Proinflammatory mesenchymal effects of the non-genotoxic hepatocarcinogen phenobarbital: a novel mechanism of antiapoptosis and tumor promotion

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Abstract

Many environmental pollutants and drugs, including steroid hormones, hypolipidemics and antiepileptics, are non-genotoxic carcinogens (NGC) in rodent liver. The mechanism of action and the risk for human health are still insufficiently known. Here, we study the effects of phenobarbital (PB), a widely used model NGC, on hepatic epithelial–mesenchymal crosstalk and the impact on hepatic apoptosis. Mesenchymal cells (MC) and hepatocytes (HC) were isolated from control and PB-treated rat livers. PB induced extensive changes in gene expression in MC and much less in HC as shown by transcriptomics with oligoarrays. In MC only, transcript levels of numerous proinflammatory cytokines were elevated. Correspondingly, ELISA on the supernatant of MC from PB-treated rats revealed enhanced release of various cytokines. In cultured HC, this supernatant caused (i) nuclear translocation and activation of nuclear factor-κB (shown by immunoblots of nuclear extracts and reporter gene assays), (ii) elevated expression of proinflammatory genes and (iii) protection from the proapoptotic action of transforming growth factor beta 1 (TGFβ1). PB treatment in vivo or in vitro elevated the production and release of tumor necrosis factor alpha from MC, which was identified as mainly responsible for the inhibition of apoptosis in HC. In conclusion, our findings reveal profound proinflammatory effects of PB on hepatic mesenchyme and mesenchymal–epithelial interactions. The resulting release of cytokines acts antiapoptotic in HC, an effect crucial for tumor promotion and carcinogenesis by NGC.

Introduction

Chemical carcinogens are categorized as genotoxic or non-genotoxic, according to their pathogenic mechanism. While genotoxicity is a key event in tumor formation by agents of the first category, the mode of action of non-genotoxic carcinogens (NGC) is still unclear (1). Many endogeneous or synthetic hormones act as NGC and account for cancer in breast, prostate or other hormone-dependent organs of various mammalian species (2). Other NGC produce mainly liver tumors in long-term rodent bioassays, as observed for antiepileptics, such as PB, hypolipidemics, antidiabetics and many non-drug agents. Considering the daily intake of these NGC by millions of humans, thorough knowledge of the mode of action is of utmost importance to estimate the health risks (1,2).

Most NGC are ligands/activators of nuclear receptors, such as hormone receptors or the constitutive androstane receptor (CAR). Following activation receptors mediate adaptive increases in specific enzymes, organelles and/or cell number in target tissues. Adaptive cell multiplication is a self-limited process and
per se not carcinogenic (3,4). However, cells carrying mutations in critical (growth regulatory) genes may show an ‘over-response’ towards NGC, i.e., selectively increased proliferation based on a continuous shift from cell death towards cell replication (5). This strong growth pressure may trigger the outgrowth of mutated cells to malignancy.

For our mechanistic investigations, we chose the CAR activator and barbiturate phenobarbital (PB), an antiepileptic drug for humans, which has been applied and studied as liver tumor promoter in rodents since more than four decades. In experimental mouse models, the key events in PB-induced liver tumor formation are considered to comprise activation of CAR, resulting in altered gene expression and increased cell proliferation, which induces growth of altered hepatic foci and ultimately the development of liver tumors (3,4). In mice, PB strongly promotes HC carrying mutations of the Ctnnb1 gene, encoding a constitutively activated version of β-catenin (6). Dong et al. (7) have shown that activation of β-catenin alone induces senescence and growth arrest. This is overcome if combined with CAR activation, resulting in uncontrolled proliferation and tumor formation.

In rats, the significance of CAR for PB-driven hepatocarcinogenesis has remained unclear due to lack of transgenic or knock-out models. While PB strongly induces DNA replication in murine liver, it is much less effective in rats—similar to human HC in primary culture or chimeric ‘humanized’ mice (8,9). Unlike in mice, in rats PB suppresses apoptosis in normal liver and more potently in preneoplastic lesions. Thereby it accelerates the outgrowth of malignancy, a fundamental mechanism of tumor promotion by this NGC in this rodent species, as shown in previous studies and reproduced also under the present experimental conditions (Supplementary Figure 1, available at Carcinogenesis Online) (3,5,8). Furthermore, in rats most PB-generated liver tumors fail to exhibit mutations of the β-catenin gene as observed in mice (unpublished observation).

These findings imply that other/additional mechanisms may play a role in tumor promotion by PB in rat liver.

Conventional notion regards the action of PB and other NGC as essentially confined to parenchymal liver cells. Accordingly, the potential role of the hepatic mesenchyme has not been investigated in detail. However, MC play a key role in carcinogenesis by genotoxic agents via eliciting proinflammatory states. For example, exposure to genotoxic carcinogens, like dietary aflatoxin-B1 or ethanol, exerts considerable cytotoxicity leading to necrosis of HC and inflammation, followed by regenerative growth and/or replacement of the dying cells by scarring (10). These processes involve an altered epithelial-mesenchymal dialogue wherein Kupffer cells (KC) initiate the inflammatory response by the release of cytokines, like interleukin 1β (IL1β) and tumor necrosis factor α (TNFα) (11). TNFα recruits further immune cells to the liver. Moreover, it induces hepatocellular apoptosis via tumor necrosis factor receptor 1 (TNFR1), an event primarily antagonized by activation of the transcription factor nuclear factor-κB (NFκB) (12–15). This balance between proapoptotic stimuli and activated NFκB plays a key role in hepatocarcinogenesis driven by inflammation, e.g., Mdr2<sup>−/−</sup> mice develop hepatic inflammation and hepatocellular carcinoma; the development of hepatocellular carcinoma, however, can be prevented by expression of an NFκB repressor transgene inducing proapoptotic pathways in HC (16). KC, activated by proinflammatory signals, also release superoxide, which appears to contribute to hepatocarcinogenesis. In previous work, p47-phox knockout mice, lacking superoxide formation by KC, were less sensitive than wild-type animals to the carcinogenic effect of cytotoxic doses of diethylnitrosamine (17).

As to NGC, a few pioneering studies have shown that these agents act not only on HC but also on hepatic MC, resulting in enhanced secretion of growth factors, proinflammatory cytokines and reactive oxygen species (11,18–20). Moreover, some of these growth factors have been found to act selectively on preneoplastic HC, serving as endogenous tumor promoters (21,22).

The present study aims at a better understanding of the role of the hepatic mesenchyme for NGC-driven hepatocarcinogenesis. In humans, PB and other barbiturates are known to interfere with the innate immune system by altering the function of leukocytes/monocytes/macrophages (23,24). Here, we show for the first time that PB induces immunological alterations in the mesenchyme of rat liver, which includes elevated secretion of proinflammatory cytokines from MC. In HC, this effect results in enhanced translocation of NFκB to the nucleus and a considerably reduced susceptibility to proapoptotic stimuli. In conclusion, the hepatic mesenchyme appears to be the actual source of the antiapoptotic signal of PB, which plays a crucial role for tumor promotion and carcinogenicity by this NGC (5).

Materials and methods

Animals and treatment

Male Han–Wistar rats, 8 weeks old, were obtained from Charles River (FRG). They were kept under standardized SPF conditions. Animals were treated with 50 mg PB (5-ethyl-5-phenylbarbituric acid sodium salt, Sigma–Aldrich) per 1000 g body weight by a single gavage or via drinking water for a period of 7 or 14 days. Controls received tap water only. All experiments were approved by the ‘Committee of Animal Protection’ of the Austrian government and performed according to Austrian regulations.

Separation of liver cells and primary cultures

Livers of untreated or PB-treated rats were perfused with collagenase (Worthington, Lakewood, NJ). The cell suspension obtained was used to separate MC from HC by low-speed centrifugation in percoll gradients. MC were further separated by selective cell adherence into an endothelial cell (EC)-enriched fraction and a fraction consisting mainly of exsudative
cd68+/ED1+ exudative monocytes/macrophages (EM) and liver-resident cd163+/ED2+ KC. For details on procedures see Böhm et al. (25). The purity of cell fractions were determined to be: 98.4 ± 0.6 for HC, 99.9 ± 0.1 for MC, 80 ± 19.4 for KC/EM and 94.5 ± 2.8 for EC (25). Stock solutions were prepared of PB, IL1ß, TNFα, TGFß1 and BAY11-7082 (BAY) and aliquots were added to the medium to provide the final concentration (Supplementary Table 1, available at Carcinogenesis Online). Treatment of cells commenced 2 h after plating (time point 0).

**Gene expression analyses**

For reverse transcriptase quantitative PCR (RT-qPCR), the extracted mRNA was processed and measured by the ABI-Prism/7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan-based assays (Applied Biosystems). For primers and assays see Supplementary Table 1, available at Carcinogenesis Online.

For whole genome expression analyses, the extracted RNA was subjected to quality control (2100 Bioanalyzer System, Agilent, St Clara, CA). Complementary DNA targets were prepared and hybridized according to the manufacturer’s procedures on high-density oligonucleotide microarrays (Affymetrix Rat 230 2.0 Array, Cleveland, OH). The microarrays were performed at the core facility ‘Genomics’ at the Medical University Vienna. For further details, see Supplementary Material, available at Carcinogenesis Online. For validation of transcriptome data by quantitative RT-qPCR, see Supplementary Figure 2, available at Carcinogenesis Online.

**Immunodetection**

Antisera and enzyme-linked immunosorbent assay (ELISA) kits used see Supplementary Table 1, available at Carcinogenesis Online.

**Immunoblotting**

Nuclei were isolated via ‘NE-PER Nuclear & Cytoplasmic Extraction Kit’ (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Proteins, harvested in RIPA buffer (500 mM NaCl, 50 mM Tris (pH 7.4), 0.1% SDS, 1% igepal CA630, 0.5% Na-deoxycholate, 0.5 mM phenylmethylsulfonylfuorid; all obtained from Sigma–Aldrich), were homogenized by sonication and centrifugation (12 000 rpm, 10 s) and loaded at equal amounts (10 μg) onto 10% SDS gels. Bands, obtained by immunostaining (‘TCL Plus Western Blotting Detection Kit’, GE Healthcare, St Gilles, GB), were evaluated by densitometry (‘Optimax 2010 X-ray film processor’ and ‘Quantity One 4.2.1’; Bio-Rad, Hercules, CA). Further details see (25).

**ELISA**

ELISAs were performed according to the manufacturer’s instructions. Material not included in the module set: tetramethylbenzidine peroxidase solution for color development (Thermo Scientific); microwell plates (Costar, Corning, NY).

**Immunohistochemistry**

Sections, obtained from Carnoy- or formalin-fixed liver tissue, were embedded in paraffin and stained, as given in detail elsewhere (25).

**Reporter gene assay**

Primary HC were seeded at 5 × 10^4 cells/cm^2; 24 h later cells were cotransfected by 500 ng plasmid DNA, coding for the NFκB-responsive luciferase vector and 25 ng of the renilla luciferase vector applying lipofectamine 2000 (Life Technologies, Carlsbad, CA). Transfections with basic PGL2- and pRL-SV40 vectors (Promega, Madison, WI) served as control. Mesenchymal supernatant was added 24 h after transfection; 24 h later firefly and renilla luciferase were detected with the ‘Dual Luciferase Reporter System’ (Promega) according to the manufacturer’s instruction. Each set of experiments was performed in triplicate and repeated thrice.

**Results**

**PB affects transcriptome profiles: multiple alterations in MC, few in HC**

To analyze the role of the liver mesenchyme in NGC-driven hepatocarcinogenesis, we treated male rats with PB once by gavage or for 2 weeks. Twenty-four hours after gavage or at the end of the 14th day period of PB application via drinking water, cells were isolated from the liver and separated into HC and MC. Cell fractions from untreated rats served as control. Subsequent analyses by oligoarrays revealed that a single dose of PB altered the expression of 64 genes in HC and 133 genes in MC. Fourteen days’ treatment changed mRNA levels of 58 genes in HC and of even 887 genes in MC (Figure 1A; Supplementary Figure 3, available at Carcinogenesis Online). Interestingly, at both time points only a few genes were commonly deregulated in both cell fractions. Overall, these data show that both hepatic cell types, but more prominently MC, are able to react extensively and specifically to PB treatment.

**PB induces a stress-response in HC via enhanced secretion of TNFα by MC**

Transcriptome data were subjected to bioinformatic analyses. Unlike in HC, in MC many different proinflammatory cytokines and respective downstream signaling pathways were found to be deregulated by PB, such as NFκB-driven pathways (Supplementary Table 2, available at Carcinogenesis Online). Furthermore, there was over-proportional upregulation of chemokines of the CCL- and CXCL-family in MC (Figure 1B), which was not seen in HC (data not shown).

To study the effect of the upregulated proinflammatory cytokines in PB-treated MC, we chose a holistic approach, i.e. we isolated MC from animals, which had been treated with PB for 7 days (‘MC-PB’), cultivated the cells, collected their supernatant (‘SN/MC-PB’) and exposed cultured HC with SN/MC-PB. Supernatants of MC from rats, which had been treated with solvent (‘SN/MC-CO’), served as control. For further details, see Supplementary Figure 4, available at Carcinogenesis Online.

Exposure of untreated HC to SN/MC-CO induced mRNA levels of proinflammatory genes, i.e. nitric oxide synthase-2 (NOS2) and TNFα expressions were elevated ~300- and ~4-fold, respectively. This effect became even more pronounced when SN/MC-PB was applied, i.e. 1400-fold (NOS2) and 100-fold (TNFα) elevated transcript levels were obtained when compared with HC in basic medium (Figure 2A). This effect was evident also after one PB application and 14 days of PB treatment (Supplementary Figure 5, available at Carcinogenesis Online). The dramatic upregulation of proinflammatory genes was confirmed for NOS2 at the protein level (Figure 2C). Probably due to the very complex post-transcriptional/post-translational regulation of this gene, the PB-induced elevation of the NOS2 protein was less pronounced than of the transcript (26).

When determining proinflammatory cytokines in the supernatants, considerable levels of IL1ß and TNFα were evident in SN/MC-CO indicating spontaneous activation of cultured MC, as shown before (27). However, considerably higher concentrations of these cytokines were found in SN/MC-PB than in SN/MC-CO (Figure 2B). To test for a causal role of these two cytokines for the supernatant effects, we added recombinant TNFα or IL1ß to HC cultures, which simulated the effect of SN/MC-PB on HC (Supplementary Figure 6, available at Carcinogenesis Online). To clarify which of the two cytokines is mainly responsible, we pre-incubated SN/MC-PB with neutralizing anti-IL1ß or anti-TNFα. Anti-TNFα abolished distinctly the supernatant effects while anti-IL1ß had little impact (Figure 2D). This implies that TNFα is the main factor in SN/MC-PB, causing the stress–response in HC.

**PB in vivo enhances anti-inflammatory effects of HC on MC**

Interestingly, the supernatant of untreated HC reduced the expression of proinflammatory genes in MC. This effect was even more pronounced with supernatant of HC from PB-treated rats (Supplementary Figure 7, available at Carcinogenesis Online). This...
suggests that PB-treated HC may counteract the proinflammatory action of the PB-activated mesenchyme. The outcome of the transcriptome analysis suggests that in the intact liver the effect of MC is predominating as indicated by upregulation of proinflammatory cytokines in MC and activation of stress–response pathways in HC (Supplementary Table 2, available at Carcinogenesis Online).

PB acts directly on MC to induce proinflammatory genes

Due to complex epithelial–mesenchymal interactions in the intact liver, it remained unclear whether PB acts directly on MC to induce proinflammatory genes. For clarification, MC were isolated from untreated rats and were separated into EC- and KC/EM-enriched fractions, both of which were kept in culture and treated with PB. As shown in Figure 3, PB in vitro elevated IL1β and TNFα concentrations in the supernatant of KC/EM, but not in EC supernatant. This indicates that PB is able to directly activate hepatic KC/EM; these cells appear to be the main source of TNFα in the PB-treated mesenchyme.

MC express hardly PB-responsive nuclear receptors (CAR, PXR) but considerable levels of glycine receptor subunits

The direct effects of PB on isolated and cultured KC/EM raised the question as to the underlying mechanism (Figure 3). We could not detect significant transcript levels of the PB-responsive nuclear receptors CAR or PXR in EC or KC/EM (Supplementary Table 3, available at Carcinogenesis Online). Then we tested for expression of receptors known to interact with PB in neuronal/non-hepatic tissues, such as γ-aminobutyric acid (GABA) GABA_A-receptors, AMPA/kainate receptors, glutamate receptors 3 and 6 and glycine receptor (28–33). We found transcripts only of the glycine receptor subunits with levels being considerably higher in MC than in HC, as described previously for KC (32). The interaction of PB with hepatic glycine receptors and the possible consequences are discussed below.

Supernatant of PB-treated MC activates NFκB in HC to exert antiapoptotic activity

Considering the bioinformatic analyses and the hepatocellular upregulation of several NFκB-target genes, such as NOS2, COX2, IL1β, IL6 and TNFα by SN/MC-PB, we checked whether this supernatant is able to induce the translocation of NFκB to HC nuclei. In fact, NFκB protein was increased about 2-fold in nuclei of HC exposed to SN/MC-CO and 3-fold elevated with SN/MC-PB (Figure 4A and B). This was reduced by BAY, which blocks irreversibly and selectively the cytokine-induced phosphorylation of NFκB-inhibitor alpha (IκBα), necessary to unmask the nuclear localization signals of NFκB (34). A somewhat elevated occurrence of NFκB-positive HC nuclei was also evident in tissue sections obtained from PB-treated liver (Figure 4C). Furthermore, SN/MC-PB was able to enhance NFκB activity in HC, as shown by a reporter-gene assay (Figure 4D).

It is well established that activation of NFκB serves as a primary mechanism in protecting HC from proapoptotic stimuli. We therefore checked whether the SN/MC-PB-induced activation of NFκB is involved in the antiapoptotic activity of PB in HC. Under the present experimental conditions 0.7 ± 0.3% of HC in primary culture underwent spontaneous apoptosis 24 h after seeding, which agrees with published data (Figure 5) (35). SN/MC-CO, SN/MC-PB and recombinant IL1β and TNFα had only marginal effects on basal apoptotic activity. In order to induce a distinct apoptotic response, HC were treated with TGFß1, which elevated the occurrence of apoptosis 3.3-fold. Coapplication of IL1β further enhanced the TGFß1 effect, while SN/MC-CO, TNFα and—most pronounced—SN/MC-PB decreased the rate of apoptosis. Thus, the elevated level of TNFα in SN/MC-PB will
support the survival of HC and thereby contribute to the tumor promoting effect of PB.

**Discussion**

The present work shows for the first time that the NGC PB (i) induces profound alterations in the transcriptome profiles of MC and much less in HC, (ii) increases the production and release of proinflammatory cytokines from MC, (iii) causes a proinflammatory reaction and nuclear translocation/activation of NFκB in HC and (iv) thereby protects HC from proapoptotic stimuli. These alterations appear to be causally involved in the carcinogenic action of this compound as outlined in the following.

We have shown in previous and the present study that the NGC PB is able to induce multiple alterations in the hepatic mesenchyme, which is reflected not only by altered transcriptome profiles but also by deregulated patterns of intracellular as well as secreted proteins (36). PB treatment elevated transcript levels of chemokines of the CC-, CXC-type, of the TNF, TGFβ and interleukin superfamily. CCL2, CCL7 and CXCL10 specifically attract monocytes and/or regulate macrophage function and were found to be elevated at both time points of investigation. To understand the impact of the altered intrahepatic chemokine patterns, we determined the occurrence of KC and EM and found no alteration between untreated and PB-treated livers (data not shown). Next, we studied the effect of PB on hepatic inflammation induced by a necrogenic dose of the genotoxic nitrosamine N-nitrosomorpholine. N-nitrosomorpholine alone reduced the frequency of KC but elevated dramatically the occurrence of EM (Supplementary Figure 8, available at Carcinogenesis Online). Application of PB appeared to delay both, the recruitment of EM to the liver and the repopulation of this organ by KC. Interestingly, also barbiturates have been documented repeatedly to impair the innate host defense in mammals. In rats, long-term treatment with PB reduced lymphoproliferative responses and anesthesia by thiopentone impaired phagocytosis of peripheral macrophages (37,38). Patients sedated by barbiturates often suffer from life-threatening bacterial infections in the post-operative period (23,24). Mechanistic studies revealed that barbiturates inhibit chemotaxis/recruitment.
and phagocytosis of human monocytes and/or macrophages (23,24,39). The upregulated CCL- and CXCL-mRNAs, as observed in the present study in MC-PB, might be a consequence of the body’s attempt to counter-regulate the impaired recruitment of KC/EM.

There were also direct effects of PB on isolated and cultured KC/EM, raising questions on the underlying mechanism (Figure 3). As we could not detect significant levels of the PB-responsive nuclear receptors CAR or PXR in EC or KC/EM, we tested for expression of receptors known to bind PB in neuronal/non-hepatic tissues. PB prolongs/potentiates the action of GABA on GABA_A receptors and at higher concentrations was shown to directly activate this receptor (28). Barbiturates also block AMPA/kainate receptors and impair the glutamine receptors 3 and 6 (28–31). However, in HC, EC and KC/EM, we could not find significant transcript levels of components of these receptors (data not shown). However, KC/EM express several glycine receptor subtypes, as described previously and confirmed in the present study (32). Froh et al. (32) showed that cell membranes of KC became hyperpolarized by glycine due to a chloride influx. This hyperpolarization prevented lipopolysaccharide-induced activation of the cells, thereby minimizing production of TNFα and various other cytokines and eicosanoids, such as prostaglandins. PB, however, was found to interfere with the response of isolated human α1-glycine receptors to glycine (33). When glycine impairs the proinflammatory function of the cells and PB inhibits the glycine effect, the final outcome might be enhanced production of cytokines under PB treatment, as seen under our present experimental conditions. This also agrees with reports on enhanced production of prostaglandins in PB-treated rat KC (20). Furthermore, when tested at blood concentrations reached during anesthetic administration, the barbiturate pentothal caused a 4-5-fold increased production of TNFα from human monocytes (40). Taken together, these data from literature agree with our finding that despite of a transiently impaired recruiting/repopulation of KC/EM in the inflamed liver by PB, PB-treated
Figure 4. Supernatant of MC from PB-treated rats enhances translocation of NFκB to HC nuclei. (A, B, D) Rats were left untreated or were treated with PB for 7 days to generate SN/MC-CO or SN/MC-PB, which were applied to cultured HC, obtained from control rats. Treatment with TNFα (10 ng/ml) served as control. BAY (10 µM) was applied to HC 1h before the addition of supernatants or TNFα. (A) Nuclear proteins of HC were separated by SDS page and immunoblotted for detection of NFκB. (B) Nuclear NFκB protein levels were evaluated by densitometry and expressed as fold medium control. (C) Immunostains of formalin-fixed and paraffin-embedded tissue sections obtained from control liver (left panel) or livers subjected to PB treatment for 7 days (right panel); magnification: ×150. (D) HC from untreated rats were cotransfected by NFκB-responsive luciferase and renilla luciferase vectors. Supernatants were added 24h after transfection for 24h. Data show luciferase activity, normalized to renilla activity and expressed as fold medium control. (B, D) Data are means ± SEM of ≥3 rats. Statistics by Wilcoxon’s t-test: medium versus BAY, SN/MC-CO or TNFα: (a) P ≤ 0.05, (b) P ≤ 0.01; SN/MC-PB treatment versus SN/MC-PB treatment + BAY: (d) P ≤ 0.05.

Figure 5. Supernatant of PB-treated MC exerts anti-apoptotic activity in HC. Rats were left untreated or were treated with PB for 7 days to generate SN/MC-CO or SN/MC-PB, which were applied to cultured HC, obtained from control rats. Parallel cultures were treated with recombinant IL1β (0.1 ng/ml), TNFα (1 ng/ml) and/or TGFβ1 (1 ng/ml). About 24h later, HC were fixed and stained with Hoechst to count apoptotic bodies: (A) shows early stage apoptosis (B) an advanced stage. (C) At least 2000 HC per experiment and treatment group were screened. Data are expressed as fold medium control and are means ± SEM of ≥3 rats. Abbreviations: Med., medium. Statistics by Wilcoxon’s test: medium versus TNFα or TGFβ1: (a) P ≤ 0.05, (b) P ≤ 0.001; TGFβ1 versus TGFβ1+SN/MC-PB: (c) P ≤ 0.05.
KC/EM per se release enhanced levels of proinflammatory cytokines. Furthermore, the human relevance of our findings on immunological effects of PB is supported as well.

With regard to the action of PB as NGC in rats, continuous tumor promotion with this compound only marginally elevated DNA replication in the premalignant hepatic lesions but distinctly suppressed the elimination of preneoplastic HC by apoptosis, leading to outgrowth of tumors (Supplementary Figure 1, available at Carcinogenesis Online) (3,5). Discontinuation of PB leads to a dramatically elevated apoptotic activity in the liver reversing not only PB-induced hyperplasia but reducing dramatically also the size of the preneoplastic lesions. This PB-induced shift from death towards renewal of preneoplastic cells was found to be the base of the tumor promoting effect of this compound (3,5).

Homeostasis in mammalian tissues is dependent on the continuous integration of cell survival and cell death signals mainly deriving from the extracellular environment. Extensive crosstalk between these antagonistic signaling pathways emanate from TNFR1 stimulation. The proapoptotic downstream signaling includes JNK-activating kinase (JNK), erk and caspase-8 activation. Antia apoptotic signaling events are IkBα phosphorylation/ubiquitination and NFκB translocation into the nucleus (41). The HC-specific deubiquitinating enzyme ubiquitin carboxy-terminal hydrolase-2 (USP2), which is involved in degrading IkBα, is considered critical, i.e. USP2 knockdown inhibited actinomycin D/TNFα-induced apoptosis of HC via elevated levels of the anti-apoptotic protein c-flip(L/S), while USP2 overexpression exerted the opposite effect. Thus, Haimerl et al. (42) suggested that TNFα-induced USP2 downregulation is an effective antia apoptotic mechanism for HC. Papa et al. (43) observed that NFκB activation promotes growth arrest and DNA-damage-inducible, beta (GADD45β) activation, which also blocks the TNFα-induced apoptosis of HC. Analogous alterations were observed in PB-exposed rat HC under our experimental conditions. A counter-regulated and thus truncated TNFα and interferon response was evident, involving USP2 downmodulation, nuclear translocation of NFκB and upregulation of GADD45β (Figure 6). To conclude, our present work provides strong evidence that mesenchyme-induced NFκB activation in HC is deeply involved also in non-genotoxic hepatocarcinogenesis, driven by PB.

Much effort has been focused on the assessment of the putative health risks for humans being continuously treated by barbiturates. For better estimation, cross-species comparison may be helpful. We analyzed the hepatic mesenchyme in PB-treated mice and failed to see significant proinflammatory alterations (Supplementary Figures 9 and 10, available at Carcinogenesis Online). This observation might be due to profound functional differences of the innate immune system between rats and mice (44–46). This is also in line with the observation that in mice apoptosis suppression appears to be of minor importance in both, PB-driven hepatocarcinogenesis (outlined above) and regulation of liver mass (8). Accordingly, withdrawal of the NGC WY-14,643 or food led to regression of the murine liver without considerable induction of the hepatocellular apoptotic activity (47). However, in rats and humans, the effects of barbiturates on the innate immune system appear to be similar, i.e. a delayed recruitment of monocytes/macrophages and an increased production of proinflammatory cytokines by these cells were observed in both species. The TNFα-mediated antiapoptotic activity in HC can easily switch towards proapoptotic effects of this cytokine. Accordingly, death ligands like TNFα or FasL are often mechanistically involved in the development of drug-induced liver injury. In fact, during long-term PB treatment a mixed pattern of hepatocellular and/or cholestatic damage is seen in a small fraction (<1%) of the patients (48). Liver biopsies from these patients often show granulomatous infiltrations but enhanced apoptosis/necrosis of HC has also been observed (49). Further research in human-relevant systems is required to clarify whether PB-treated human hepatic mesenchyme produces elevated levels of TNFα which might lead to induction of apoptosis and drug-induced liver injury or apoptosis suppression and tumor promotion.

To conclude, our findings imply that the carcinogenic activity of NGC may not, or not always, result primarily or exclusively from effects on the parenchyma as generally assumed. Rather, direct effects on the hepatic mesenchyme seem to play...
important roles in NGC carcinogenesis. This new concept needs to be tested with other NGC as well as with other organs and species. Eventually, new insight generated along these lines will improve concepts of risk assessment of NGC.

Supplementary material
Supplementary Figures 1–11 and Tables 1–3 can be found at http://carcin.oxfordjournals.org/.

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