of denaturation at 95° C for 0 s, annealing at 58°C (*CD1*) or 59°C (*p21*$^{WAF1/Cip1}$) for 15 s, and elongation at 72°C for 17 s (*CD1*) or 11 s (*p21*$^{WAF1/Cip1}$). The relative concentration of PCR product derived from the target gene was calculated using software of the LightCycler System. A standard curve for each run was constructed by plotting the crossover point against the log (number of starting molecules). The number of target molecules in each sample was then calculated automatically by reference to this curve. Results were expressed relative to the number of *GAPDH* transcripts used as an internal control. Some amplification products performed in the LightCycler were checked by electrophoresis on 3% ethidium bromide-stained agarose gels. All experiments were performed in quintuplicate.

Statistical analysis

Statistical analysis of our data was performed using the Student's *t*-test according to Welch. All analyses were performed using the StatView-J 4.5 program (Berkeley, CA).

Results

Production of oxidative stress

To assess oxidative changes induced by PB administration in rat liver we measured hydroxyl radical levels and total content of cytochrome P-450 along with protein and activity of its two isoenzymes, CYP2B1/2 and CYP3A2, in the microsomal fraction. Formation of hydroxyl radicals was detected by the ESR technique. Figure 1A shows ESR spectra for DMPO–OH spin adducts in liver microsomal fraction at day 8 after beginning administration of 0.05% PB. The spectrum is in agreement with that reported earlier for spectrum of DMPO–OH adduct (34). The signal intensity of the third OH· peak in the quartet was normalized relative to the standard signal intensity of the MnO peak used as an internal control. As shown in the Figure 1B, DMPO–OH adduct was significantly enhanced by day 4 of PB exposure, reached a maximum by day 8 (82.06 ± 5.99%; $P < 0.001$), and thereafter remained stationary. Only at day 8, very low levels of DMPO–OH adduct were detected in liver microsomes of PB-treated rats without addition of NADPH to the reaction mixture (Figure 1A).

Spectrophotometric and western blot analyses of liver microsomes revealed a time-dependent significant elevation of total P-450 content and protein levels of two P-450 isoenzymes inducible by PB, CYP2B1/2 and CYP3A2 (data not shown). Similar to hydroxyl radical levels, CYP2B1/2 and CYP3A2 activity was rapidly increased until day 8, and thereafter remained constant with insignificant deviation (Figure 2). The activity of CYP3A2 appeared to be higher than that of CYP2B1/2 in contrast to the protein levels of these P-450 isoforms.

Concomitant elevation of 8-OHdG and P-450

Results of immunohistochemical examinations are summarized in Table II. Significant increase in 8-OHdG-specific immunoreactivity was detected on days 4, 6, 8, 10 and 12 post-treatment with 0.05% PB (Table II). Positively stained hepatocytes were mainly localized around the central vein (Figure 3A and C). Unexpectedly, on day 8, the level of 8-OHdG was enhanced

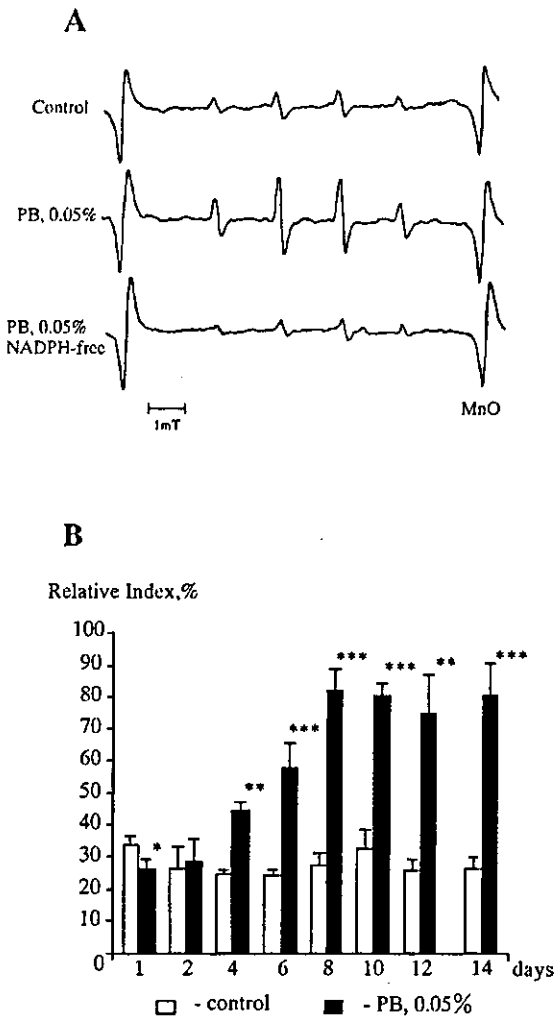

Fig. 1. Generation of hydroxyl radicals determined by ESR. DMPO was used as a spin-trapping agent. (A) DMPO–OH spectra with liver microsomal fraction from rats treated with 0.05% PB for 8 days. Very low levels of DMPO–OH adduct were detected in liver microsomes of PB-treated rats without addition of NADPH to the reaction mixture. (B) DMPO–OH levels after application of PB. The signal intensity of the third OH· peak in the quartet was normalized relative to the standard signal intensity of MnO peak used as an internal control. Values are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (Student's *t*-test).

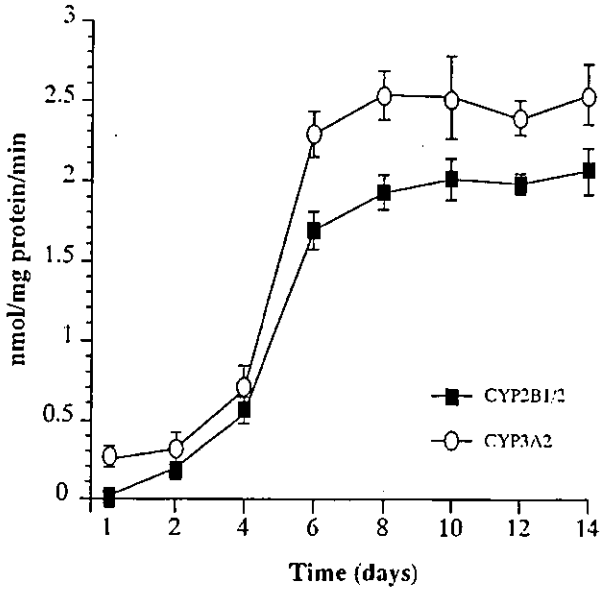

Fig. 2. Induction of cytochrome P-450 isoenzymes CYP2B1/2 and CYP3A2 by administration of 0.05% PB. Activity levels were evaluated in terms of P-450-mediated hydroxylation of testosterone. The results are mean ± SD values ($n = 3$).

up to the value of 18.79 ± 3.78 number/1000 cells, and 2 days later dramatically reduced, returning to the control level (0.14 ± 0.03 number/1000 cells) by day 14 (Table II and Figure 4). Furthermore, alterations of hydroxyl radicals and 8-OHdG levels were not strictly in parallel. Markedly increased until day 8, DMPO–OH adduct levels thereafter remained unchanged, thus demonstrating a striking difference from the observed alteration of 8-OHdG (Figure 1B and Table II). To check the reproducibility of our immunohistochemical findings a further experiment was performed. Rats were treated with 0.05% PB for 6, 8 and 10 days and liver sections were immunohistochemically analyzed. Accumulation of 8-OHdG in the nuclei of hepatocytes at day 8 post-treatment and its reduction 2 days thereafter exactly reproduced the experimental results described above. Furthermore, elevation of 8-OHdG on days 4 (1.27 ± 0.19 8-OHdG/10^6 dG; $P < 0.05$), 6 (1.41 ± 0.14 8-OHdG/10^6 dG; $P < 0.01$), 8 (2.8 ± 0.05 8-OHdG/10^6 dG; $P < 0.0001$), 10 (1.25 ± 0.19 8-OHdG/10^6 dG; $P < 0.05$) and 12 (0.96 ± 0.16 8-OHdG/10^6 dG; $P < 0.05$) with the peak on day 8 was detected using the HPLC method and a good correlation with the results of immunohistochemistry was found. This was done in order to confirm that the original 8-OHdG levels in hepatocytes were detected by immunoassay and no damage was induced by H_2O_2 treatment used in the protocol of the immunohistochemical examination. Moreover, to check the possibility of the immunohistochemical detection of cells that might be susceptible to oxidation by H_2O_2 liver sections were pre-treated with different concentrations of H_2O_2, however, no difference in staining intensity was observed.

Double staining for 8-OHdG and P-450 revealed an increase of CYP2B1/2 and CYP3A2 proteins in pericentrally localized hepatocytes, accompanied by 8-OHdG elevation in the nuclei (Figure 3A–D). Immunohistochemistry clearly demonstrated that an increase of 8-OHdG occurs primarily in hepatocytes strongly staining for CYP2B1/2 (Figure 3A and B) and CYP3A2 (Figure 3C and D) suggesting that the formation of oxidative base modifications is functionally dependent on the protein and activity levels of these P-450 isozymes.

Evaluation of apoptosis (ssDNA)

As shown in Table II, 2.5- and 10-fold increase in the number of apoptotic cells was found at days 6 and 8, respectively, following PB administration. Only the nuclei of hepatocytes localized in the pericentral region were positively stained for ssDNA (Figure 3E and F). Correlations were clearly apparent with alterations in 8-OHdG (Table II and Figure 4). Furthermore, the double staining for 8-OHdG and apoptosis revealed, that nuclei positively stained for apoptosis were always positive for 8-OHdG. However, only 70% of 8-OHdG-stained cells showed positive immunoreaction with antibody against ssDNA (Figure 5). Apoptotic degradation of DNA was well associated with DNA oxidation. Similar to the case with nuclear 8-OHdG

Table II. Immunohistochemical assessment of 8-OHdG, apoptosis (ssDNA) and PCNA in liver sections of 0.05% PB-treated and normal rats

Days of treatment	8-OHdG positivity (number/1000 cells)		Apoptosis (ssDNA) (number/1000 cells)		PCNA (number/1000 cells)	
	Control	PB	Control	PB	Control	PB
1	0.04 ± 0.01	0.02 ± 0.01	0.28 ± 0.19	0.15 ± 0.04	30.64 ± 13.78	31.44 ± 12.93
2	0.07 ± 0.03	0.36 ± 0.23	0.21 ± 0.05	0.19 ± 0.12	44.42 ± 14.98	47.05 ± 17.48
4	0.09 ± 0.01	0.46 ± 0.16[a]	0.66 ± 0.60	0.24 ± 0.12	59.16 ± 3.44	62.29 ± 17.80
6	0.10 ± 0.07	0.51 ± 0.15[a]	1.20 ± 0.39	3.65 ± 1.02[a]	50.69 ± 12.03	117.11 ± 16.03[b]
8	0.05 ± 0.03	18.79 ± 3.78[c]	1.11 ± 0.56	13.09 ± 4.28[b]	46.45 ± 23.70	4.01 ± 1.08[a]
10	0.23 ± 0.10	1.20 ± 0.39[a]	1.16 ± 0.24	0.45 ± 0.03[b]	41.69 ± 9.49	30.75 ± 10.94
12	0.25 ± 0.01	0.78 ± 0.26[a]	0.77 ± 0.25	0.61 ± 0.08	40.15 ± 5.35	40.15 ± 9.50
14	0.14 ± 0.03	0.14 ± 0.02	0.72 ± 0.47	0.79 ± 0.25	35.30 ± 10.86	22.25 ± 5.26

Data presented are a mean ± SD values (Student's *t*-test).
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

levels, enhancement of ssDNA formation at day 8 post-PB exposure was followed by sudden reduction. On day 10, the average number of apoptotic cells had decreased to a value significantly lower than that in the control group (Table II). However, at days 12 and 14 no significant differences in the apoptotic index between control and experimental groups were detected.

PCNA

A 2-fold elevation of the PCNA index reflecting changes in cell proliferation was found at day 6 after starting PB treatment (Table II). Unexpectedly, it declined significantly at day 8. However, from day 10 of PB exposure, PCNA values slowly increased again.

Induction of Ogg1 mRNA expression

In order to elucidate the reason for the sudden reduction of nuclear 8-OHdG levels observed in our study, we investigated mRNA expression for the 8-OHdG repair enzyme, Ogg1. RT–PCR semi-quantitative analysis revealed the time-dependent induction of Ogg1 mRNA (Figure 6). Only small amounts of Ogg1 mRNA were detected in the non-treated controls. Significant increase of Ogg1 mRNA was observed beginning from day 6 of PB exposure, and the highest levels of expression were found at days 12 and 14.

Cell-cycle arrest

To determine whether PB exerts its carcinogenic effect in part by altering cell-cycle regulation, we investigated an expression of CD1 and $p21^{WAF1/Cip1}$ mRNA by LC-RT–PCR. Results were expressed relative to the number of *GAPDH* transcripts used as an internal control. The highest induction of CD1 mRNA expression at day 4 (0.45 ± 0.01; $P < 0.0005$) preceded the highest elevation of PCNA indices on day 6 (Figure 7A and Table II). This enhancement of CD1 mRNA expression was followed by lowest expression at day 6, prior to suppression of the PCNA index observed in our experiment at day 8 (Figure 7A and Table II). At days 8 and 10 the average copy numbers of *CD1* transcripts again increased, reaching the control level, and thereafter again showed a tendency for enhancement. $p21^{WAF1/Cip1}$ mRNA expression was significantly induced only at day 6 of PB exposure (0.42 ± 0.07; $P < 0.005$) at the time of increase in the PCNA index and prior to the elevation of 8-OHdG and apoptosis determined by immunohistochemistry (Figure 7B and Table II). These results are in good correlation with those observed by double staining for 8-OHdG and apoptosis, being an indication that $p21^{WAF1/Cip1}$ mRNA expression might be increased in a certain percentage of hepatocytes, which are positively stained for both, 8-OHdG and apoptosis on day 8. Alterations of both CD1 and $p21^{WAF1/Cip1}$ mRNA indicated that activity of ROS induced by PB treatment at a carcinogenic dose results in enhancement of cellular proliferation followed by cell-cycle arrest and apoptosis regulated by *CD1* and *$p21^{WAF1/Cip1}$*, which occur simultaneously with the increase of 8-OHdG.

Discussion

The present study demonstrated a range of changes in cell proliferation, DNA damage and apoptosis-related parameters dependent on the period of PB exposure. While no formation of 8-OHdG in the rat liver was earlier found after up to 22 weeks of PB administration at a high dose (35), marked enhancement 8 days after starting the application was evident here. Previously, Murkofsky *et al.* (36) reported that treatment with 0.05% PB for 5 days in F344 rats induced cell proliferation, however, it did not affect steady-state levels of 8-OHdG in liver DNA. Our results indicated a conspicuous but short time 8-OHdG increase of nuclear localization in hepatocytes, which was associated with the rise in the intracellular hydroxyl radical level and in the beginning, with the elevation of cellular proliferating activity. In the present study, PB treatment was continued for 2 weeks with killing performed every 2 days, while the duration of previously published short-term experiments was no longer than 1 week. That gave us an opportunity to reveal the correlative changes in oxidative stress, proliferation, apoptosis, DNA damage and its repair, which occur in the rat liver during continuous PB exposure. Induction of Ogg1 mRNA expression suggested that one of the reasons for subsequent 8-OHdG decrease might be activation of the 8-OHdG repair. This has been shown to be activated in response to DNA damage generated by hydroxyl radicals and is attributable to glycosylase, endonuclease and lyase activity (22,37,38). Activation of 8-OHdG repair processes might explain the failure to detect 8-OHdG formation in the rat liver up to 22 weeks of PB application. Furthermore, changes of oxidative base modifications level observed in the present experiment are consistent with results demonstrating reversible alteration to liver DNA 8-OHdG after administration of genotoxic carcinogens, which is suggested to be potentially involved in initiation of hepatocarcinogenesis in rats (16,18,19). On the

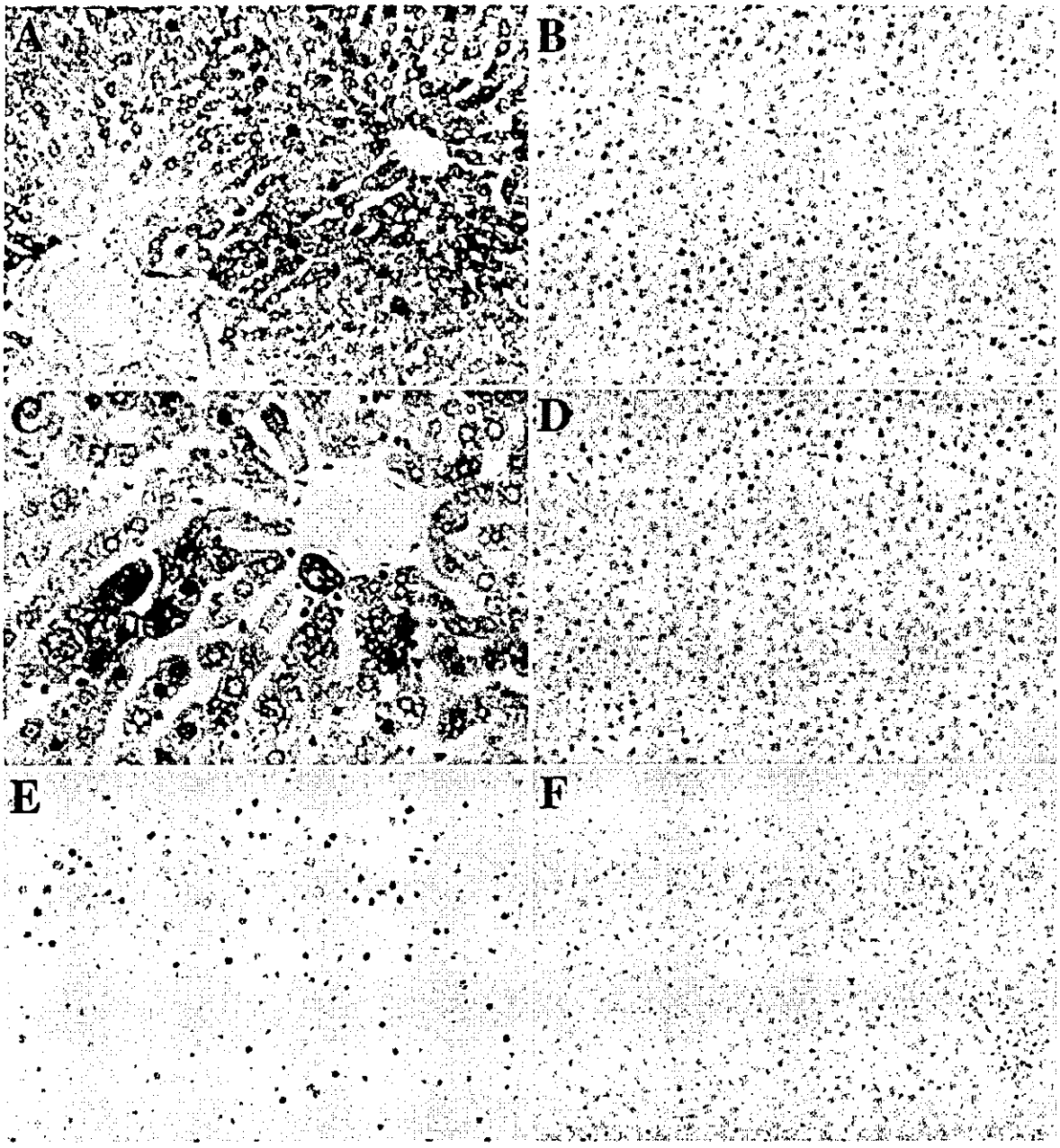

Fig. 3. Double immunohistochemistry for 8-OHdG and P-450, and immunohistochemical assessment of apoptosis (ssDNA) in the livers of F344 rats treated for 8 days with 0.05% PB. (A) Immunohistochemistry for 8-OHdG and CYP 2B1/2 in a PB-treated (×100) and (B) control animal (×100); (C) 8-OHdG and CYP3A2 in PB-treated (×400) and a (D) control animal (×100). The red color cytoplasm of hepatocytes reflects binding of rabbit polyclonal primary antibody against cytochrome P-450, while brown or black stained nuclei showed positive immunoreactions for monoclonal primary antibody against 8-OHdG. Note: the increase of 8-OHdG in the nuclei of pericentrally localized hepatocytes most strongly stained for CYP2B1/2 and CYP3A2. (E) Immunohistochemical assessment of apoptosis (ssDNA) in PB-treated (×100) and (F) normal F344 rats (×100). As with 8-OHdG, positive immunoreactivity for ssDNA was found in the nuclei of pericentrally localized hepatocytes.

other hand, an induction of a significant and steady elevation of 8-OHdG is thought to be essential for the activation of carcinogenic properties of the cells (21). Recently, the longer maintenance of high levels of 8-OHdG in liver DNA is explained by the exhaustion and/or disturbance of the DNA repair system by the administration of carcinogens (16). It is thus conceivable that early increment of 8-OHdG in the nuclei of hepatocytes, induced by non-genotoxic chemicals via generation of oxidative stress, might influence the carcinogenic potential of initiated cells with already disrupted DNA repair producing stronger damage to DNA and promoting hepatocarcinogenesis.

Coordinated accumulation of 8-OHdG in the nucleus and CYP2B1/2 and CYP3A2 in the cytoplasm implied that increase of DNA damage after treatment with 0.05% PB in rat is caused by production of oxidative stress, related to increase in protein

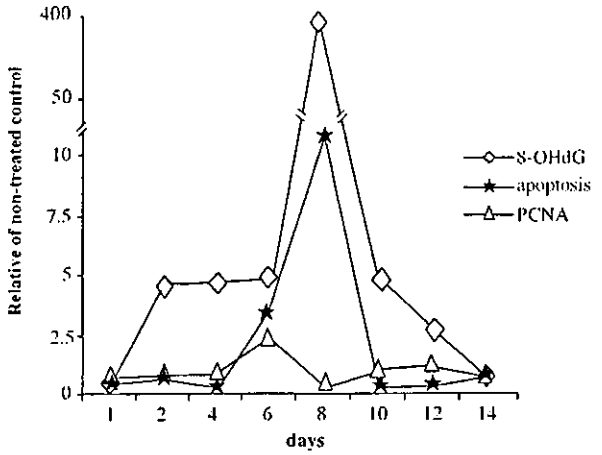

Fig. 4. Co-ordinated changes of 8-OHdG, apoptosis (ssDNA) and PCNA in the livers of rats treated with 0.05% PB.

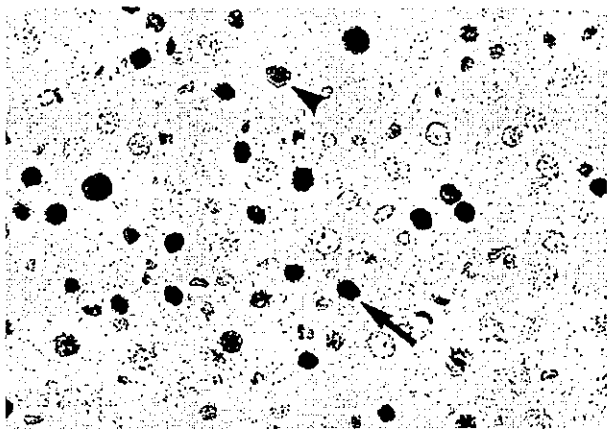

Fig. 5. Double immunohistochemistry for 8-OHdG and apoptosis (ssDNA) (×400). Brown and blue-stained nuclei reflected binding of primary antibody against 8-OHdG and ssDNA, respectively, while deep black staining showed positive immunoreaction with both 8-OHdG and ssDNA. Long arrow—nuclei positive for 8-OHdG and apoptosis, Arrow head—8-OHdG positive cells.

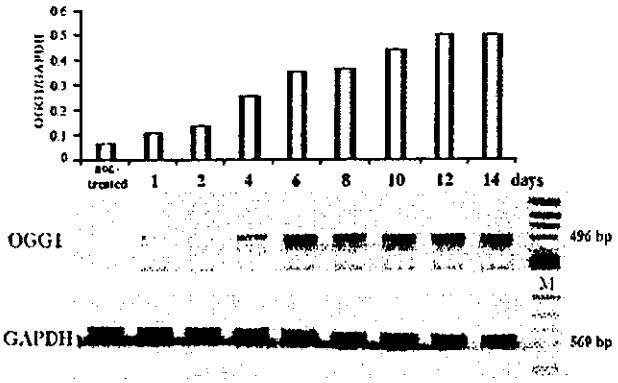

Fig. 6. Ogg1 mRNA levels in rat liver after administration of 0.05% PB. Significant increase of Ogg1 mRNA was observed beginning from day 6 of PB exposure, and the highest levels of expression were detected at days 12 and 14.

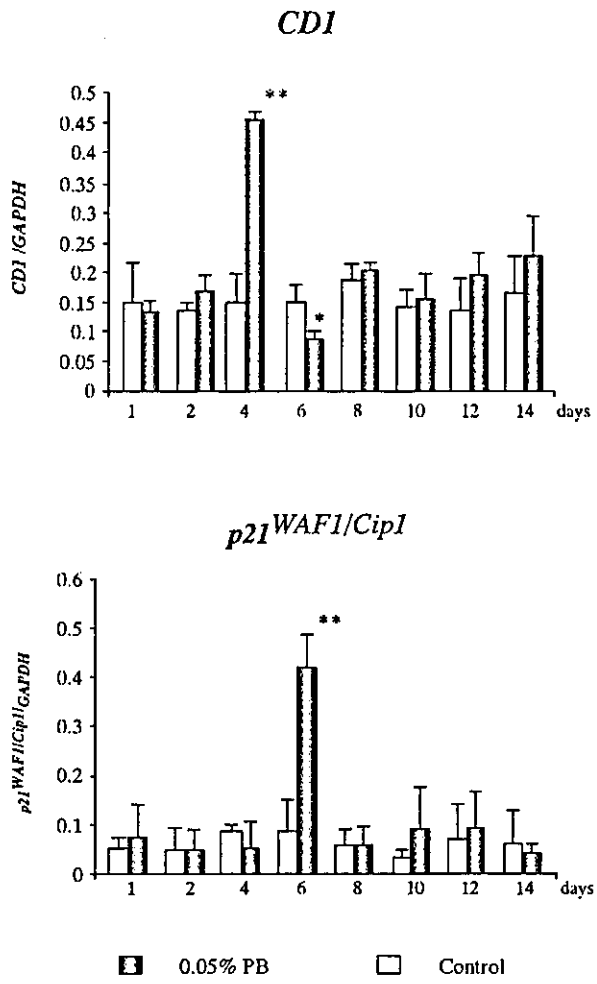

Fig. 7. (A and B) CD1 and p21 $^{WAF1/Cip1}$ mRNA expression, respectively, in the livers of rats treated with 0.05% PB detected by real-time 2-step quantitative LightCycler PCR. *GAPDH* was used as an internal control. The number of target molecules in each sample was calculated automatically with reference to the standard curve ($r = 0.99$). Results were expressed initially as relative to the number of *GAPDH* transcripts used as an internal control. The data are mean ± SD values. $*P < 0.01$, $**P < 0.005$, $***P < 0.0005$.

and activity levels of these two P-450 isoenzymes. Our results obtained by biochemical examination corresponded with the data reported previously on the induction of CYP2B1/2 and CYP3A2 by PB (10).

Interestingly, immunohistochemical examination of rat livers after 8 days of PB treatment showed the high levels of concordance between induction of 8-OHdG and apoptosis (ssDNA), which were inversely correlated with the PCNA labeling index (Figure 4). Furthermore, double immunohistochemistry for 8-OHdG and apoptosis demonstrated, that in ~70% of cells with damaged DNA nuclei were apoptotic, suggesting that 8-OHdG elevation induced DNA fragmentation. However, the observation that oxidative DNA damage is mostly observed in the apoptotic cells could also mean that the oxidative degradation of DNA is the consequence rather than the cause of apoptosis. To investigate the mechanisms we analyzed mRNA expression of *CD1* and *p21*$^{WAF1/Cip1}$, genes involved in cell-cycle regulation and cellular response to DNA damage.

CD1 plays a key regulatory role during G_1 phase of the cell cycle and its gene is amplified and overexpressed in many cancers. Recent studies have indicated that the induction of both CD1 and $p21^{WAF1/Cip1}$ may contribute to wild type p53-mediated G_1 growth arrest (39). $p21^{WAF1/Cip1}$, a cyclin-dependent kinase (CDK) inhibitor and downstream effector of p53-mediated growth suppression, which is capable of linking the DNA damage response pathway with the cell-cycle machinery, contributing to DNA repair and maintaining genomic integrity (40,41). Recent studies have shown that it is also regulated through a p53-independent pathway (42,43). A unique feature of $p21^{WAF1/Cip1}$, which distinguishes it from other CDK inhibitors, is its ability to associate with PCNA and induce cell-cycle arrest in G_1 and G_2 phases (44,45). It has been further shown to function as a tumor suppressor through its ability to control cell-cycle progression (46).

In this investigation, analysis with LC-RT–PCR revealed the increased mRNA expression of CD1 and $p21^{WAF1/Cip1}$ by 4 and 6 days, respectively, after starting 0.05% PB administration. $p21^{WAF1/Cip1}$ mRNA alteration was observed during significant increase of PCNA, and prior to the elevation of 8-OHdG and apoptosis. These results suggested that application of 0.05% PB to non-initiated rats for 1 week results in an induction of CD1 mRNA and cellular proliferation, which are followed by $p21^{WAF1/Cip1}$-dependent G_1 and G_2 arrest. The last phenomenon is likely to be related to the $p21^{WAF1/Cip1}$ capacity for direct binding to PCNA and suppression of CDK complexes. Double immunohistochemical examination for 8-OHdG and apoptosis clearly indicated that the induction of $p21^{WAF1/Cip1}$ might be related to the oxidative DNA damage, which occur in pericentrally localized hepatocytes. Biochemical and molecular biological data of this study corresponded to the results of our immunohistochemical analysis. However, the exact mechanisms deserve further investigations.

Recently, an increase of bromodeoxyuridine (BrdU) labeling indices, 1 week post-treatment with 0.05% PB, was demonstrated in the livers of c-*myc*/TGF-α double-transgenic and C57BL/10J mice (47,48). Another short-term study, performed with AP Wistar rats, revealed enhancement of cell proliferation after 3 days of treatment, and thereafter return to the control level (49). Rise in the PCNA index in our study at day 6 post-treatment with PB is thus generally in line with the literature (Figure 4). Recently, the generation of intracellular ROS is reported to result in the elevation of mitogen activated kinases activities and DNA synthesis, what might be related to the increase of CD1 mRNA expression and PCNA index observed in the present experiment (50).

The fact of a reduced apoptotic index at day 10 possibly suggests a link to activation of 8-OHdG repair mechanisms. On the other hand, depression of apoptosis by PB has been recently explained on the basis of its ability to inhibit *p53* (51), *p21*$^{WAF1/Cip1}$ (52) and enhance the *bcl-2* gene family expression (53). Furthermore, PB may co-operate with c-*myc* and TGF-α in the selective inhibition of apoptosis through diverse molecular pathways (54).

In conclusion, our results indicate that application of non-genotoxic liver tumor promoter PB at a high dose to rats induces conspicuous but reversible alteration to DNA oxidative base modifications in the nuclear of hepatocytes via generation of oxidative stress and affects expression of genes involved in cell-cycle regulation with a sequence of events leading first to cell proliferation, then to cell-cycle arrest, apoptosis and finally apparent accommodation. How the treatment with non-genotoxic chemicals impacts on these in rats after initiation of hepatocarcinogenesis with genotoxic carcinogen is the subject for our further investigations.

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Phenobarbital at low dose exerts hormesis in rat hepatocarcinogenesis by reducing oxidative DNA damage, altering cell proliferation, apoptosis and gene expression

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Our recent research indicated that phenobarbital (PB) may inhibit the development of *N*-diethylnitrosamine (DEN)-initiated pre-neoplastic lesions at low doses in a rat liver medium-term bioassay (Ito test), while high doses exhibit promoting activity. This raises the question of whether treatment with low doses of PB might reduce cancer risk. For clarification, male 6-week-old F344 rats were treated with PB at doses of 0, 2, 15 and 500 p.p.m. in the diet for 10 or 33 weeks after initiation of hepatocarcinogenesis with DEN. In a second, short-term experiment, animals were given PB at doses of 2, 4, 15, 60 and 500 p.p.m. for 8 days. Formation of glutathione *S*-transferase placental form (GST-P) positive foci and liver tumors was inhibited at 2 p.p.m. Generation of oxidative DNA damage marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG), cellular proliferation within the areas of GST-P positive foci and apoptosis in background liver parenchyma were suppressed. Suppression of 8-OHdG formation by PB at low dose might be related to the enhanced mRNA expression of 8-OHdG repair enzyme, oxoguanine glycosylase 1 (Ogg1). Moreover, as detected by cDNA microarray analysis, PB treatment at low dose enhanced mRNA expression of glutamic acid decarboxylase (GAD65), an enzyme involved in the synthesis of gamma-aminobutyric acid (GABA), and suppressed MAP kinase p38 and other intracellular kinases gene expression. On the contrary, when PB was applied at a high dose, GST-P positive foci numbers and areas, tumor multiplicity, hydroxyl radicals and 8-OHdG levels were greatly elevated with the increase in CYP2B1/2 and CYP3A2 mRNA, protein, activity and gene expression of GST, nuclear tyrosine phosphatase, NADPH-cytochrome P-450 reductase and guanine nucleotide binding protein G(O) alpha subunit. These results indicate that PB exhibits hormetic effect on rat hepatocarcinogenesis initiated with DEN by differentially altering cell proliferation, apoptosis and oxidative DNA damage at high and low doses.

Introduction

It is generally believed that rodent carcinogens are human carcinogens, despite the existence of exceptions, the prevailing paradigm is that even tiny doses can induce cancer (1). However, many studies have shown benefits, not harm, from low-level exposure to toxicants, this phenomenon being known as hormesis (2). Furthermore, it has been observed that for some substances tested, carcinogens are similar to other toxicants in improving health at low doses, although, the mechanism of their action remains unclear (3).

Phenobarbital (PB), a sedative and anti-convulsant used as an anti-epilepsy drug in humans, is also a non-genotoxic carcinogen and a well-known promoter of hepatocarcinogenesis *in vivo* and *in vitro* (4–9). The promoting effect of PB at a high dose on hepatocarcinogenesis in rodents has been extensively studied, but reasons for its carcinogenic action have yet to be unequivocally clarified. Increased reactive oxygen species (ROS) due to the activity of detoxifying enzymes and induction of oxidative stress are suggested to be possible mechanisms by which non-genotoxic chemicals may exert carcinogenicity (10,11). Oxygen radicals attack DNA bases and deoxyribose residues, producing damaged bases and single strand breaks, or oxidize lipid and protein molecules, generating intermediates which can react with DNA and form adducts. Among the more abundant types of base modifications, 8-hydroxy-2′-deoxyguanosine (8-OHdG), produced by the oxidation of deoxyguanosine, is considered as the most sensitive and useful marker of oxidative DNA adducts (12). It has been shown that 8-OHdG is closely associated with various diseases, including cancer, and is produced by exposure to various carcinogens (12). It is known to cause mutations, predominantly G to T transversions (13,14).

Our previous research indicated that PB administered at doses from 60 to 500 p.p.m. dose-dependently increases the numbers and areas of glutathione *S*-transferase placental form (GST-P) positive foci, whereas, the dose 15–30 p.p.m. did not appear to have any effect in a medium-term rat liver bioassay (Ito test) (15). Interestingly, in the same study, inhibition activity of PB on the development of GST-P positive lesions was apparent for doses in the range of 1–7.5 p.p.m. with significance at 1 and 2 p.p.m. (15). In past studies a dose–response effect of PB on the increase of the hepatic cancer was observed, exhibiting a threshold at low doses (16–18).

Anticarcinogenic effects of PB might result from stimulation of hepatic drug-metabolizing enzymes, which detoxify carcinogens (19). In the post-initiation phase the mechanisms

Abbreviations: CYP2B1/2, CYP3A2 and CYP2C11, 2B1/2, isoenzymes of cytochrome P-450, respectively; DEN, *N*-diethylnitrosamine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMPO–OH, spin adduct of DMPO and hydroxyl radicals; ESR, electron spin resonance; GAPDH, glyceraldehyde-3-P dehydrogenase; GABA, gamma-aminobutyric acid; GAD65, glutamic acid decarboxylase; GST-P, glutathione *S*-transferase placental form; HCC, hepatocellular carcinoma; HPLC, high performance liquid chromatography; Ogg1, 8-oxoguanine DNA glycosylase 1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; OR, NADPH cytochrome P-450 reductase; PB, phenobarbital; P-450, cytochrome P-450; PCNA, proliferating cell nuclear antigen; ssDNA, single-stranded DNA.

underlying modulation are not clear but hormesis-type responses may represent modest overcompensation to a disruption of homeostasis (20). Furthermore, a number of studies have revealed that biphasic dose–response appears to result from direct stimulation at low doses and inhibition at high doses without involvement of the above-mentioned compensatory responses (20).

In the present study, the effects of PB application at 2, 15 and 500 p.p.m. were investigated with reference to lesion development, oxidative stress, DNA damage, cellular proliferation and apoptosis in the rat liver. For this purpose the combination of high performance liquid chromatography (HPLC), electron spin resonance (ESR), competitive RT–PCR and double immunohistochemistry was employed. In addition, mRNA expression of 8-OHdG repair enzyme, oxoguanine DNA glycosylase 1 (Ogg1), was analysed by real-time PCR. We also made use of cDNA microarray technology to gain a global view of the pool of genes that might play important roles in inhibition of hepatocarcinogenesis by PB at low dose.

Materials and methods

Chemicals

PB sodium salt (CAS no. 57-30-7) (purity ≥98%) and other reagents were purchased from Wako Pure Chemicals Industries (Osaka, Japan). *N*-Diethylnitrosamine (DEN) was from Sakai Research Laboratory (Fukui, Japan). The spin trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan).

Animals

A total of 168, 5-week-old male Fisher 344 rats (Charles River, Japan, Hino, Shiga, Japan) were quarantined for 1 week before the start of the experiment. They were housed in an animal facility maintained on a 12 h (07:00–19:00) light/dark cycle, at a constant temperature of 23 ± 1°C and relative humidity of 44 ± 5%, and were given free access to tap water and food (Oriental MF pellet diet, Oriental Yeast Co., Tokyo, Japan).

Experimental design

In the first experiment, 138 F344 rats were divided into six experimental groups. After 1 week on basal diet they underwent i.p. injection of DEN (100 mg/kg body wt) dissolved in saline to initiate hepatocarcinogenesis. This was performed three times, once per week. After 1 further week on basal MF diet, animals were administered diet containing PB at a dose of 0 (control), 2, 15 or 500 p.p.m. for 10 (10 rats per group) or 33 (20 rats per group) weeks. Two groups were injected with saline instead of DEN and administered PB at doses of 0 or 500 p.p.m. for 10 (five rats per group) and 33 (four rats per group) weeks. At experimental weeks 13 and 36 the animals were killed.

In the second, short-term experiment, animals were divided into six experimental groups, five rats each. After 1 week on basal diet they were administered diet containing PB at doses of 0 (control), 2, 4, 15, 60 or 500 p.p.m. for 8 days. The duration of the treatment was chosen from the results of our previous study, in which PB administered to F344 rats at a dose of 500 p.p.m., showed the highest induction of hydroxyl radicals (OH•) and 8-OHdG in the rat liver DNA after 8 days of application (21).

In experiment 1, immunohistochemical demonstration of GST-P positive foci and HE staining for assessment of the incidence, multiplicity and histopathological classification of liver tumors were carried out at weeks 13 and 36, respectively. Separate portions of normal-appearing liver tissue and tumors at week 36 were fixed in Bouin's solution and 10% phosphate-buffered formalin.

At death animals were anesthetized with diethyl ether. In experiments 1 (week 10) and 2, liver perfusion was performed *in situ* at 2 ml/min with ice-cold 1.15% KCl buffer (1.15% KCl, 1 mM EDTA, 0.25 mM PMSF) at room temperature (21). The perfusion was continued for 8 min.

After finishing the perfusion, livers were immediately removed. Separate portions were fixed in Bouin's solution and 10% phosphate-buffered formalin, partially frozen in liquid nitrogen and stored at −80°C for molecular assessment. The remainder of the liver tissue was immediately processed for microsomes isolation, as described elsewhere (22). The liver microsomal fraction was stored at −80°C for subsequent ESR examination of hydroxyl radical generation and cytochrome P-450 content, P-450 mediated hydroxylation of testosterone by HPLC for activity determination and western blotting (23–26). SDS–PAGE was performed by the method of Laemmli (27). NADPH-cytochrome P-450 reductase (OR) activity in rat liver microsomes was measured using the artificial electron acceptor cytochrome C (28).

ESR

DMPO was employed as a spin trapping agent and signals were assessed with a quartz flat cell (inner size, 60 × 10 × 0.31 mm) using a JES-TE200 ESR Spectrometer (Japan Electronics Datum Co., Osaka, Japan) as described (21). After recording, the signal intensity of the spin adducts of DMPO and OH• (DMPO–OH) was evaluated from the peak height of the third signal of the quartet and normalized as relative height against the standard signal intensity of the manganese oxide marker.

8-OHdG analysis

Rat liver DNA 8-OHdG levels were determined by an HPLC-ECD method (29). Furthermore, immunohistochemical staining of 8-OHdG was accomplished as described previously (21).

Immunohistochemistry for GST-P positive foci

Immunohistochemical assessment of GST-P was performed with the avidin–biotin complex method as described by Kitano *et al.* (15) using an anti-rat GST-P primary (1:2000) (IgG, 100 μg/ml) antibody diaminobenzidine tetrahydrochloride as the substrate to demonstrate sites of peroxidase binding. Quantitation of GST-P positive foci was done using two-dimensional evaluation (30). The numbers and areas of foci >0.2 mm in diameter, and total areas of liver sections, were measured using a color image processor (IPAP; Sumica Technos, Osaka, Japan) to give values per cm^2 of liver section.

Double immunohistochemistry for proliferating cell nuclear antigen and GST-P

Double staining for GST-P positive foci and proliferating cell nuclear antigen (PCNA) was performed using the polyclonal rabbit anti-rat GST-P antibody at 1:2000 dilution and an anti-PCNA mouse monoclonal (PC-10, IgG2a; DAKO, Kyoto, Japan; 1:500) antibody. Immunohistochemical detection of GST-P was accomplished according to the protocol described above. The sites of peroxidase binding were demonstrated with alkaline phosphatase [Vectastain ABC-AP kit, Vector Red (SK-5100)] solution. Thereafter, sections were sequentially treated with 0.2 M glycine, pH 2.2, for 2 h to remove immune complexes. Immunohistochemistry for PCNA was essentially performed as described previously (21). GST-P positive foci were stained red, while blue staining of nuclei showed a positive immunoreaction of monoclonal primary antibody with PCNA. The PCNA indices were estimated for GST-P positive areas of a same size (foci consisting of 20–30 cells) and in background liver parenchyma as numbers of positive nuclei per 1000 cells.

Double immunohistochemistry for GST-P and apoptosis

Double immunohistochemistry for GST-P and apoptosis [single-stranded DNA (ssDNA)] was performed with Bouin-fixed sections as described above for GST-P and PCNA using alkaline phosphatase (Vectastain ABC-AP kit, Vector Red/Vector Blue) solutions for the immunohistochemical detection of GST-P and apoptosis (21,31). Blue-stained nuclei reflected binding of primary antibody against ssDNA, while GST-P positive foci were stained red. Apoptotic index was assessed as for PCNA.

RNA preparation

Total RNA was isolated from rat liver (pieces <5 mm in diameter) using ISOGEN (Nippon Gene, Toyama, Japan) (32,33). RNA was isopropanol precipitated, dissolved in DEPC-treated distilled water and kept at −80°C until use. RNA concentrations were determined with a spectrophotometer (Ultraspec 3000, UV/Visible Spectrophotometer; Pharmacia Biotech, Tokyo, Japan). Reverse transcription of 3 μg of total RNA was performed with Oligo-dT primer, and cDNA samples were stored at −20°C until assayed.

cDNA microarray analysis

One hundred micrograms of pooled aliquots of total RNA from 10 rats in each group were treated with DNase I and processed for PolyA$^+$ RNA enrichment and generation of ^{32}P-cDNA probes using the Atlas Pure RNA Labeling System (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Radiolabeled cDNA was purified and eluted through a NucleoSpin Extraction Column (centrifuged 14 000 r.p.m.). Each Atlas Rat 1.2 Array (Clontech) was pre-hybridized with 5 ml of ExpressHyb buffer (Clontech) and 0.5 mg of denatured DNA from sheared salmon testes at 68°C for 1 h with continuous agitation. The probes synthesized from the treatment samples, and their corresponding controls were then added. After overnight hybridization and a

high-stringency wash the arrays were scanned with a BAS-2500 Imager (Fujifilm Medical Systems, Stamford, CT) after 1 day exposure. Hybrid intensity was normalized to values for house keeping genes with Atlas Image Software. To minimize the effects of measurement variation introduced by artificial sources during the experiments, we only included spots with up-regulation or down-regulation at least 2-fold. CYP2B1, CYP3A2, CYP2C11 isoenzymes of P-450 and Ogg1 were not included in cDNA microarray analysis and their expression was investigated with the use of competitive or real-time RT–PCR. Microarray analysis was repeated twice to check the reproducibility of the data. Furthermore, results obtained by cDNA microarray for expression of *GAD65* (glutamic acid decarboxylase) were checked by quantitative RT–PCR analysis using a set of primers (forward: GGCTCTGGCTTTTGGTCCTTC and reverse: TGCCAATTCCCAATTATACTCTTGA) as described previously (34).

Competitive RT–PCR

As CYP2B1/2, CYP3A2, CYP2C11 isoforms of cytochrome P-450 were not included in the microarray analysis, the determination of their mRNA expression and the house keeping gene cyclophillin was performed using a Rat Cytochrome P-450 Competitive RT–PCR set and a Takara RNA LA PCR TM Kit (Takara Biomedicals, Japan) according to previously reported protocols and the data were expressed as a relative density of CYP2B1/2, CYP3A2 or CYP2C11 bands as compared with the band density of RNA competitor (35).

Real-time LightCycler PCR

Using RNA extracted from the livers of rats without any chemical treatment as a template, *OGG1* and *GAPDH* (glyceraldehyde-3-P dehydrogenase) were amplified by RT–PCR and subcloned into a pT-AdV vector plasmid using an AdvanTAge™ PCR cloning Kit (Clontech Laboratories, USA) as described previously (21). Serial dilutions ranging from 10^9 to 10^2 molecules were then prepared. Real-time quantitative LightCycler PCR (LC-PCR) for *OGG1* and *GAPDH* was performed using a LightCycler™ DNA Master SYBR Green I FastStart Kit (Roche Molecular Biochemicals, Germany) and a set of primers for *OGG1* and *GAPDH* as reported (21). Results were expressed relative to the number of *GAPDH* transcripts used as the internal control. Some amplification products were checked by electrophoresis on 3% ethidium bromide-stained agarose gels. All examinations were performed in quintuplicate.

Statistical analysis

Statistical analysis was performed with the Fisher's exact probability test for the incidence of liver tumors and the two-tailed Dunnet's test for the remainder of our data using the StatView-J 5.0 program (Berkeley, CA).

Results

General observations

All treatment diets were well tolerated and there were no significant differences among the groups with regard to food and water consumption or body weight gain (data not shown). Two rats died before the termination of the experiment without discernible cause. Final liver/body weight ratios were increased significantly only in the groups administered PB at a dose of 500 p.p.m. for 10 weeks (4.8 ± 0.6%, $P < 0.0001$) and 33 weeks (5.9 ± 1.6%, $P < 0.0001$) following DEN initiation as compared with values for the control groups, (3.5 ± 0.3%) and (3.1 ± 0.3%), respectively. No change in liver/body weight ratio was induced by administration of PB at a dose of 2 and 15 p.p.m. as compared with initiation control group.

Inhibitory and promoting effects of PB on formation of GST-P positive foci and liver tumors

Data on effects of PB application at different doses on formation of GST-P positive foci at week 13 and liver tumors at week 36 in experiment 1 are shown in Table I. After 10 weeks of continuous PB administration to rats initiated with DEN a significant decrease in number and area of GST-P positive foci was found in the group treated with PB at a dose of 2 p.p.m. as compared with the DEN initiation control group (Table I). In contrast, values were highly elevated in animals administered PB at a dose of 500 p.p.m.

Histological examination of liver tumors at week 36 in the experiment 1 demonstrated a significant reduction in the multiplicity of hepatocellular carcinomas (HCCs) and total tumors in the group treated with PB at a dose of 2 p.p.m. as compared with the DEN control group. Furthermore, a tendency for decreased incidences of adenomas and HCCs was also evident. In contrast, HCC and total tumor multiplicity values in the group treated with PB at 500 p.p.m. were highly elevated as compared with the controls. Most tumors developing in animals administered 500 p.p.m. of PB were well differentiated HCCs. However, in the 2 p.p.m. PB-treated group ~70% of tumors were adenomas.

Dose-dependent production of oxygen radicals

The observed hydroxyl radical spectrum was in agreement with that reported earlier for the DMPO–OH adduct (23). Dose-dependent elevation of DMPO–OH spectra was found in the rat liver microsomal fraction at week 10 of PB administration (Table II). A slight but significant increase of DMPO–OH spectra was also evident in the 2 p.p.m. treated group after 8 days of PB application in experiment 2 (data not shown). Hydroxyl radical levels were highly elevated in the liver microsomal fraction by the treatment with PB at a dose of 500 p.p.m. in both long (Table II) and short-term (data not shown) studies.

Table I. Quantitative data for GST-P positive foci and liver tumors in rats undergoing initiation with DEN followed by treatment with PB

Treatment	No. of rats	GST-P (13 weeks) (≥0.2 mm)		No. of rats	Liver tumors (36 weeks)				
					Incidence (%)		Multiplicity		
		Numbers (No./cm²)	Areas (mm²/cm²)		HCC	Adenoma	HCC	Adenoma	Total
DEN[a]	10	128.04 ± 16.51	1.41 ± 0.54	20	19 (95)	20 (100)	3.00 ± 1.95	3.65 ± 1.90	6.65 ± 2.64
DEN → PB, 2 p.p.m.	10	96.57 ± 15.36[c]	0.97 ± 0.35[b]	19	14 (74)	16 (84)	1.21 ± 0.98[c]	2.84 ± 2.04	4.05 ± 2.04[c]
DEN → PB, 15 p.p.m.	10	104.87 ± 23.07	1.11 ± 0.49	19	19 (100)	18 (95)	3.32 ± 1.60	2.37 ± 1.42	5.69 ± 2.71
DEN → PB, 500 p.p.m.	10	161.91 ± 36.59[c]	3.08 ± 0.92[d]	20	20 (100)	20 (100)	9.60 ± 3.15[d]	4.10 ± 2.86	13.7 ± 3.53[d]
Vehicle control	5	0	0	4	0	0	0	0	0
PB, 500 p.p.m.	5	1.34 ± 0.90[c]	0.004 ± 0.002[c]	4	0	0	0	0	0

[a]Data are mean ± SD values.
[b–d]Significantly different from the DEN control group; [b]$P < 0.05$; [c]$P < 0.01$; [d]$P < 0.0001$.
[c]Significantly different from the vehicle control group; $P < 0.05$.

Table II. Induction of oxidative stress in the livers of rats treated with PB for 10 weeks after initiation hepatocarcinogenesis with DEN

Treatment	No. of rats	8-OHdG 8-OHdG/10^6dG	Ogg1 mRNA Ogg1/GAPDH	P-450 content nmol/mg protein	Hydroxyl radicals RI, %
DEN[a]	10	1.86 ± 0.14	0.024 ± 0.002	0.59 ± 0.12	57.79 ± 12.19
DEN → PB, 2 p.p.m.	10	1.16 ± 0.26[b]	0.069 ± 0.031[e]	0.64 ± 0.13	58.73 ± 12.94
DEN → PB, 15 p.p.m.	10	1.49 ± 0.12	0.071 ± 0.035[e]	0.68 ± 0.13	65.99 ± 11.79
DEN → PB, 500 p.p.m.	10	2.31 ± 0.73[d]	0.044 ± 0.011[d]	2.03 ± 0.38[e]	95.44 ± 17.88[e]
Vehicle control	5	1.31 ± 0.24[d]	0.033 ± 0.013	0.85 ± 0.08[d]	57.03 ± 10.62
PB, 500 p.p.m.	5	2.63 ± 0.73[g]	0.058 ± 0.016[f]	2.12 ± 0.21[g]	88.49 ± 7.96[g]

[a]Data are mean ± SD values.
[b–e]Significantly different from the DEN control group: [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$; [e]$P < 0.0001$.
[f,g]Significantly different from the vehicle control group: [f]$P < 0.01$; [g]$P < 0.0001$.

Influence on 8-OHdG formation

HPLC analysis demonstrated 8-OHdG levels in rat liver DNA to be significantly decreased in the group administered PB at a dose of 2 p.p.m. in experiment 1 (Table II). Similar results were obtained in the short-term experiment (data not shown). Alteration of 8-OHdG showed a similar pattern with that of GST-P positive foci numbers, areas and incidence and multiplicities of liver tumors (Table I). Formation of oxidative base modifications was increased in the initiation control group as compared with vehicle controls and significantly enhanced by application of PB at a dose of 500 p.p.m. (Table II). The results of immunohistochemical examination and HPLC analysis for 8-OHdG showed good concordance (data not shown).

Increase of Ogg1 mRNA expression

In an attempt to provide an explanation for the observed inhibition of oxidative DNA damage by PB at low dose, we analyzed mRNA expression for the 8-OHdG repair enzyme, Ogg1, by real-time LC-PCR. Significant elevation was noted with PB application at 2 p.p.m., 15 and 500 p.p.m. in experiment 1 (Table II). Therefore, the difference in 8-OHdG levels observed in this experiment might be dependent on the balance between the production of ROS, formation of 8-OHdG and its elimination by Ogg1.

Alteration to cell proliferation

Double staining of GST-P and PCNA revealed an increase in number of positively stained cells within the area of GST-P positive foci in the 15 and 500 p.p.m. PB dose groups. Several hepatic foci, consisting of > 20 cells, contained a large number of PCNA positive nuclei (Figure 1A and B). The biggest foci were strongly positive for PCNA. On the contrary, small foci were almost negative for PCNA in all groups. For that reason we estimated PCNA index within the areas of GST-P foci of the same size (20–30 cell foci). In the livers of rats treated with PB at a dose of 2 p.p.m., the PCNA index for GST-P positive foci was significantly lower than in the DEN control group (Table III and Figure 1C). Alterations to cell proliferation in the areas of hepatic foci and oxidative base modifications in the rat liver DNA were similar in the low dose group (Figure 2). Furthermore, cell proliferation was also slightly suppressed in the background liver parenchyma of 2 p.p.m. PB treated rats (Table III). Administration of PB at 500 p.p.m. without DEN initiation resulted in a non-significant decrease in PCNA positive cells in normal-appearing tissue (Table III).

Evaluation of apoptosis (ssDNA)

Analysis by double immunohistochemistry for GST-P and ssDNA demonstrated significant increase of apoptosis in the livers of rats treated with DEN alone and DEN followed by PB at 500 p.p.m., predominantly in the centralobular region. Hepatic foci were often localized in the areas positive for ssDNA (Figure 1D). In contrast, in the livers of rats given DEN and PB at a dose of 2 p.p.m. only a few apoptotic cells in the normal-appearing background liver tissue were found in line with the lowering of 8-OHdG (Figure 1E, Figure 2 and Table III). Immunohistochemistry for 8-OHdG and double staining for GST-P and apoptosis performed with serial sections of the liver tissue revealed ssDNA staining consistently in the regions positive for 8-OHdG (Figure 1D and F). No significant changes in apoptosis were apparent within areas of GST-P positive foci between initiation control, low, median and high dose PB groups. All GST-P positive foci were almost negative for apoptosis (Table III, Figure 1D and E).

Activation of detoxifying enzymes

Similar to the results for DMPO–OH spectrum, dose-dependent elevation of cytochrome P-450 total content in the liver microsomal fraction was found in experiments 1 (Table II) and 2 (data not shown). Significant decrease of liver P-450 was found in the DEN control group as compared with the vehicle controls. Slight but significant increase of CYP3A2-2β (0.16 ± 0.01 nmol/mg/min), CYP3A2-6β (0.64 ± 036 nmol/mg/min), CYP2C11 (0.86 ± 0.39 nmol/mg/min) and OR (0.07 ± 0.003 nmol/mg/min) activities was evident after 8 days treatment with PB at 2 p.p.m. in the short-term experiment (Figure 3A). In the long-term study, PB administration at 2 p.p.m. after DEN initiation enhanced activities of CYP3A2 and CYP2C11 without significance (data not shown). However, no change in mRNA expression or protein levels of CYP2B1/2, CYP3A2 and CYP2C11 was found after treatment with the low dose of PB in experiments 1 (data not shown) and 2 (Figure 3B–D). In contrast, when animals were administered PB at a dose of 15 p.p.m. and higher, a significant increase in CYP2B1/2 and CYP3A2 mRNAs, proteins and activities was detected (Figure 3 and data not shown). In addition, CYP2C11 protein and activity levels were decreased after 8 days of PB administration at a dose of 500 p.p.m. The data were consistent with the previously published results (36). However, in experiment 1 CYP2C11 protein and mRNA were significantly enhanced after application of PB for 10 weeks, suggesting that short-term treatment results in CYP2C11 inhibition, however, continuous application leads to

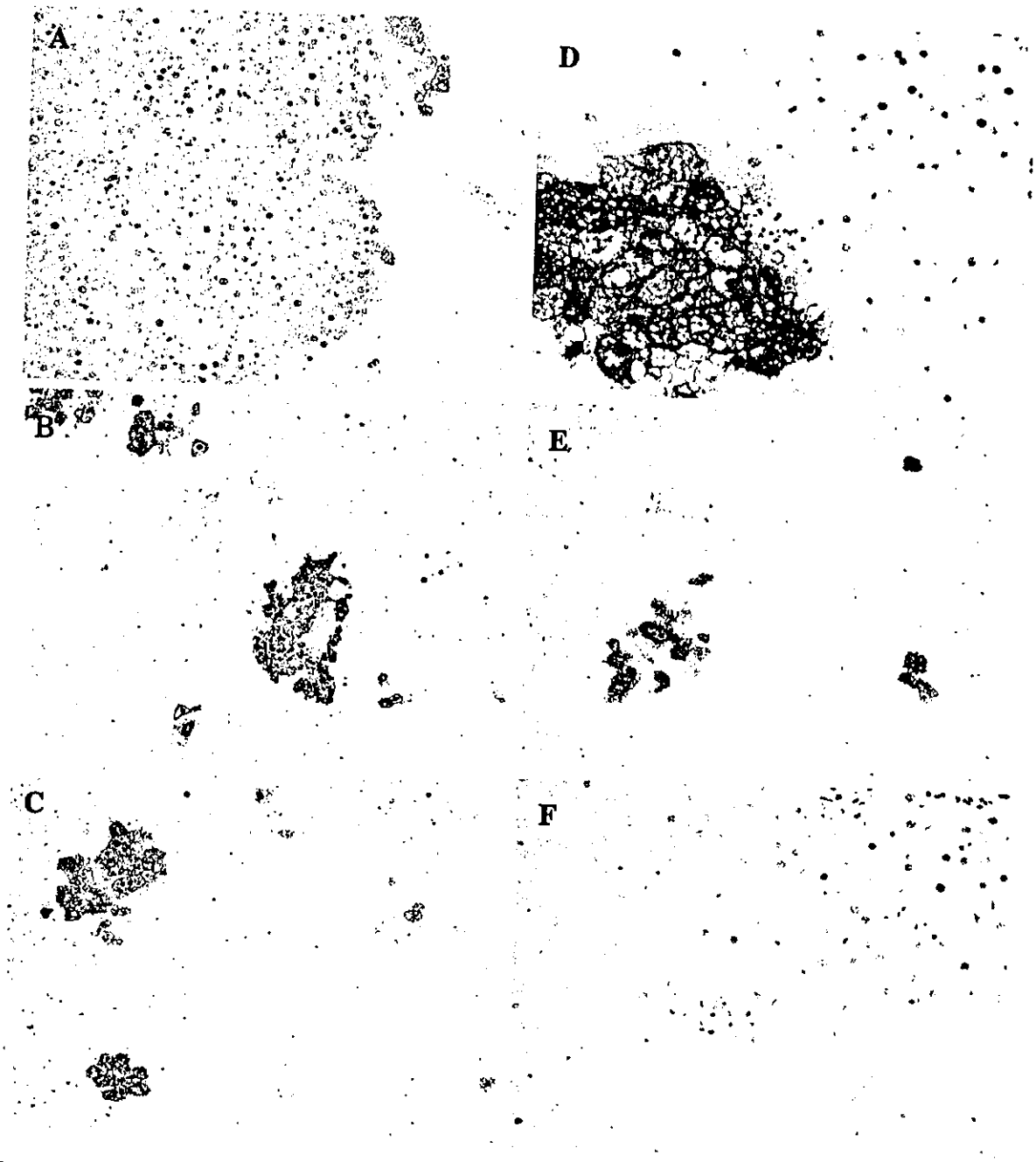

Fig. 1. Double immunohistochemistry for GST-P (red) and PCNA (blue), GST-P and apoptosis (ssDNA) (blue) and immunohistochemical assessment of 8-OHdG in the livers of F344 rats treated with PB for 10 weeks after DEN initiation. (A–C) GST-P and PCNA after PB at 500 p.p.m. (×100), 15 p.p.m. (×200) and 2 p.p.m. (×100), respectively. (D and E) GST-P and apoptosis after PB at 500 (×200) and 2 p.p.m. (×100), respectively. (F) 8-OHdG immunohistochemistry in the liver of the rat treated with PB at 500 p.p.m. (×200). Note, that (D) and (F) are serial liver sections and the good concordance between 8-OHdG and ssDNA stainings.

its elevation due to an increase of mRNA expression. Alterations of CYP3A2-2β, CYP3A2-6β and CYP2C11 activities were clearly apparent with that of OR, suggesting enzymes of the hepatic detoxification system to be activated by PB treatment at a dose of 2 p.p.m., but without any change in mRNA or protein levels of P-450 isoforms.

cDNA microarray analysis

The results of Atlas 1.2 Rat cDNA microarray analysis of differentially expressed genes, which were up- or down-regulated at least 2-fold in the livers of rats, obtained in experiment 1 after 10 weeks of PB administration are presented in Table IV. Ogg1, CYP2B1, CYP3A2 and CYP2C11 genes were not included in the analysis and their mRNA expression was detected by real-time and competitive RT–PCRs. Reproducibility and specificity of the data was confirmed by repeating microarray analysis two times. The results obtained from three determinations were compared with Atlas Image Software and the strong concordance of the results was found. Furthermore, the data obtained by cDNA microarray on

Table III. PCNA and apoptotic indices for livers of rats treated with PB for 10 weeks after initiation hepatocarcinogenesis with DEN

Treatment	No. of rats	PCNA (No./1000 cells)		Apoptosis (No./1000 cells)	
		GST-P	Surrounding area	GST-P	Surrounding area
DEN[a]	10	14.9 ± 5.1	4.3 ± 1.8	0.7 ± 0.5	35.9 ± 5.6
DEN → PB, 2 p.p.m.	10	9.6 ± 2.9[b]	3.9 ± 1.6	0.9 ± 0.7	12.3 ± 3.5[e]
DEN → PB, 15 p.p.m.	10	22.2 ± 6.8[c]	4.6 ± 0.7	0.8 ± 0.7	19.3 ± 10.2[d]
DEN → PB, 500 p.p.m.	10	26.3 ± 7.6[d]	4.1 ± 1.3	1.1 ± 0.3	69.7 ± 8.1[e]
Vehicle control	5	0	3.3 ± 0.7	0	1.6 ± 0.9[e]
PB, 500 p.p.m.	5	0	2.7 ± 0.4	0	4.2 ± 2.21

[a]Data are mean ± SD values.
[b–e]Significantly different from the DEN control group; [b]$P < 0.05$; [c]$P < 0.01$; [d]$P < 0.001$; [e]$P < 0.0001$.

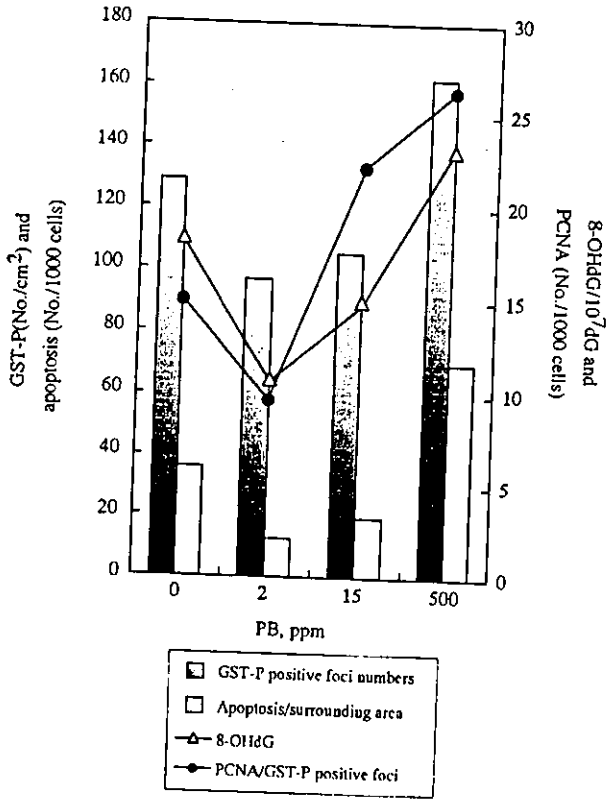

Fig. 2. Co-ordinated change in GST-P positive foci numbers and PCNA indices, for apoptosis and 8-OHdG levels in background liver parenchyma after DEN then PB for 10 weeks. Three preparations of the liver tissue for each rat were used in the analysis of GST-P positive foci numbers. 5000 cells on the normal-appearing liver tissue and total cells in the GST-P positive foci areas were checked for the analysis of PCNA and apoptotic indices. Note the inhibitory effects of PB at the dose of 2 p.p.m. and elevation at 500 p.p.m.

the expression of *GAD65* were checked by quantitative RT–PCR analysis, which showed the same pattern (data not shown). In the livers of animals in the DEN control group, not administered PB, over-expression of GST-P subunit (*GST7-7*), *rac-beta* serine/threonine kinase (*rac-PK-beta*), alcohol dehydrogenase class 1 (*ADH1*), plakoglobin, annexin V and interleukin-4-receptor was found. Furthermore, genes including *GAD65*, thymidylate synthase (*TS*), gamma aminobutyric acid (GABA-A) receptor beta subunit, guanylyl cyclase, proteasome subunit RC 10-II and orphan nuclear receptor TR_4 were down-regulated. PCNA and others related to cell proliferation genes were slightly up-regulated, however, without significance.

Comparison between groups treated with DEN alone and DEN followed by PB at a dose of 2 p.p.m. revealed increased expression of *GAD65*, phospholipase C delta and chloride channel CIC-7 in the latter (Figure 4 and Table IV). Mitogen-activated protein kinase p38 (*p38*), *Lyn* tyrosine protein kinase, *rac-beta* serine/threonine kinase (*rac-PK-beta*), calponin and calcium/calmodulin-dependent protein kinase type IV genes were down-regulated. When animals were administered PB at a dose of 15 p.p.m., an enhancement of cytochrome P-450 isoenzyme 3A1, nuclear tyrosine phosphatase PRL-1, presenilin and reduction of plakoglobin gene expression were detected. Furthermore, results of cDNA microarray showed that treatment with PB at a dose of 500 p.p.m. after DEN initiation resulted in up-regulation of the hepatic detoxifying enzymes including cytochrome P-450 isoforms 3A1, 2C7 and 4A8, NADPH P-450 cytochrome reductase and GST-P and -Ya subunits, and nuclear tyrosine phosphatase PRL-1, guanine nucleotide binding protein G(O) alpha subunit, DOPA decarboxylase, DOPA/tyrosine phosphatase and presenilin 2 genes. Administration of PB at a dose of 500 p.p.m. without DEN initiation led to the increase in expression of the GST-Ya subunit, cytochrome P-450 isozymes 3A1 and B5, NADPH P-450 cytochrome reductase, guanine nucleotide binding protein G(O) alpha subunit and calponin.

Discussion

The present study demonstrated a hormetic effect of PB treatment on rat hepatocarcinogenesis, inhibited development of GST-P positive foci and liver tumors by PB at a dose of 2 p.p.m. being evident after administration for 10 and 33 weeks, respectively. The number and area of GST-P positive foci in the low dose group were decreased by 25% and 31%, respectively. Furthermore, an impressive inhibition in the multiplicity of HCC was observed, although the multiplicity of adenoma and incidences of HCC and adenoma were decreased without significance. In contrast, the chemical administered to rats at a dose of 500 p.p.m. resulted in promotion in line with the literature (4–9).

8-OHdG, a marker of oxidative DNA damage, potentially involved in carcinogenesis in various experimental models, is known to cause mutations (12–14,37,38). Induction of a significant and steady elevation of 8-OHdG is thought to play an

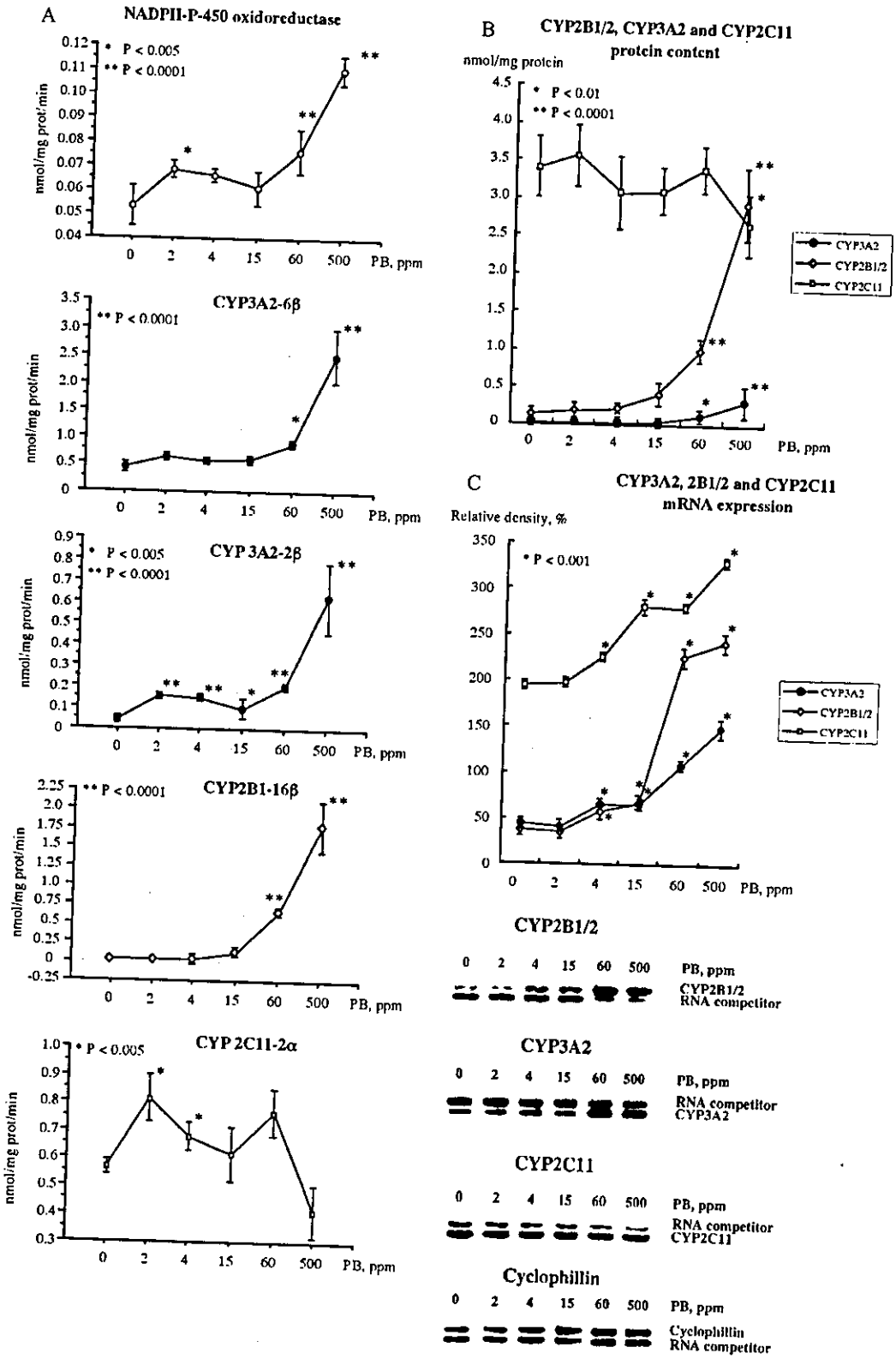

Fig. 3. Alterations of cytochrome P-450 isoenzymes CYP3A2, CYP2B1/2, CYP2C11 and OR induced by administration of PB at different doses for 8 days in experiment 2. (A) Activity, (B) protein and (C) mRNA levels were evaluated in terms of P-450 mediated hydroxylation of testosterone, western blotting and competitive RT–PCR. (D) Demonstration of the data obtained by competitive RT–PCR. The results are mean ± SD values ($n = 5$).

integral role in carcinogenesis (39). Significant increase of 8-OHdG levels in the DEN initiation group over the vehicle control observed in the present study supported this concept. Therefore, the suppression of GST-P positive foci and tumor development by PB at low dose might be related to the observed reduction in 8-OHdG level in the DNA of hepatocytes. Conspicuous increase of GST-P positive foci numbers and areas after 10 weeks of PB administration at a dose of 500 p.p.m., on the other hand, was associated with elevation of intracellular OH• and 8-OHdG levels in the rat liver DNA. Enhanced protein and activity levels of P-450 isoenzymes CYP2B1/2 and CYP3A2 might be related to accumulation of OH• and 8-OHdG with the high dose (21).

The induction of *OGG1* mRNA expression observed in this study suggested that a reason for the 8-OHdG decrease in the background liver parenchyma in the low dose group might have been activation of 8-OHdG repair. This has been shown to be elevated in response to DNA damage generated by hydroxyl radicals and is attributable to glycosylases, endonucleases and lyase activity (40,41). Our results revealed the enhancement of Ogg1 mRNA expression after treatment at 2 p.p.m. as well as 15 and 500 p.p.m. dose of PB. However, the levels of hydroxyl radicals and oxidative DNA base modifications differed between the low and high dose groups. OH• increased dose-dependently, while 8-OHdG generation was suppressed by PB at a dose of 2 p.p.m. and markedly enhanced by the high dose treatment. It is thus possible that Ogg1, induced by generation of OH•, might swing the balance between generation and elimination of 8-OHdG in DNA. Hydroxyl radicals and 8-OHdG levels were presumably very high due to the increase of cytochrome P-450 CYP2B1/2 and CYP3A2 in the livers of rats treated with PB at a dose of 500 p.p.m., and the repair of oxidative base modifications was insufficient to prevent the elevation of 8-OHdG.

The present data demonstrated an enhancement of cell proliferation particularly in the foci consisting of more than 20 cells in the groups treated with 15 and 500 p.p.m. dose of PB. However, the PCNA index was significantly suppressed with the low dose. As detected by cDNA microarray analysis, PB treatment at low dose enhanced gene expression of GAD65, an enzyme involved in the synthesis of GABA. Furthermore, the expression of *GAD65* was reduced in the livers of animals of the initiation group as compared with the vehicle control, but no significant differences in *GAD65* expression were observed in 15 and 500 p.p.m. dose groups. Therefore, the induction of *GAD65* might be specific for PB activity at a dose of 2 p.p.m. Recently, it has been reported about the negative correlation between the expression of GABA-A receptors in hepatocytes and thymidine incorporation in liver specimens but without evidence of causal relationship, and GABA-B receptor subtype involvement in mechanisms of hepatocyte DNA synthesis and mediation of growth stimulation (42–44). Therefore, the increased mRNA expression of GAD65 by the low dose of PB observed in the present study may reflect something interesting; however, the role of GABA and the subtypes of GABA receptors in liver proliferation is not at all clear and needs further investigation. Furthermore, suppression of gene expression of signal transduction modulators, such as MAP kinase p38, *Lyn* tyrosine kinase, calcium-calmodulin-dependent protein kinase and *rac-beta* serine/threonine kinase detected with the use of cDNA microarray analysis might have been related to the inhibitory effect of PB on cell proliferation. To support this idea, the

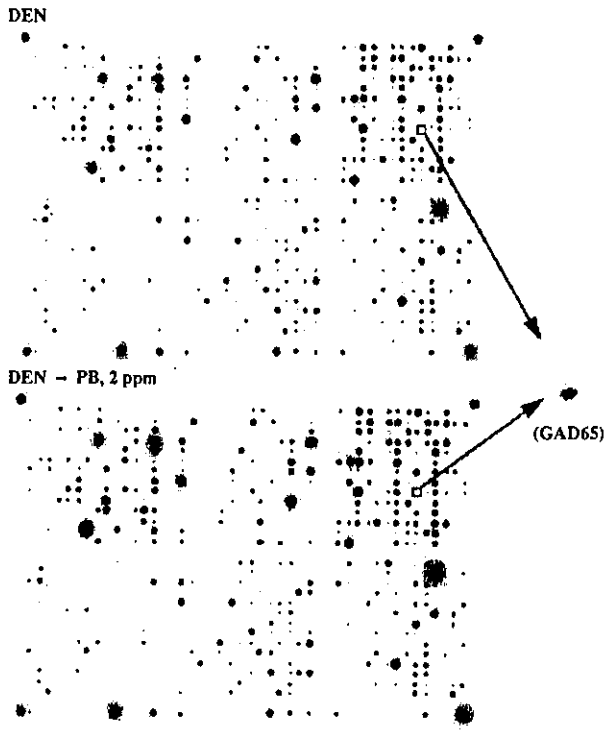

Fig. 4. Representative photographs of cDNA microarray phosphoimages. (Top) Gene expression in the DEN control group. (Bottom) 2 p.p.m. PB-treated group. Note the up-regulation of *GAD65*.

analysis of the levels of the active (phosphorylated) forms of kinases will be performed in our future investigations.

Kitano *et al.* (15) have reported that development of transforming growth factor-α (TGF-α) positive foci in the livers of rats treated with PB at different doses is basically similar to that of GST-P positive foci. Hence, the suppression of cell proliferation detected by double immunohistochemical staining in the areas of hepatic foci by the treatment with low dose of the chemical and its elevation in the groups treated with 15 and 500 p.p.m. suggests a link to the previously observed effect of PB administration on formation of TGF-α positive foci. Rise of the PCNA labeling index within the GST-P areas observed in our study 10 weeks post-treatment with 15 and 500 p.p.m. dose of PB is generally in line with the previously published results regarding hepatocarcinogenesis (45). Furthermore, as detected by cDNA microarray analysis and competitive RT–PCR, PB treatment at high dose enhanced gene expression of enzymes involved in xenobiotic metabolism in the liver consistently with previously reported data (46). Its administration at 15 and 500 p.p.m. after DEN initiation increased expression of nuclear tyrosine phosphatase PRL-1, affecting the cell growth. Moreover, PB at 500 p.p.m. enhanced gene expression of guanine nucleotide binding protein G(O) alpha subunit, which may result in activation of GTPase signal transduction pathway and induction of cell proliferation (47).

A reduction of apoptosis in the normal-appearing liver tissue surrounding the GST-P positive foci due to the inhibition of oxidative DNA damage after treatment with low dose PB might suppress their enlargement. Apoptosis of the normal-appearing tissue might be one of the factors regulating the size

Table IV. Differentially expressed genes in the livers of rats treated with PB for 10 weeks (Exp. 1)

Treatment	Function	Gene	Accession no.	Difference	Ratio
DEN → PB, 2 p.p.m.[b]	GABA synthesis				DEN → PB/DEN
		Glutamic acid decarboxylase (GAD65)	M72422	10	2.43
	Voltage-gated ion channels				
		Chloride channel CLC-7	Z67744	5	2.25
	Signal transduction modulators and effectors				
		Phospholipase C delta	D50455	4	2.11
		MAP kinase p38	U73142	−3	0.50
		Ca-CAM-dependent PK	M63334	−3	0.50
		Lyn tyrosine protein kinase	AF000300	−4	0.43
		Rac-beta serine/threonine kinase (*rac*-PK-beta)	D30041	−5	0.44
		Dipeptidyl peptidase, Gp 110 glycoprotein	J02997	−4	0.50
		Calponin	M10161	−5	0.38
DEN → PB, 15 p.p.m.[b]	Xenobiotic metabolism				
		Cytochrome P450 3A1	X71071	246	2.01
	Cell growth				
		Nuclear tyrosine phosphatase PRL-1	L27843	35	2.03
	Signal transduction modulators and effectors				
		Presenilin 2	AB004454	7	2.17
		Plakoglobin	U58858	−9	0.44
DEN → PB, 500 p.p.m.[b]	Xenobiotic metabolism				
		Cytochrome P450 3A1	X71071	1029	5.24
		GST-P subunit (GST7-7)	X02904	184	5.28
		GST-Ya subunit (GST YA)	K01931	260	2.10
		Cytochrome P450 2C7	M18774	626	3.37
		Cytochrome P450 4A8	M37828	32	2.03
		NADPH-cytochrome P450 oxidoreductase	M12516	37	2.76
	Cell growth				
		Nuclear tyrosine phosphatase PRL-1	L27843	39	2.15
	Amino acid and energy metabolism				
		DOPA decarboxylase (DDC)	M27716	12	2.09
		DOPA/tyrosine sulfotransferase	U38419	255	2.45
		Triacylglycerol lipase precursor	M16235	−11	0.42
	Signal transduction modulators and effectors				
		Guanine nucleotide binding protein alpha subunit (GNAO)	M17526	171	4.23
DEN[a]	Xenobiotic metabolism				DEN/vehicle
		GST-P subunit (GST7-7)	X02904	16	3.0
	Energy and nucleotide metabolism				
		Alcohol dehydrogenase A subunit (ADH1)	M15327	66	2.35
		Thymidylate synthase (TYMS, TS)	L12138	−110	0.45
	Signal transduction modulators and effectors				
		Rac-beta serine/threonine kinase (rac-PK-beta)	D30041	3	2.5
		Guanylyl cyclase (membrane form)	M74535	−3	0.50
		Plakoglobin	U58858	6	3.0
	GABA synthesis and GABA receptors				
		Glutamic acid decarboxylase (GAD65)	M72422	−6	0.33
		GABA receptor beta subunit	X15466	−3	0.40
	Nuclear receptors				
		Orphan nuclear receptor TR4	L27513	−17	0.49
	Proteasomal proteins				
		Proteasome subunit RC10-II	D21800	−10	0.47
	Cytokines and cytokines related components				
		Interleukin-4 receptor	X69903	3	2.0
	Membrane channels, transporters				
		Annexin V (ANX5)	M21730	4	2.0
PB, 500 p.p.m.[a]	Xenobiotic metabolism				
		GST-Ya subunit (GST YA)	K01931	435	2.63
		Cytochrome P450 3A1	M10161	2341	4.77
		Cytochrome P450 B5	D13205	562	2.25
		NAPDH-cytochrome P450 oxidoreductase	M12516	30	2.25
	Signal transduction modulators and effectors				
		Guanine nucleotide binding protein alpha subunit	M17526	414	5.36
		Calponin	X71071	19	2.36
		Mannose-6-phosphate/insuline like growth II receptor	U59809	51	2.96
	Membrane channels, transporters				
		Apolipoprotein A-IV precursor	M00002	−403	0.50

[a]Genes that were up-regulated (+) or down-regulated (−) versus vehicle control.
[b]Versus DEN control group.

of foci, as the enlargement of GST-P positive foci probably is more difficult in the case of non-apoptotic surrounding tissue. The results of the immunohistochemical examination of GST-P, ssDNA and 8-OHdG in serial sections showed that 8-OHdG and ssDNA positive hepatocytes were located in the same area. Furthermore, in our previous study, double immunohistochemistry for 8-OHdG and apoptosis in the livers of rats treated for 8 days with PB at a dose of 500 p.p.m. revealed high levels of concordance between induction of 8-OHdG and apoptosis (ssDNA) (21). Thus, the data of the present experiment are in agreement with our previous results. Moreover, recently, depression of apoptosis by PB has been explained on the basis of its ability to inhibit *p53* (48), $p21^{WAF1/Cip1}$ (49), and enhance *bcl-2* gene family expression (50). PB may also co-operate with *c-myc* and TGF-α in the selective inhibition of apoptosis through diverse molecular pathways (51).

A decrease of P-450 expression in liver nodules induced by DEN has been found as compared with the normal tissue (52). The data are consistent with the results of the present experiment showing a reduction of P-450 total content in the liver microsomal fraction of the DEN control animals. Furthermore, it has been reported that suppressive effect of PB on the development of pre-neoplastic lesions might be due to the stimulation of hepatic drug-metabolizing enzymes, which detoxify carcinogens (17). Therefore, activation of P-450 isoenzymes CYP2C11, CYP3A2 and OR in the liver microsomes observed in the present study after administration of PB at low dose, if not accompanied by induction of their gene expression leading to the tremendous elevation of $OH^{\bullet}$, might have a protective effect. No significant differences in mRNA or protein levels of CYP3A2, CYP2B1/2 and CYP2C11 were detected in the low-dose group in both experiments, suggesting that those enzymes of the detoxification system were activated, but their gene expression was not altered. In contrast, P-450 isoenzymes mRNA and protein levels were conspicuously elevated in the high dose-treated groups, and the data were in line with the previously noted results (11). In addition, an inhibitory effect of PB on development of GST-P positive foci in a medium-term rat liver bioassay was reported previously to correlate with changes in CYP3A2 isoenzyme protein content, which was suppressed by the low and enhanced by high doses of PB (15). However, in this study we did not observe any effect of PB at low dose either on the levels of CYP3A2 mRNA or protein expression, possibly due to differences in experimental models.

In conclusion, PB, a promoter of hepatocarcinogenesis in rodents at high dose, at low dose inhibited the formation of GST-P positive foci and liver tumors. Our data demonstrate the presence of a threshold for the promoting effects of PB and the lack of linearity in the low-dose area of the dose–response curve, rather providing evidence of a J-shape. Suppression of 8-OHdG formation and apoptosis in background normal-appearing liver parenchyma, cell proliferation in the areas of hepatic foci, and alterations in the expression of genes related to control of cell proliferation, might explain in part the hormetic inhibition of hepatocarcinogenesis by PB at low dose.

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