[CANCER RESEARCH 54, 3145–3152, June 15, 1994]

Colocalized Alterations in Connexin32 and Cytochrome P450IIB1/2 by Phenobarbital and Related Liver Tumor Promoters[1]

Mark J. Neveu,[2] Karlee L. Babcock, Elliot L. Hertzberg, David L. Paul, Bruce J. Nicholson, and Henry C. Pitot[3]

McArdle Laboratory for Cancer Research and Center for Environmental Toxicology, University of Wisconsin, Madison, Wisconsin 53706-1599 [M. J. N., K. L. B., H. C. P.]; Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461 [E. L. H.]; Department of Anatomy and Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 [D. L. P.]; and Department of Biological Sciences, SUNY-Buffalo, Buffalo, New York 14260 [B. J. N.]

ABSTRACT

Direct intercellular signal transduction is achieved by the passage of small molecules through gap junctions (GJ). Previous studies in our laboratory showed that the liver tumor promoter phenobarbital (PB) reversibly decreases the abundance of the GJ protein connexin32 (Cx32) in both preneoplastic-altered hepatic foci and centrolobular hepatocytes (M. J. Neveu *et al.*, Cancer Commun., *2:* 21–31, 1990). Because the inhibitory effects of PB on GJ intercellular communication are prevented by the nonspecific cytochrome P-450 inhibitor SKF-525A (J. E. Klaunig, *et al.*, Toxicol. Appl. Pharmacol., *102:* 533–563, 1990), we investigated whether alterations in Cx32 are coincident with changes in the major PB-inducible cytochrome P-450, termed b/e or IIB1/2.

Immunostaining of liver cryosections from rats fed dietary PB demonstrated that centrolobular hepatocytes that exhibit reduced Cx32 express enhanced cytochrome P450IIB1/2 protein. In contrast, no change in the periportal distribution of connexin26 immunoreactivity was found in PB-treated rats. In addition, rats were treated with the structurally related barbiturates pentobarbital, amobarbital, barbital, and barbituric acid. We found that the extent of the hepatic lobule occupied by coincident centrolobular alterations in Cx32 and P-450 staining correlates with the ability of the compounds to promote liver oncogenesis.

To determine the molecular mechanisms responsible for the modification in Cx32 staining, we examined the mRNA and protein levels of Cx32 and P450IIB1/2 in total-tissue homogenates from PB-treated rats. Northern blotting demonstrated that dietary PB dramatically induced P-450IIB1 mRNA, but the same RNA samples failed to show alterations in Cx32 steady-state transcripts. Consistent with these findings, the level of Cx32 protein in total liver homogenates did not change in rats chronically fed PB. Examination of Cx32 solubility in 20 mM NaOH demonstrated that PB treatment results in the generation of a NaOH-soluble form of Cx32 (*i.e.*, 47 kDa). In addition, trypsinized paraffin-embedded liver sections from PB-treated rats exhibited diffuse cytoplasmic Cx32 staining that was restricted to centrolobular cells. Our results show that PB and related barbiturate tumor promoters reversibly down-regulate punctate Cx32 staining in centrolobular hepatocytes posttranslationally, possibly through modification(s) in the transport, assembly, and/or turnover of GJs.

INTRODUCTION

GJs[4] are aggregates of transmembrane pores that mediate intercellular signal transduction by regulating the intercellular transfer of ions, metabolites, second messengers, and other molecules less than 1000 daltons in mass (1, 2). Cloning studies have identified a family of GJ proteins, termed connexins, that are distinguished by their predicted molecular mass (2). Many normal biological processes including tissue homeostasis, embryonic development, glandular secretion, cellular differentiation, and growth control are modulated by GJIC (2–4).

Alterations in the number of GJs or the level of GJIC have been found in preneoplastic and neoplastic cells from several rodent and human tissues. Furthermore, many tumor promoters, oncogenic proteins, and growth factors modulate GJIC (2–7). In addition, transfection of tumor cells with either Cx32 or Cx43 results in reduced growth rates in cell culture and in nude mice (3, 4).

In rodent and human liver, the abundance of GJs, the level of GJIC, and the expression of Cx32 are diminished in most preneoplastic altered hepatic foci and carcinomas (4, 6, 7). In addition to these changes in mutated cells, the liver tumor promoter PB decreases the morphological abundance of GJs (8) and the level of punctate Cx32 staining in centrolobular hepatocytes (9). Furthermore, physiological studies demonstrated that PB rapidly inhibits hepatocyte GJIC in primary cultures (7, 10) and liver tissue slices (11). The PB-induced alterations in both GJIC and Cx32 staining are rapidly reversible after withdrawal of PB (9, 10). In this report we examined whether the PB-induced changes in Cx32 staining in centrolobular hepatocytes associate with other previously characterized changes in gene expression.

Administration of PB to rodents induces a program of adaptive changes in centrolobular hepatocytes. The modifications include increased levels of smooth endoplasmic reticulum, cytochrome P450IIB1/2, epoxide hydrolase, and isozymes of glutathione *S*-transferase (12, 13). A strong association exists between the ability of a subclass of compounds, including barbiturates, to promote hepatocarcinogenesis and the ability to induce cytochrome P450IIB1/2 enzymatic activity (14). Relatively minor modifications in the structure of barbiturates can modify these activities (see Fig. 1). Amobarbital, pentobarbital, and barbituric acid are weak inducers of P450IIB1/2 enzymatic activity and are ineffective promoters of rat hepatocarcinogenesis (15–20). Furthermore, the inducibility of P450IIB1/2 activity correlates with the susceptibility of various strains of rodents to PB promotion (14, 22).

The present study examines whether alterations in Cx32 and P450IIB1/2 protein expression occur in livers from rats treated with PB, amobarbital, barbituric acid, pentobarbital, or barbital. Because the inhibitory effect of PB on GJIC in primary hepatocytes is prevented by the cytochrome P-450 inhibitor SKF-525A (23, 24), changes in Cx32 and P450IIB1/2 may colocalize. In support of this hypothesis, PB is a weak or ineffective inhibitor of GJIC in fibroblasts (V79) and in liver epithelial cells (WB-F344) that exhibit low levels of microsomal enzyme activity (7). To determine which molecular mechanisms were responsible for centrolobular alterations in Cx32 staining, we examined the expression of Cx32 mRNA and protein after dietary PB. Contrary to a previous report, which suggested that PB reduces the level of Cx32 mRNA (25), our results show that PB induces posttranslational alterations in the

Received 6/23/93; accepted 4/13/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The studies described in this investigation were supported by grants from the National Cancer Institute (CA-07175 and CA-45700), USPHS. M. J. N. was a predoctoral trainee in Environmental Toxicology of the National Institute of Environmental Health Sciences (ESO-7015).

[2] Present address: Pfizer Central Research, Eastern Point Rd., Groton, CT 06340.

[3] To whom requests for reprints should be addressed, at McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, 1400 University Ave., Madison, WI 53706-1599.

[4] The abbreviations used are: GJ, gap junction; GJIC, gap junctional intercellular communication; Cx32, connexin32; Cx26, connexin26; Cx43, connexin43; PB, phenobarbital; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; RT, room temperature; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

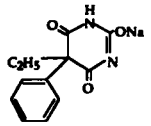
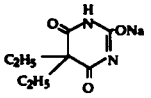
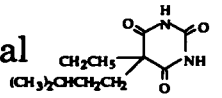
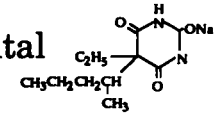
	Structure	Effects on Hepatocyte GJIC	Induction P450 IIB1/2	Liver Tumor Promoter
Barbituric acid		No Effect[12]	Negative[21]	Negative[19,20,21]
Phenobarbital		Inhibition[12]	Positive[16,21] ++++	Positive[17-21] ++++
Barbital		Inhibition[12]	Positive[16,21] ++++	Positive[19-21] ++++
Amobarbital		No Effect[12]	Positive[16,21] +	Negative[17,20,21] Weak[18,19]
Pentobarbital		Unknown	Positive[16,21] ++	Positive[18,19,21] ++

Fig. 1. Changes in GJIC and P450IIB1/2 during liver tumor promotion.

assembly and/or turnover of Cx32 that are colocalized with alterations in P450IIB1/2 expression.

MATERIALS AND METHODS

Animal Experiments. Male Fischer F344 rats (Harlan-Sprague-Dawley, Inc., Madison, WI) were maintained at 27°C on a 14-h light/10-h dark cycle and given AIN-76 diet (Teklad Test Diets, Madison, WI) and water *ad libitum*. Three rats per group were maintained on either AIN-76 diet (26) alone or supplemented with 0.01, 0.05, or 0.1% PB (free acid form; Sigma Chemical Co., St. Louis, MO) for 30, 60, or 90 days. Rats were also examined after 3, 5, and 9 days following 0.05% PB administration. In addition, rats were treated with an AIN-76 diet supplemented with 0.05% pentobarbital, amobarbital, barbital (sodium salt), or barbituric acid for 11 weeks (see Fig. 1). All barbiturates were obtained from Sigma. Rats were also treated for 6–12 months with an NIH-07 diet supplemented with either tamoxifen (500 mg/kg), CI Solvent Yellow (1250 ppm), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (0.01 μg/kg), 2,5,2′,5′-tetrachlorobiphenyl (100 ppm), WY-14,643 (0.05%), or ciprofibrate (0.01%) (9, 27). Following their respective regimens, rats were sacrificed by decapitation, and the livers were quickly excised, rinsed in saline, blotted dry, and weighed. Livers were then sliced with a razor blade, and portions from each lobe were frozen rapidly on solid CO_2 for staining studies or in liquid nitrogen for mRNA and protein isolations.

Antibodies. Antibodies generated against different epitopes of Cx32 and Cx26 were utilized to examine connexin protein expression. In Cx32 studies we used a mouse monoclonal antibody generated against purified rat liver GJ (M12.13) (21), a rabbit polyclonal antibody reactive with amino acids 98–124 of rat Cx32 (Lola) (21), and affinity-purified sheep polyclonal antibodies against rat liver GJ (594) (28). For Cx26 we used two different affinity-purified rabbit polyclonal antibodies raised against amino acids 101–119 (Lulu) (29) and amino acids 105–119 of rat Cx26 (a19) (from E. Hertzberg). In addition, an affinity-purified rabbit polyclonal antibody to purified cytochrome P450IIB1/2 served as a positive control for the PB-induced response (30). Nonspecific ascites fluid and preimmune serum were used as controls. As an additional control, antibodies generated against Cx32 were preabsorbed overnight at 4°C with highly purified rat liver GJs (31), spun at 12,000 × *g*, and then incubated with tissue sections.

Immunocytochemistry. Immunostaining of cryosections (6–8 μm) was performed as described previously (9). Briefly, after the blocking of endogenous biotin and peroxidase activity, the acetone-fixed sections were incubated with primary antibody overnight at 4°C. Antibody-antigen complexes were visualized by the biotin-streptavidin-peroxidase method with aminoethylcarbazole as the chromogen (Zymed Laboratories, Inc., San Francisco, CA). Double staining with P450IIB1/2 was performed with biotin-conjugated goat anti-rabbit antibody, followed by avidin conjugated to β-galactosidase and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma) as the chromogen (9). Paraffin-embedded tissues were prepared by immersing fresh liver in acetone for 24 h at 4°C, 100% ethanol for 7 h, and 100% xylene for 6 h, followed by infiltration medium (IF-200; Surgipath, Grayslake, IL), and then embedding medium (IF-400; Surgipath). Deparaffinized sections were air dried and extracted with 0.05% Triton X-100 in phosphate-buffered saline for 10 min at RT. Sections were washed with distilled water, air dried, and postfixed in acetone for 10 min at RT. Air-dried sections were washed in phosphate-buffered saline and then treated with trypsin (Zymed Histo-Kit) for 1 h at RT. Subsequently, the sections were blocked for nonspecific protein binding with 1% bovine serum albumin (Sigma) and 5% normal goat serum (Zymed) and incubated with primary antibody, and protein-antibody complexes were visualized as described above. Nuclei were counterstained with Mayer's hematoxylin, and sections were coated with Crystal/Mount (Biomedia Corp., Foster City, CA). All slides were viewed with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) and photographed with either light-field, Nomarski and/or dark-field microscopy. Semi-quantitative analyses of the acinar distribution of Cx32 (yellow dots) were performed by counting punctate immunoreactive spots in 1000 hepatocytes from periportal and centrolobular regions of three different rat livers. P450IIB1/2 expression (purple) was quantified simultaneously with dark-field illumination.

Immunoblot Analyses. Crude homogenates were prepared by disrupting approximately 50 mg of tissue for 30 s with a Brinkmann homogenizer (Brinkmann, Westbury, NY). Isolation buffer contained 4 mM $NaHCO_3$, 2 mM phenylmethylsulfonyl fluoride (Sigma), 2 μg/ml aprotinin (Sigma), 2 mM EDTA, 5 mM diisopropylfluorophosphate (Sigma), and the phosphatase inhibitors 100 mM sodium fluoride, 10 mM sodium phosphate, and 2 mM sodium *o*-vanadate (Sigma). For analysis of base solubility, tissues were homogenized in 20 mM NaOH followed by brief sonication at 50% power with a Branson

sonifier 250 (VWR, Chicago, IL). NaOH-insoluble pellets were recovered by centrifugation at 12,000 × *g* for 15 min, resuspended in 20 mM NaOH, repelleted, and then suspended in isolation buffer (32). The pH of supernatant fractions was adjusted to 6.8 with Tris-HCl prior to protein quantitation. Crude liver membranes were isolated as described previously (31). Protein quantitation was performed as described by Peterson (33).

Samples were solubilized in 2% SDS (Gallard-Schlesinger, Carle Place, NY) buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 50 mM DTT (Sigma) for 30 min at RT. Solubilized samples were separated by SDS-PAGE (34) with 3–5% stacking and 12.5% separating gels cast in a minigel apparatus (Bio-Rad, Richmond, CA). Transfer of the protein to positively charged Immobilon-P membranes (Millipore, Bedford, MA) was carried out in modified Towbin transfer buffer (10% methanol) at 300 mA for 90 min at 4°C (35). Residual gels were stained with Coomassie Blue R-250 (Bio-Rad) to evaluate equal loading and transfer of proteins.

Nonspecific protein binding of the membranes was blocked with filtered Blotto (5% Carnation nonfat dry milk powder in 40 mM Tris-HCl (pH 7.4–0.1% Tween 20–0.05% sodium azide) for 1 h at RT. Primary antibodies were incubated with the blots for 2 h at RT and then washed several times in 50 mM Tris-HCl (pH 7.4, 0.9% NaCl-0.05% sodium azide, followed by incubation with the appropriate affinity-purified rabbit secondary antibody (Chappel, Malvern, PA) for 1 h at RT. Antibody-antigen complexes were determined with 430 μCi/ml ^{125}I-protein A (ICN; specific activity, >70 μCi/μg). Rainbow ^{14}C-methylated-protein markers were used for molecular weight determination (Amersham, Arlington Heights, IL). Autoradiography with XAR-5 film (Eastman Kodak, Rochester, NY) was performed at −70°C with an intensifying screen. The relative amounts of immunoreactive band(s) were evaluated with an LKB soft laser densitometer (Biomed Instruments, Chicago, IL).

Northern Blot. Total RNA was isolated from pulverized snap-frozen tissues by the acid-guanidinium method (36), and Northern blots were performed with 10 μg total RNA per lane (37, 38). Ethidium bromide at a concentration of 0.2 μg/ml was added to the gels before electrophoresis to verify the integrity of the RNA as well as equal loading before and after transfer to GeneScreen Plus (DuPont, Boston, MA). *Eco*RI fragments of the complementary DNAs corresponding to near full-length coding mRNA for Cx32 (1.5 kilobases) (21) and Cx26 (1.1 kilobases) (28) were isolated from plasmid sequences by agarose electrophoresis and purified by GeneClean (Bio101, Inc., La Jolla, CA). Radiolabeled probes [^{32}P]dCTP, 3000 Ci/mmol; Amersham) were generated with the multiprime DNA labeling system (Amersham) followed by G-50 Quickspin purification (Boehringer Mannheim, Indianapolis, IN). Prehybridization, hybridization, and posthybridization washes were performed as described previously (37). The expression of P450IIB1 was examined with a 22-base pair oligonucleotide probe as described previously (38). Blots were exposed to XAR-5 X-ray film (Eastman Kodak) at −70°C with a Lighting Plus intensifying screen (DuPont). The relative amount of connexin mRNA was evaluated by an LKB soft laser densitometer. Multiple exposures were taken of the blots to verify that the intensity of the bands was in a linear response range of XAR-5 film.

RESULTS

A homogeneous pattern of punctate Cx32 immunoreactivity was observed throughout the hepatic lobule in livers from rats maintained on an AIN-76 diet (Fig. 2*A*). These hepatocytes exhibited ordered punctate immunoreactivity located between juxtaposed hepatocytes. In agreement with a previous report (9), we found a pronounced zonal reduction in Cx32 staining in centrolobular hepatocytes after 30 days of dietary 0.1% PB. Double immunostaining demonstrated that alterations in Cx32 staining were coincident with induction of cytochrome P450IIB1/2 immunoreactivity (Fig. 2*B*). A similar colocalization of reduced Cx32 staining and induction of epoxide hydrolase was observed in the livers of PB-treated rats (Ref. 12; data not shown). Semiquantitative determination of the average number of Cx32 immunoreactive spots in 1000 periportal hepatocytes from three rat livers showed no significant difference between rats maintained on AIN-76 [6.2 ± 2.2 (SE)] and those rats on a diet supplemented with 0.05% PB (6.8 ± 2.7). In contrast, punctate Cx32 staining in centrolobular hepatocytes from rats treated with dietary 0.05% PB (1.4 ± 1.2) decreased 4-fold when compared with centrolobular hepatocytes from AIN-76 control livers (7.8 ± 2.1). Unlike the homogeneous staining of Cx32 in normal rat liver, Cx26 staining is restricted to periportal hepatocytes (39). Staining of serial cryosections from rats treated for 30 days with 0.1% PB showed that Cx26 staining was not altered in periportal hepatocytes (Fig. 2*C*) that continue to express Cx32 (Fig. 2*D*).

The proportion of the liver lobule, defined by the distance between the portal tract and the terminal hepatic vein, exhibiting alterations in Cx32 and P450IIB1/2 depends upon the concentration and duration of PB exposure. Whereas 74 ± 7% of the liver lobule exhibits coincident changes after 30 days of 0.1% PB (Fig. 2*D*), similar treatment with 0.01% PB resulted in only 13 ± 5% of the lobule showing altered staining. Temporal analysis demonstrated that the portion of the liver lobule occupied by alterations in P450IIB1/2 and Cx32 staining increased with time from 20 ± 3% at 60 h, to 34 ± 5% at 120 h, and to 47 ± 7% after 9 days of 0.05% dietary PB. The changes observed after 9 days were comparable to changes in those livers maintained on 0.05% PB for 4, 8, and 11 weeks.

Fig. 1 compares the published effects of various barbiturates on GJIC (primary rodent hepatocytes), induction of cytochrome P-450 (pentoxy- and benzyloxyresorufin activity), and rat liver tumor-promoting ability. The percentages of the liver lobule occupied by changes in Cx32 and P450IIB1/2 were examined after 11 weeks of continuous dietary exposure (4.3 mM; equivalent to 0.1% PB) of the compounds listed in Fig. 1. In accordance with studies by Nims *et al.* (15), we found that PB and sodium barbital increased the liver:body weight ratio, whereas amobarbital, barbituric acid, and pentobarbital were ineffective (Table 1). Coincident alterations in P450IIB1/2 and Cx32 were observed in rats treated with all compounds except barbituric acid. The extent of the liver lobule occupied by the changes varied among different PB congeners. In agreement with the properties listed in Fig. 1, the extent of the liver lobule showing alterations correlated with previously reported abilities to promote rat hepatocarcinogenesis (phenobarbital > barbital > pentobarbital > amobarbital).

We also examined P450IIB1/2 and Cx32 staining in livers treated with a diet supplemented with the liver tumor promoters tamoxifen, CI Solvent Yellow, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,5,2′,5′-tetrachlorobiphenyl, Wy-14,643, or ciprofibrate (see "Materials and Methods"). Coincident changes similar to those in Fig. 2*B* were observed in livers from rats treated with either tamoxifen, CI Solvent Yellow, or 2,5,2′,5′-tetrachlorobiphenyl, whereas no change in Cx32 or P450IIB1/2 staining was observed in animals treated with the other tumor promoters. These results further support the association between alterations in P450IIB1/2 expression with modifications in Cx32 staining in centrolobular hepatocytes.

Because the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate decreases GJIC by posttranslational alterations in connexin43 (40, 41), we pretreated frozen and paraffin-embedded sections prior to primary antibody incubation to test whether Cx32 antigenic sites are masked in PB-treated livers (see "Materials and Methods"). Pretreatment of paraffin-embedded sections with trypsin and Triton X-100 unmasked Cx32 immunoreactivity in centrolobular hepatocytes of PB-treated rats (Fig. 2*E*). Nomarski optics were used to show more detailed cellular architecture than standard transmitted light microscopy. Whereas punctate Cx32 staining outlined periportal hepatocytes (Fig. 2*F*), cytoplasmic immunoreactivity and occasional punctate staining were observed in centrolobular hepatocytes after PB treatment (Fig. 2*G*). Intracellular staining was absent in the livers of rats maintained on AIN-76 (data not shown) and in the periportal hepatocytes from PB-treated livers (Fig. 2*F*). The intercellular and punctate patterns of Cx32 staining observed in PB-treated livers were

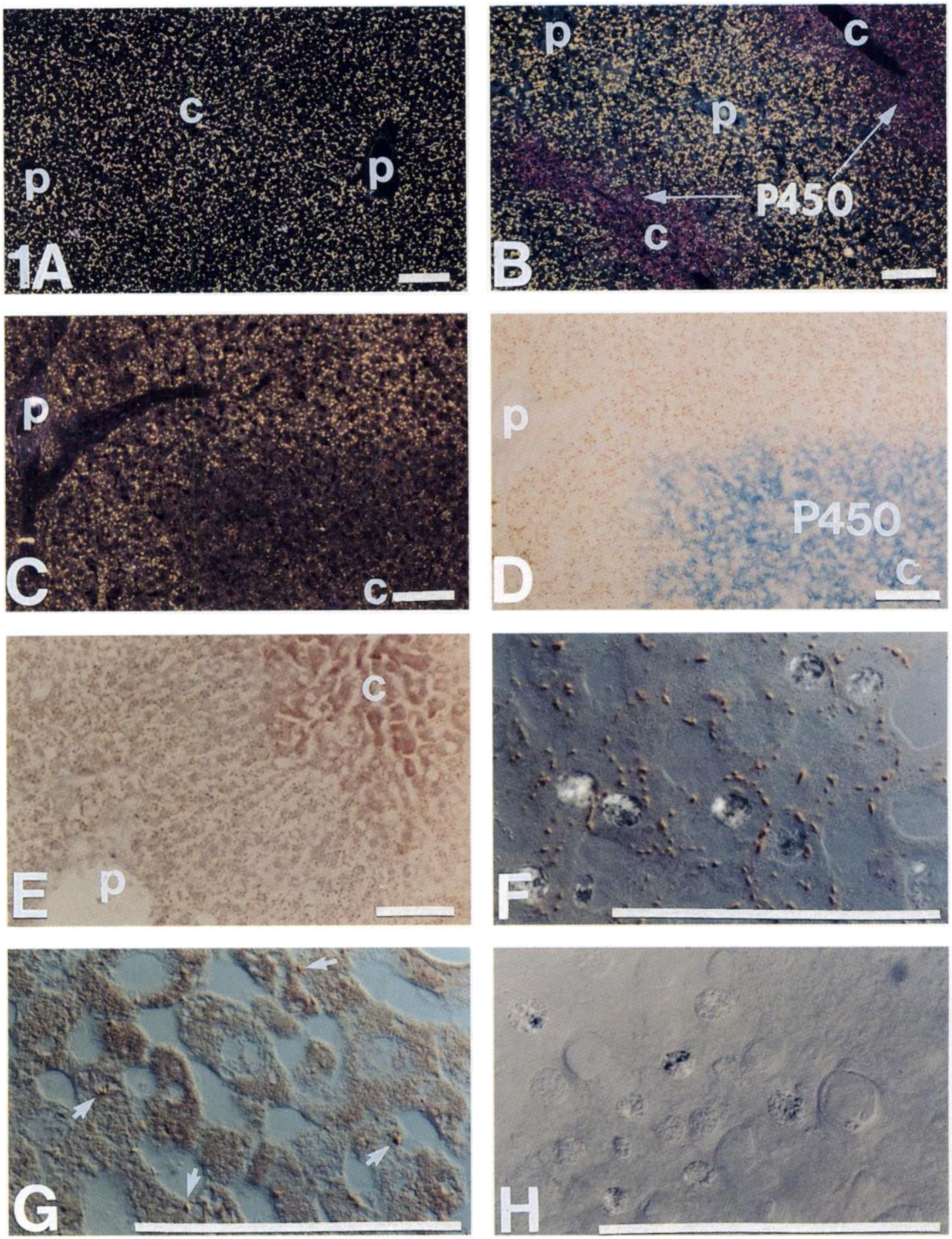


Fig. 2. Alterations in Cx32 and cytochrome P450IIB1/2 immunostaining in centrolobular hepatocytes induced by dietary PB. Frozen (*A–D*) and paraffin-embedded (*E–H*) sections were incubated with mouse monoclonal anti-Cx32 (M12.13; *A*, *E–G*), a mixture of M12.13 plus rabbit anti-P450IIB1 and IIB2 (*B*, *D*), anti-Cx26 (Lulu; *C*), or M12.13 presorbed with purified rat liver gap junctions (*H*) (see "Materials and Methods"). Sections were viewed by either dark-field (*A–C*), light-field (*D*, *E*), or Nomarski microscopy (*F–H*). (*A*) Whereas punctate Cx32 staining (yellow dots) is distributed in hepatocytes throughout the hepatic lobule in control rats, (*B*) liver from rats treated for 30 days with 0.01% dietary PB exhibited decreased Cx32 staining in centrolobular hepatocytes that colocalizes with induction of cytochrome P450IIB1/2 expression (purple). No change in the periportal distribution of Cx26 (*C*) or Cx32 (*D*) staining was observed after 4 weeks of 0.1% dietary PB. Trypsin digestion of paraffin-embedded sections demonstrated centrolobular cytoplasmic Cx32 staining in rats treated with PB (*E*). Closer examination of this section demonstrated that periportal cells exhibit characteristic punctate staining (*F*), whereas centrolobular cells display diffuse cytoplasmic staining with only occasional punctate staining (*G*). No staining was observed when anti-Cx32 (M12.13) antibody was presorbed with purified rat liver gap junctions (*H*). *p*, portal tract; *c*, central vein; *P450*, cytochrome P450IIB1/2; *bar*, 50 μm.

specific because presorbing anti-Cx32 antibody (M12.13 or 594) with highly purified rat liver GJs prevented staining (Fig. 2*H*).

Consistent with immunostaining results, densitometric scanning of Northern blots did not show a significant change in the abundance of Cx32 mRNA in total RNA samples isolated from rats treated for 30, 60, or 90 days with AIN-76 diet or a diet supplemented with 0.05% or 0.1% PB (Fig. 3). Three animals were examined for each group, and the Northern blots were performed twice. Fig. 4 shows that strong induction of P450IIB1 mRNA transcripts was detected in total liver RNA samples isolated from rats treated for 60 days with either 0.05% or 0.1% PB. However, the same RNA samples did not show alterations in the steady-state levels of Cx32 and Cx26 mRNAs. In contrast to chronic studies, a 50% reduction in Cx32 mRNA was observed in RNA samples isolated from rats treated with 0.1% PB for only 3 days (60 h). The results show that alterations in steady-state levels of Cx32 mRNA are not maintained in livers from rats chronically treated with various doses and durations of dietary PB.

An immunoblot of Cx32 and P450IIB1/2 expression in total liver homogenates is depicted in Fig. 5. Although enhancement of P450IIB1/2 expression was evident in crude total liver homogenates from rats treated for 60 days with 0.1% PB, no alteration in the abundance or SDS-PAGE migration pattern of Cx32 immunoreactivity was detected. Equivalent amounts of monomer (27-kDa) and dimer (47 kDa) forms of Cx32 were present in samples from untreated or PB-treated rats. In addition, no significant change in the level of Cx32 protein was apparent in NaOH-insoluble fractions from untreated or 0.1% PB-treated rats. Instead, we observed a 47-kDa band present in NaOH-soluble fraction from PB-treated rats that is not present in untreated animals. Increased abundance of the 47-kDa dimer Cx32 band was also found in crude liver membranes isolated from PB-treated rats. Analysis of Cx32 in crude liver homogenates from animals treated with the various barbiturates listed in Fig. 1 (at 4.3 mM for 11 weeks; equivalent to 0.1% PB) also failed to demonstrate quantitative changes in Cx32 protein expression (Fig. 4*B*).

Table 1 *Percentage of the hepatic lobule displaying coincident changes in Cx32 and P450IIB1/2 immunostaining*

Rats were fed AIN-76 diet supplemented with either phenobarbital, pentobarbital, sodium barbital, barbituric acid, or amobarbital (4.3 mM; equivalent to 0.1% PB.) Sections were doubly stained with anti-Cx32 (M12.13) and P450IIB1/2 as described in "Materials and Methods".

Treatment[a]	Mean liver:body wt ratio[b]	% of lobule with altered Cx32/P450[c]
Control diet	4.61 ± 0.16	Negative
Barbituric acid	4.67 ± 0.24	Negative
Phenobarbital	5.73 ± 0.31	74 ± 9
Barbital	5.33 ± 0.19	56 ± 6
Amobarbital	4.76 ± 0.18	9 ± 2
Pentobarbital	4.90 ± 0.20	17 ± 3

[a] Compounds (4.3 mM; equivalent to 0.05% phenobarbital) were fed *ad libitum* in AIN-76 diet for 4 weeks. Values are mean ± SD for three male F344 rats per treatment.
[b] Units of liver/100 g body weight.
[c] Values represent the percentage of the liver lobule between portal tract and terminal hepatic vein showing alterations in P450IIB1/2 and Cx32 (M12.13) immunoreactivity as determined by planimetry.

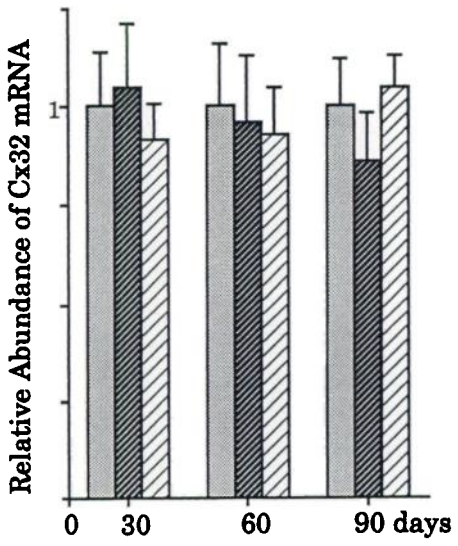


Fig. 3. Analysis of Cx32 mRNA levels in total RNA samples isolated from age-matched rats fed AIN-76 diet alone (■) or AIN-76 supplemented with 0.05% PB (▨) or 0.1% PB (▨) for 30, 60, or 90 days. Total RNA was isolated, and the samples were analyzed by Northern blotting (see "Materials and Methods"). *Columns* mean densitometry values of Cx32 mRNA from three animals; *bars*, SE.

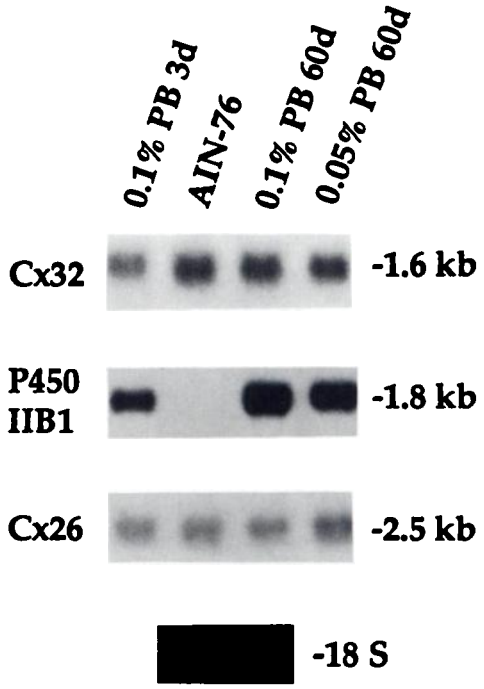


Fig. 4. Effect of chronic PB administration on Cx32 and P450IIB1 mRNA expression. Male F344 rats were maintained on AIN-76 diet or diet supplemented with 0.05% or 0.1% PB. Total RNA was isolated, and the samples were analyzed by Northern blotting (see "Materials and Methods"). Whereas P450IIB1 mRNA was enhanced after acute (3 days) or chronic dietary PB (60 days), reduced levels of Cx32 mRNA were observed only 3 days after starting dietary PB. *kb*, kilobases.

DISCUSSION

In this report we show that dietary PB induces alterations in Cx32 and P450IIB1/2 immunostaining that colocalize to centrolobular hepatocytes. In agreement with the observations of Bars and Elcombe (42), we observed that the proportion of the hepatic lobule exhibiting altered Cx32 and P450IIB1/2 expression expanded as the dose of dietary PB was increased from 0.01% (Fig. 2*B*) to 0.1% (Fig. 2*D*). Our findings that a subset of hepatocytes (*i.e.*, centrolobular) exhibits altered Cx32 staining may explain why PB treatment results in only partial inhibition of GJIC in primary hepatocytes (11). As observed *in vivo*, treatment of cultured hepatocytes with PB results in heterogeneous induction of P-450 immunoreactivity (43). Independent studies have shown that treatment of primary hepatocytes with the maximum noncytotoxic dose of PB (2 mM) induces alterations in P450IIB1/2 expression and GJIC in only 30–35% of the mixed cell population (11, 43). The observed coincidence of changes in Cx32 and P450IIB1/2 staining may be physiologically important for effective

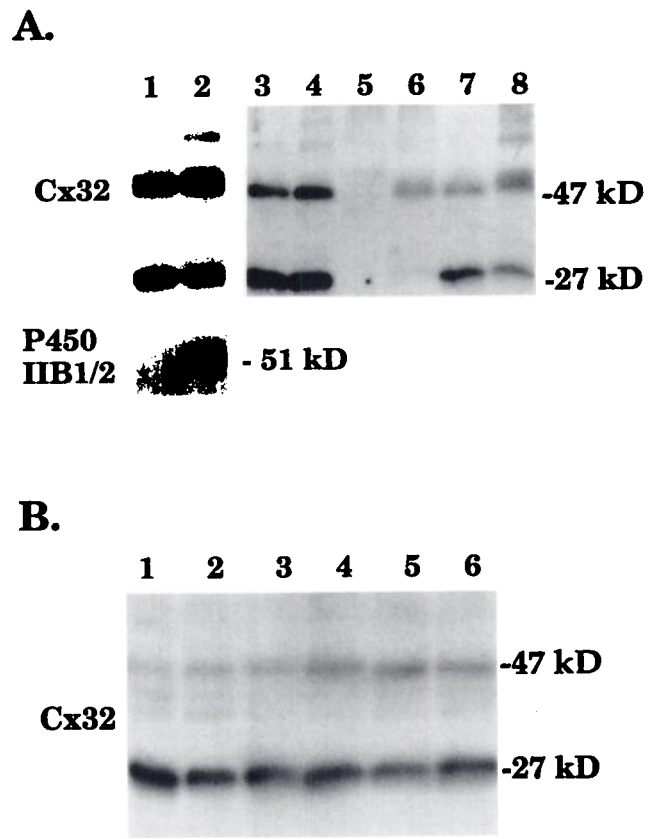


Fig. 5. (*A*) Immunoblot detection of Cx32 and P450IIB1/2 in livers from uninitiated rats treated with AIN-76 (*Lanes 1, 3, 5, 7*) or diet supplemented with 0.1% PB (*Lanes 2, 4, 6, 8*). Crude homogenates (35 μg/lane; *Lanes 1, 2*); NaOH-insoluble pellets (4 μg/lane; *Lanes 3, 4*); NaOH-soluble proteins (30 μg/lane; *Lanes 5, 6*); and crude liver membranes (15 μg/lane, *Lanes 7, 8*) were solubilized and electrophoretically separated as described in "Materials and Methods". Migration of monomer [27 kDa (*kD*)] and dimer (54 kDa) forms of Cx32 are shown. Note that the level of Cx32 immunoreactivity does not change after PB treatment in crude homogenates, but that Cx32 becomes soluble in 20 mM NaOH in PB-treated rats. Increased levels of Cx32 aggregates (>54 kDa) were observed in crude liver membranes isolated from PB-treated rats (*Lane 8*). (*B*) Immunoblot detection of Cx32 in total liver homogenates from rats treated for 11 weeks with AIN-76 (*Lane 1*), phenobarbital (*Lane 2*), amobarbital (*Lane 3*), sodium barbital (*Lane 4*), pentobarbital (*Lane 5*), or barbituric acid (*Lane 6*) (4.3 mM; equivalent to 0.1% PB) (see Table 1). Crude homogenates (35 μg/lane) were solubilized and electrophoretically separated as described in "Materials and Methods." No change in the level of monomer (27 kDa) or dimer (54 kDa) forms of Cx32 was detected.

metabolism of xenobiotics by limiting the cytoplasmic diffusion of reactive intermediates (44) and glutathione (45).

Whereas chronic PB treatment does not increase proliferation in normal centrolobular hepatocytes (9), a diminution of GJIC in preneoplastic hepatocytes that contain mutations in their growth-regulatory genes may allow proliferation by removing cytoplasmic growth constraints of surrounding quiescent hepatocytes. If this hypothesis is valid, decreased Cx32 staining in noninitiated rats may represent a short-term assay for "PB-like" tumor promoters. In support of this suggestion, treatment of rats with equimolar amounts of barbiturates structurally related to PB demonstrated that the extent of the hepatic lobule with altered Cx32/P450IIB1/2 staining correlated with their ability to promote hepatocarcinogenesis (see Fig. 1 and Table 1). Only sodium barbital induced a change in Cx32 and P450IIB1/2 comparable to that of PB. Dietary treatment with the weak tumor promoters amobarbital or pentobarbital induced colocalizing alterations that were restricted to one to two layers of hepatocytes surrounding the central vein. These results, consistent with the findings of Lubet *et al.* (14), illustrate that compounds which cause 20–40% of maximal induction of P450IIB1/2 exhibit minimal promoting activity.

Several liver tumor promoters that are structurally unrelated to PB, including tamoxifen, 2,5,2′,5′-tetrachlorobiphenyl, and CI Solvent Yellow, were also found to induce coincident changes in Cx32 and P450IIB1/2 staining in centrolobular hepatocytes. In separate reports, 2,5,2′,5′-tetrachlorobiphenyl has also been found to inhibit GJIC in human liver cell strains (46) and to induce P450IIB1/2 staining (27). Similar to PB, CI Solvent Yellow does not inhibit GJIC in V79 cells that exhibit low microsomal enzyme activity (47). We also observed that several liver tumor promoters that do not induce P450IIB1/2 (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, WY-14,643, or ciprofibrate) fail to modify Cx32 staining in noninitiated rats.

The changes in punctate Cx32 immunostaining in centrolobular hepatocytes after chronic dietary PB do not result from a reduction in steady-state Cx32 mRNA transcripts. Although increased P450IIB1 mRNA was detected in total RNA samples isolated from rats treated with 0.05% or 0.1% dietary PB for 60 days, no change was observed in Cx32 steady-state mRNA transcripts (Fig. 4). Similar findings were observed in samples of total RNA isolated from rats treated with PB for 8 or 11 weeks (Fig. 3). In support of these observations, mRNA *in situ* hybridization analysis of liver sections from rats initiated with diethylnitrosamine and promoted with PB showed that most altered hepatic foci down-regulate Cx32 staining independent of changes in mRNA abundance.[5] In contrast to chronic studies, a 50% decrease in Cx32 mRNA levels was apparent in total RNA isolated 3 days (60 h) after starting 0.1% dietary PB (Fig. 4). The differences in Cx32 mRNA abundance between acute and chronic studies may result from the transient surge of hepatocyte proliferation that occurs 3 days after PB administration (19). A reduction in Cx32 mRNA is associated with compensatory hyperplasia induced in rat liver by a 70% partial hepatectomy (37). Similar to our findings with chronic dietary PB, centrolobular hepatocytes of postpartum rats exhibited a diminution in centrolobular Cx32 staining independent of changes in Cx32 mRNA abundance (48).

In agreement with our Northern blotting results, no change in the abundance or SDS-PAGE migration pattern of Cx32 protein was observed in total liver homogenates isolated from rats treated with 0.1% PB for 4 weeks (Fig. 5). Both monomer (27,000) and dimer (47,000) forms of Cx32 were observed in control and PB-treated livers. In addition, no change in Cx32 protein levels was seen in crude homogenates isolated from livers treated with either 0.05% or 0.1% PB for 8 or 11 weeks. Analysis of the base solubility of Cx32 demonstrated that a NaOH-soluble 47,000 band was present in PB-treated livers exclusively. Alterations in the conformation of Cx32 in centrolobular hepatocytes may allow for preferential solubilization of Cx32 in NaOH and rapid dimerization in SDS. Consistent with equivalent levels of Cx32 protein in control and PB-treated livers, diffuse intracellular Cx32 staining was observed in centrolobular hepatocytes of PB-treated rats (Fig. 2, *E, G*). The staining was specific because preincubation of anti-Cx32 (M12.13 or 594) with purified rat liver GJs inhibits punctate and intracellular staining. The diffuse pattern of immunoreactivity suggests that intracellular Cx32 is not restricted to lysosomes or the Golgi apparatus but is possibly present in endoplasmic reticulum that increases in hepatocytes after PB administration (49).

The observations that several antioxidants, as well as SKF-525A, prevent PB-inhibitory effects on GJIC suggest that oxidative stress may have a role in the post-translational regulation of Cx32 (23, 50–52). Interestingly, an increase in the production of reactive oxygen species was found to associate with P450IIB1/2 induction in centrolobular hepatocytes and neoplastic nodules (53). Furthermore, sev-

[5] M. J. Neveu, J. R. Hully, K. L. Babcock, E. C. Hertzberg, B. J. Nicholson, D. L. Paul, H. C. Pitot. Multiple mechanisms are responsible for altered expression gap junction genes during oncogenesis in rat liver, submitted for publication.

eral other free radical-generating compounds including paraquat, benzoyl peroxide, 12-*O*-tetradecanoylphorbol-13-acetate, carbon tetrachloride, DDT, and 4-hydroxynonenal inhibit GJIC in various cell types in culture (54–57). A report by Saez *et al.* (55) showing that reducing agents could diminish the uncoupling effects of carbon tetrachloride on GJIC in primary hepatocytes suggests that oxidation of sulfhydryl groups of Cx32 may be responsible for the inhibition.

Alternatively, PB may act through other posttranslational modifications to alter Cx32 staining. Treatment of primary hepatocytes with PB has been found to induce a transient decrease in cyclic AMP levels (58). Interestingly, increasing cyclic AMP levels in hepatocytes (dibutyryl cyclic AMP or caffeine) can reverse PB-inhibitory effects of GJIC (23, 58). Cyclic AMP may modify the phosphorylation of Cx32 or change the expression/activity of another protein involved in GJ assembly or turnover. For example, PB alters the distribution of microfilament and microtubule proteins that regulate the abundance of GJs in hepatocytes (2, 4, 24).

Acute induction of P450IIB1/2 expression by itself is not sufficient to inhibit GJIC. Whereas DDT and cyclodiene pesticides (endosulfan, chlordane, and heptachlor) induce PB-specific microsomal enzyme activity (14), their inhibitory effects on GJIC are not preventable by SKF-525A (23, 52). The mechanisms of GJIC inhibition by PB and DDT appear to be different because the latter inhibits communication in primary hepatocytes (express Cx32 and Cx26), V79 fibroblasts (express Cx43), and WBF344 liver epithelial cells (express Cx43 and Cx26), whereas PB only inhibits GJIC in hepatocytes (4). Similar to PB-like promoters, antioxidants or cyclic AMP can prevent the inhibitory effects of DDT on GJIC (50). Therefore, induction of oxidative stress may be a better predictor for the ability of a compound to inhibit GJIC and promote rat hepatocarcinogenesis. In accord with this hypothesis, structural analogues of DDT (fenarimol) and diazepam (clonzepam) induce P-450 activity without inhibiting GJIC (V79 and F344 cells) or promoting rat hepatocarcinogenesis (59, 60). Despite acute induction of P-450 activity by clonzepam, chronic treatments resulted in only a marginal induction that may not be sufficient for tumor promotion (14, 59). Additional studies are necessary to determine the precise molecular mechanisms responsible for the capability of antioxidants and cyclic AMP to prevent PB-inhibitory effects on hepatocyte GJIC. The capability of PB and related barbiturates to posttranslationally down-regulate punctate Cx32 staining in centrolobular hepatocytes provides a reproducible *in vivo* model to examine the mechanisms of PB-like tumor promoters as well as antipromoters.

ACKNOWLEDGMENTS

The authors express their gratitude to Colin Jefcoate for cytochrome P450IIB1/2 antibodies, to Jennifer Vaughn for expert animal experimentation and care, and to Lacinda J. Burchell for developing and performing many of the paraffin-embedding procedures. Critical editing of the manuscript by Dr. Ilse Riegel is gratefully acknowledged by the authors.

REFERENCES

1. Loewenstein, W. R. Junctional intercellular communication: the cell-to-cell membrane channel. Physiol. Rev., *61:* 830–913, 1981.
2. Bennett, M. V. L., Barrio, L. C., Bargiello, T. A., Spray, D. C., Hertzberg, E., and Sáez, J. C. Gap junctions: new tools, new answers, new questions. Neuron, *6:* 305–320, 1991.
3. Loewenstein, W. R., and Rose, B. The cell-cell channel in the control of growth. Semin. Cell Biol., *3:* 59–79, 1992.
4. Neveu, M. J., and Bertram, J. Modulation of intercellular communication during oncogenesis. *In:* E. C. Hertzberg (ed.), Advances in Molecular and Cell Biology, Vol. 6. Connecticut: JAI Press, 1993.
5. Trosko, J. E., Chang, C. C., and Madhukar, B. V. Cell-to-cell communication: relationship of stem cells to the carcinogenesis process. *In:* Mouse Liver Carcinogenesis: Mechanisms and Species Comparison, pp. 259–276, 1990.
6. Yamasaki, H. Gap junctional intercellular communication and carcinogenesis. Carcinogenesis (Lond.), *11:* 1051–1058, 1990.
7. Klaunig, J. E. Alterations in intercellular communication during the stage of promotion. Proc. Soc. Exp. Biol. Med., *198:* 688–692, 1991.
8. Sugie, S., Mori, H., and Takahashi, M. Effect of *in vivo* exposure to the liver tumor promoters phenobarbital or DDT on the gap junctions of rat hepatocytes: a quantitative freeze-fracture analysis. Carcinogenesis (Lond.), *8:* 45–51, 1987.
9. Neveu, M. J., Hully, J. R., Paul, D. L., and Pitot, H. C. Reversible alteration in the expression of the gap junctional protein connexin 32 during tumor promotion in rat liver and its role during cell proliferation. Cancer Commun., *2:* 21–31, 1990.
10. Ruch, R. J., and Klaunig, J. E. Kinetics of phenobarbital inhibition of intercellular communication in mouse hepatocytes. Cancer Res., *48:* 2519–2523, 1988.
11. Klaunig, J. E., Ruch, R. J., Weghorst, C. M., and Hampton, J. A. Role of inhibition of intercellular communication in hepatic tumor promotion. *In Vitro* Toxicol., *3:* 91–107, 1990.
12. Wolf, C. R., Moll, E., Friedberg, T., Oesch, F., Buchmann, A., Kuhlmann, W. D., and Kunz, H. W. Characterization, localization and regulation of a novel phenobarbital-inducible form of cytochrome P450, compared with three further P450-isozymes, NADPH P450-reductase, glutathione transferases and microsomal epoxide hydrolase. Carcinogenesis (Lond.), *5:* 993–1001, 1984.
13. Jungermann, K., and Katz, N. Functional specialization of different hepatocyte populations. Physiol. Rev., *69:* 708–764, 1989.
14. Lubet, R. A., Nims, R. W., Ward, J. M., Rice, J. M., and Diwan, B. A. Induction of cytochrome P450b and its relationship to liver tumor promotion. J. Am. Coll. Toxicol., *8:* 259–268, 1989.
15. Nims, R. W., Devor, D. E., Henneman, J. R., and Lubet, R. A. Induction of alkoxyresorufin *O*-dealkylases, epoxide hydrolase, and liver weight gain: correlation with liver tumor-promoting potential in a series of barbiturates. Carcinogenesis (Lond.), *8:* 67–71, 1987.
16. Shinozuka, H., Lombardi, B., and Abomobi, S. E. A comparative study of the efficacy of four barbiturates as promoters of the development of γ-glutamyltranspeptidase-positive foci in the liver of carcinogen treated rats. Carcinogenesis (Lond.), *3:* 1017–1020, 1982.
17. Diwan, B. A., Rice, J. M., Ohshima, M., Ward, J. R., and Dove, L. F. Comparative tumor-promoting activities of phenobarbital, amobarbital, barbital sodium, and barbituric acid on livers and other organs of male F344/NCr rats following initiation with *N*-nitrosodiethylamine. J. Natl. Cancer Inst., *74:* 509–515, 1985.
18. Pelkonen, O., and Karki, N. T. Effect of physicochemical and pharmacokinetic properties of barbiturates on the induction of drug metabolism. Chem-Biol. Interact., *7:* 93–99, 1973.
19. Peraino, C., Fry, R. J. M., Staffeldt, E., and Christopher, J. P. Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin, and dichlorodiphenyltrichloroethane on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res., *35:* 2884–2890, 1975.
20. Pereira, M. A., Savage, R. E., Herren, S. L., and Guion, C. W. Comparison of enhancement of GGTase-positive foci and induction of ornithine decarboxylase in rat liver by barbiturates. Carcinogenesis (Lond.), *3:* 147–150, 1982.
21. Paul, D. L. Molecular cloning of cDNA from rat liver GJ protein. J. Cell Biol., *103:* 123–134, 1986.
22. Wolff, G. L., Leakey, J. E. A., Bazare, J. J., Harmon, J. R., Webb, P. J., and Law, M. G. Susceptibility to phenobarbital promotion of hepatotumorigenesis: correlation with differential expression and induction of hepatic drug metabolizing enzymes in heavy and light male (C3H × VY) F_1 hybrid mice. Carcinogenesis (Lond.), *12:* 911–915, 1991.
23. Klaunig, J. E., Ruch, R. J., and Weghorst, C. M. Comparative effects of phenobarbital, DDT, and lindane on mouse hepatocyte gap junctional intercellular communication. Toxicol. Appl. Pharmacol., *102:* 553–563, 1990.
24. Decloitre, F., Lafarge-Frayssinet, C., Martin, M., and Frayssinet, C. Modifications of microfilaments and microtubules induced by two hepatic tumor promoters, phenobarbital and biliverdin in non-transformed and transformed hepatic cell lines. Cell Biol. Toxicol., *6:* 23–34, 1990.
25. Mesnil, M., Fitzgerald, J. D., and Yamasaki, H. Phenobarbital specifically reduces gap junction protein mRNA level in rat liver. Mol. Carcinog., *1:* 79–81, 1988.
26. Dragan, Y. P., Rizvi, T., Xu, Y.-H., Hully, J. R., Bawa, N., Campbell, H. A., Maronpot, R. R., and Pitot, H. C. An initiation-promotion assay in rat liver as a potential complement to the 2-year carcinogenesis bioassay. Fundam. Appl. Toxicol., *16:* 525–547, 1991.
27. Sargent, L., Dragan, Y. D., Erickson, C., Laufer, C. J., and Pitot, H. C. Study of the separate and combined effects of the non-planar 2,5,2′,5′- and the planar 3,4,3′,4′-tetrachlorobiphenyl in liver and lymphocytes *in vivo*. Carcinogenesis (Lond.), *12:* 793–800, 1991.
28. Yamamoto, T., Hertzberg, E. L., and Nagy, J. I. Epitopes of gap junction proteins localized to neuronal subsurface cisterns. Brain Res., *527:* 135–139, 1990.
29. Zhang, J. T., and Nicholson, B. J. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J. Cell Biol., *109:* 3391–3401, 1989.
30. Wilson, N. M., Christou, M., and Jefcoate, C. R. Differential expression and function of three closely related phenobarbital-inducible cytochrome P-450 isozymes in untreated rat liver. Arch. Biochem. Biophys., *256:* 407–420, 1987.
31. Hertzberg, E. L. A detergent-independent procedure for the isolation of gap junctions from rat liver. J. Biol. Chem., *259:* 9936–9943, 1984.
32. Hertzberg, E. L., and Skibbens, R. V. A protein homologous to the 27,000 dalton liver gap junction protein is present in a wide variety of species and tissues. Cell, *39:* 61–69, 1984.
33. Peterson, G. L. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem., *83:* 346–356, 1977.

34. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
35. Kadle, R., Zhang, J. T., and Nicholson, B. J. Tissue-specific distribution of differentially phosphorylated forms of Cx43. Mol. Cell. Biol., *11:* 363–369, 1991.
36. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol/chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
37. Beer, D. G., Neveu, M. J., Paul, D. L., Rapp, U. R., and Pitot, H. C. Expression of the c-*raf* proto-oncogene, γ-glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. Cancer Res., *48:* 1610–1617, 1988.
38. Dunn, T. J., Koleske, A. J., Lindahl, R., and Pitot, H. C. Phenobarbital-inducible aldehyde dehydrogenase in the rat. J. Biol. Chem., *264:* 13057–13065, 1989.
39. Traub, O., Look, J., Dermietzel, R., Brummer, F., Hulser, D., and Willecke, K. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. J. Cell Biol., *108:* 1039–1051, 1989.
40. Asamoto, M., Oyamada, M., El Aoumari, A., Gros, D., and Yamasaki, H. Molecular mechanisms of TPA-mediated inhibition of gap-junctional intercellular communication: evidence for action on the assembly or function but not the expression of connexin 43 in rat liver epithelial cells. Mol. Carcinog., *4:* 322–327, 1991.
41. Berthoud, V. M., Ledbetter, M. L. S., Hertzberg, E. L., and Saez, J. C. Connexin43 in MDCK cells: regulation by tumor-promoting phorbol ester and Ca^{2+}. Eur. J. Cell Biol., *57:* 40–50, 1992.
42. Bars, R. G., and Elcombe, C. R. Dose-dependent acinar induction of cytochromes P450 in rat liver. Biochem. J., *277:* 577–580, 1991.
43. Bars, R. G., Mitchell, A. M., Roland Wolf, C., and Elcombe, E. R. Induction of cytochrome P-450 in cultured rat hepatocytes: the heterogeneous localization of specific isoenzymes using immunocytochemistry. Biochem. J., *262:* 151–158, 1989.
44. Jongen, W. M. F., van der Leede, B. J. N., Chang, C. C., and Trosko, J. E. The transport of reactive intermediates in a co-culture system: the role of intercellular communication. Carcinogenesis (Lond.), *8:* 1239–1243, 1987.
45. Kavanagh, T. J., Martin, G. M., Livesey, J. C., and Rabinovitch, P. S. Direct evidence of intercellular sharing of glutathione via metabolic cooperation. J. Cell. Physiol., *137:* 353–359, 1988.
46. Swierenga, S. H., Yamasaki, H., Piccoli, C., Robertson, L., Bourgon, L., Marceau, N., and Fitzgerald, D. J. Effects on intercellular communication in human keratinocytes and liver-derived cells of polychlorinated biphenyl congeners with differing *in vivo* promotion activities. Carcinogenesis (Lond.), *11:* 921–926, 1990.
47. Elmore, E., Milman, H. A., and Wyatt, G. P. Applications of the Chinese hamster V79 metabolic cooperation assay in toxicology. *In:* Biochemical Mechanisms and Regulation of Intercellular Communication, pp. 270–292. Princeton, NJ: Princeton Scientific Publishing, 1987.
48. Risek, B., Guthrie, S., Kumar, N., and Gilula, N. B. Modulation of gap junction transcript and protein expression during pregnancy in the rat. J. Cell Biol., *110:* 269–282, 1990.
49. Feldman, D., Swarm, R. L., and Becker, J. Ultrastructural study of rat liver and liver neoplasms after long-term treatment with phenobarbital. Cancer Res., *41:* 2151–2162, 1981.
50. Ruch, R. J., and Klaunig, J. E. Antioxidant prevention of tumor promoter induced inhibition of mouse hepatocyte intercellular communication. Cancer Lett., *33:* 137–150, 1986.
51. Ruch, R. J., Cheng, S. J., and Klaunig, J. E. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis (Lond.), *10:* 1003–1008, 1989.
52. Ruch, R. J., Fransson, R., Flodstrom, S., Warngard, L., and Klaunig, J. E. Inhibition of hepatocyte gap junctional intercellular communication by endosulfan, chlordane and heptachlor. Carcinogenesis (Lond.), *11:* 1097–1101, 1990.
53. Scholz, W., Schutze, K., Kunz, W., and Schwarz, M. Phenobarbital enhances the formation of reactive oxygen in neoplastic rat liver nodules. Cancer Res., *50:* 7015–7022, 1990.
54. Slaga, T. J., Klein-Szanto, A. J. P., Triplett, L. L., Yotti, L. P., and Trosko, J. E. Skin tumor-promoting activity of benzoyl peroxide, a widely used free radical-generating compound. Science (Washington DC), *213:* 1023–1025, 1981.
55. Saez, J. C., Bennett, M. V. L., and Spray, D. C. Carbon tetrachloride hepatoxic levels blocks reversibly gap junctions between rat hepatocytes. Science (Washington DC), *236:* 967–969, 1987.
56. Ruch, R. J., and Klaunig, J. E. Inhibition of mouse hepatocyte intercellular communication by paraquat-generated oxygen free radicals. Toxicol. Appl. Pharmacol., *94:* 427–436, 1988.
57. Radu, A., and Moldovan, N. 4-Hydroxynonenal reduces junctional communication between endothelial cells in culture. Exp. Cell Res., *196:* 121–126, 1991.
58. Klaunig, J. E., and Ruch, R. J. Role of cAMP in the inhibition of mouse hepatocyte intercellular communication by liver tumor promoters. Toxicol. Appl. Pharmacol., *91:* 159–170, 1987.
59. Diwan, B. A., Lubet, R. A., Nims, R. W., Klaunig, J. E., Wegnorst, C. M., Henneman, J. R., Ward, J. M., and Rice, J. M. Lack of promoting effect of clonazepam on the development of *N*-nitrosodiethylamine-initiated hepatocellular tumors in mice is correlated with its inability to inhibit cell-to-cell communication in mouse hepatocytes. Carcinogenesis (Lond.), *10:* 1719–1724, 1989.
60. Flodstrom, S., Hemming, H., Warngard, L., and Ahlborg, U. G. Promotion of altered hepatic foci development in rat liver, cytochrome P450 enzyme induction and inhibition of cell-cell communication by DDT and some structurally related organohalogen pesticides. Carcinogenesis (Lond.), *11:* 1413–1417, 1990.