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The effect of phenobarbital on the methylation level of the *p16* promoter region in rat liver

Grażyna Kostka*, Katarzyna Urbanek, Jan K. Ludwicki

Department of Environmental Toxicology, National Institute of Hygiene, Chocimska 24, 00-791 Warsaw, Poland

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Abstract

It has been suggested that non-genotoxic carcinogens (NGCs) may cause modification of the DNA methylation status. We studied the effects of phenobarbital (PB) – a non-genotoxic rodent liver carcinogen – on the methylation level of the promoter region of the *p16* suppressor gene, as well as on hepatomegaly, DNA synthesis, and DNA-methyltransferase (DNMTs) activity in the rat liver. Male Wistar rats received PB in 1, 3 or 14 daily oral doses (at 24-h intervals), each equivalent to 1/10 of the LD_{50} value. The study showed that PB has caused persistent elevation in relative liver weight (RLW) as well as a transient increase in DNA synthesis. This suggests that the PB-induced increase in RLW was due to a combination of both hyperplasia and hypertrophy of liver cells. The effect of PB on DNA synthesis corresponded to an increase in the methylation pattern of the *p16* promoter sequence. Methylation of cytosine in the analyzed CpG sites of the *p16* gene was found after short exposure of the animals to PB. Treatment of rats with PB for 1 and 3 days also produced an increase in nuclear DNMTs activity. After prolonged administration (14 days), DNA synthesis declined, returning to the control level. No changes in methylation of the *p16* gene nor in DNMTs activity were observed. The reversibility of early induced changes in target tissues is a mark characteristic of tumor promoters. Thus, transient changes in methylation of the *p16* gene, although their direct role in the mechanisms of PB toxicity, including its carcinogenic action, remains doubtful, may therefore be a significant element of such processes.

Keywords: *p16*; DNA methylation; DNA methyltransferase; DNA synthesis; Phenobarbital; Liver

1. Introduction

A large proportion of known chemical carcinogens are agents whose carcinogenic activity is not related to the direct damage of genetic material (Ashby, 1992; Yamasaki et al., 1996; Yoshikawa, 1996). These compounds, referred to as non-genotoxic carcinogens (NGCs), form a heterogeneous group both in terms of chemical structure and of biological and pharmacological activity. They include numerous industrial pollutants, solvents, drugs and plant protection products. The mechanisms of NGCs activity have not been elucidated completely, which largely impedes the evaluation of human health hazards resulting from exposure to this category of carcinogens.

The data available on NGCs indicates that these compounds increase the incidence of tumors in rodents and that their common feature is the ability to stimulate cell proliferation in target organs and tissues and to suppress

* Corresponding author. Tel.: +48 2254 21 356; fax: +48 2284 97 441.
E-mail address: gkostka@pzh.gov.pl (G. Kostka).

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apoptosis (Grasl-Kraupp et al., 2000; Plant et al., 1998; Roberts et al., 1997; Schulte-Hermann et al., 1995). They cause an imbalance between cell replication and death, causing a disturbance in cellular homeostasis (Cohen and Ellwein, 1990; Roberts et al., 1997; Schulte-Hermann et al., 1999), which may in consequence lead to neoplastic transformation. This demonstrates the extreme importance of the mechanisms that control both the cell cycle and apoptosis—mechanisms exercised by many proteins encoded by protooncogenes and suppressor genes.

Actually, changes in DNA methylation are considered one of the mechanisms of action for NGCs. DNA methylation directly follows replication and plays an important role in the regulation of gene expression (Goodman and Watson, 2002; Novik et al., 2002; Watson et al., 2003). From the biochemical point of view, DNA methylation is based on the coupling of a methyl group to cytosine, with *S*-adenosyl methionine (SAM) serving as the methyl group donor. The reaction is catalyzed by enzymes belonging to the family of DNA methyltransferases (DNMTs) (Bestor, 2000; Pereira et al., 2001). The resulting products are 5-methylcytosine (5-MeC) and *S*-adenosyl homocysteine (SAH).

One possibility of an altered methylation profile of a particular gene is the hypermethylation or hypomethylation of its promoter region. Such alterations are frequently found in protooncogenes and suppressor genes. Both hyper- and hypomethylation may contribute to carcinogenesis via silencing of tumor suppressor genes, activating protooncogenes, and/or decreasing genome stability (Goodman and Watson, 2002; Watson et al., 2003).

Many types of cancer cells (both human and rodent) have been found to exhibit increased CpG sequence methylation in gene promoter regions, especially for genes whose protein products take part in cell cycle control (Esteller et al., 2001; Pogribny and James, 2002; Reik et al., 2001).

The aim of the present study was to evaluate the effect of phenobarbital (PB) on the methylation level of the promoter region of the tumor suppressor gene *p16*. The selection of this compound was justified by its toxicological characteristic. IARC has classified PB as a non-genotoxic agent, definitely carcinogenic in animals and possibly carcinogenic in humans—Group 2B (IARC, 2001). Further, we chose for our studies the tumor suppressor gene *p16*, which encodes a protein involved in cell cycle control. This protein acts by inhibiting the activity of complexes formed by cyclin D with the cyclin-dependent kinases CDK4 and CDK6 (Belinsky, 2005). Hypermethylation of *p16* leads to inactivation of the gene and has been found in many types of cancer cells (Belinsky, 2005; Wong et al., 1999). Moreover, Belinsky et al. (1998) and Watson et al. (2003) have reported that aberrant methylation of the promoter of the *p16* tumor suppressor gene is an early event in most cancers, including liver cancer.

The expected changes in the methylation status of the promoter region of the *p16* gene were correlated with changes in DNA synthesis (S-phase), DNA methyltransferase (DNMTs) activity, and liver weight (i.e. target tissue).

2. Materials and methods

2.1. Chemicals

PB (95%) was from "Galenus", Poland; 6 [^{3}H]-thymidine (specific activity 1020 MBq/mol) was provided by the Institute for Research, Production and Use of Radioisotopes, Prague, Czech Republic; [^{3}H-methyl] *S*-adenosyl-L-methionine (SAM) (specific activity 362.6 GBq/mmol) was obtained from Perkin-Elmer LAS, Inc.; Genomic DNA Purification Kit, the HpaII restriction enzyme and phage λ DNA were purchased from Fermentas, USA; FastStart Taq Polymerase was obtained from Roche, Germany.

Other chemicals were obtained from Sigma Chemicals, UK, and were of the highest quality and purity.

2.2. Animals and outline of the experiments

Male Wistar rats (8 weeks) of our breed (Pzh:WIS) were used. Prior to use, the rats were acclimatized for 14 days at a controlled temperature of 22 ± 1 °C and relative air humidity of 50 ± 10%, with a 12-h light/dark cycle. Groups of rats (body weight of $200\,g \pm 10$%; five rats/group) received PB in single or repeated daily doses (at 24-h intervals, for 1, 3 or 14 days, respectively) of 92,8 mg/kg b.w. day^{-1}, which corresponded to 1/10 of the LD_{50} value. This PB dosage was chosen because it was the concentration that increased DNA synthesis in male Wistar rats (IARC, 2001). The acute oral LD_{50} of PB, determined according to the method of Weill and Deichman (OECD 401), ranged between 835 and 1003 mg/kg b.w. (927.7) for male rats (Pzh:WIS). PB was administered by gavage in an olive oil suspension between 8.00 and 9.00 a.m.; control animals received only the olive oil vehicle. During the adaptation period and throughout the experiment, the animals were fed a commercial diet and received tap water *ad libitum*.

The animals were observed daily and their body weight as well as food and water consumption were monitored. For estimation of DNA synthesis, [^{3}H]-thymidine (1.2 MBq per rat) was injected intraperitoneally (i.p.) into all animals 1 h before sacrifice.

Livers were immediately excised, rinsed, blotted to dryness, weighed, frozen in liquid nitrogen and stored at −70 °C. Rep-