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THE EFFECT OF PHENOBARBITAL ON GENE EXPRESSION LEVELS OF *p53* AND *Dnmt1* IN THE LIVER OF *WISTAR* RATS

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ABSTRACT

Background. Our previous studies have shown that short-term treatment with phenobarbital (PB) resulted in cytosine methylation of CpG sites on the *p53* gene promoter in male *Wistar* rats' liver. Furthermore, PB induced DNA-methyltransferases (DNMTs) activity was also demonstrated; being the enzymes that catalyze methyl group transfer to cytosine in CpG dinucleotides.

Objective. Since DNA methylation is involved in regulating gene transcription and that DNMT1 is implicated in regulating DNA methylation, this study assessed whether PB-induced hypermethylation of the *p53* promoter region was associated with an altered expression of *p53* and *Dnmt1* genes.

Material and methods. Male *Wistar* rats received PB in three daily oral doses (at 24-h intervals) of 92,8 mg/kg b.w. x day^{-1}. Levels of mRNA for *p53* and *Dnmt1* and levels of relevant proteins were respectively examined by Real-Time PCR and Western blot analysis.

Results. Gene expression analysis revealed that exposure of *Wistar* rats to PB caused statistically significant alternations in the expression of tested genes. We found that both mRNA and protein expression of p53 was down-regulated, whereas expression of *Dnmt1* (both mRNA and protein) was up-regulated after PB treatment.

Conclusions. Suppression of *p53* mRNA and protein expression, which is probably a result of epigenetic changes, (in particular aberrant *p53* promoter hypermethylation), can be associated with tumour promoting activity of phenobarbital.

Key words: *phenobarbital, p53, Dnmt1, genes expression, liver, rats*

STRESZCZENIE

Wprowadzenie. Nasze wcześniejsze badania wykazały, że krótkoterminowe narażenie szczurów *Wistar* na fenobarbital (PB) stymulowało metylację cytozyny w badanych sekwencjach rejonu promotorowego genu *p53*. Ponadto stwierdzono wzrost aktywności metylotransferaz DNA (DNMT), enzymów które katalizują przenoszenie grupy metylowej do cytozyny w dinukleotydach CpG.

Cel badań. Z uwagi że metylacja DNA pełni istotną rolę w ekspresji genów, a DNMT1 uczestniczy w regulacji metylacji DNA, w prezentowanych badaniach oceniano czy indukowana PB hipermetylacja rejonu promotorowego genu *p53* była związana ze zmianami ekspresji genów *p53* i *Dnmt1*.

Materiał i metody. Samce szczurów szczepu *Wistar* otrzymywały PB w dawce 92,8 mg/kg m.c. x dzień$^{-1}$, 3-krotnie w odstępach dobowych. Analizę poziomu transkryptów i białek badanych genów przeprowadzano odpowiednio metodą Real-Time PCR i Western blot.

Wyniki. W wyniku oddziaływania PB wykazano obniżoną ekspresję genu *p53* i wzrost ekspresji metylotranserazy 1 (DNMT1).

Wnioski. Supresja ekspresji *p53* (na poziomie mRNA i białka) będąca prawdopodobnie wynikiem zmian epigenetycznych, w szczególności hipermetylacji jego rejonu promotorowego może być związana z promocyjną aktywnością fenobarbitalu.

Słowa kluczowe: *fenobarbital, p53, Dnmt1, ekspresja genów, wątroba, szczury*

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INTRODUCTION

In eukaryotes, it is well known that the degree of chromatin compaction has an inherent role in regulating DNA accessibility for transcription factors. In condensed forms of chromatin (heterochromatin), most genes are inactive. The main mechanisms of regulating this gene expression are DNA methylation and post--transcriptional modifications of histones resulting in the reorganisation of chromatin structure. On the other hand, mounting evidence suggests that epigenetic alterations are early indicators of environmental chemicals' exposure [2, 5, 16].

From the biochemical point of view, DNA methylation is based on the coupling of methyl group to C^5 of cytosine ring found in CpG dinucleotides, where S--adenosyl methionine (SAM) serves as the methyl group donor. The reaction is catalysed by a group of DNA methyltransferases (DNMTs) [6]. In the mammalian cell there are three known active DNMTs (DNMT1, DNMT3a and DNMT3b). Among them, DNMT1 is responsible for maintaining the normal methylation pattern in repeated cycles of replication preceding cell divisions.

In our previous studies [14], during short-term treatment of *Wistar* rats with PB (it being the most widely used anticonvulsant worldwide and classical nongenotoxic rodent liver carcinogen), altered DNA methylation of the *p53* gene was observed. Cytosine hypermethylation in the analysed CpG sites of the *p53* gene promoter was associated with increased DNMTs' activity as well as DNA synthesis.

Since methylation changes in the promoter region of genes is one of the pathways for regulating gene expression [11], the present studies focus on evaluating whether PB-induced hypermethylation of *p53* gene can affect transcriptional activities of this tumour suppressor gene. We thus estimate the effects of PB on *p53* as well as *Dnmt1* expression (both mRNA and protein) in the liver of male *Wistar* rats.

MATERIAL AND METHODS

Animals and treatment

Twenty male *Wistar* rats (aged 5 weeks, 110-130 g) were purchased from the Center of Experimental Medicine, Medical University of Bialystok, Poland. Before treatment, the animals were housed (five rats per cage) at a temperature of 22± 1°C, relative humidity of 50 ±10% and on a 12-h light:12-h dark cycle. The animals were allowed unrestricted access to tap water and a standard rodent diet. The animals were also permitted to acclimatise for at least 2 weeks at the described conditions under which they were maintained throughout the study. Rats weighing 200-220 g were randomly distributed into two groups. PB was administered by oral gavage in an olive oil suspension (92,8 mg/kg b.w. x day^{-1}) between 08:00 h and 09:00 h, for 3 days (at 24-h intervals). Control animals received only the olive oil suspension. Sections of the liver's right lobes were removed, frozen in liquid nitrogen and stored at -80° C.

All procedures involving animals were performed according to national animal welfare regulations after receiving authorisation by the Local Ethic Committee for the conduct of research studies on live vertebrates (No 23/2010).

Real-Time PCR for p53 and Dnmt1 genes

Total RNA was extracted from frozen liver tissue using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA were synthesised from 1 μg total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Clontech, USA). The cDNA was then analysed by quantitative Real-Time PCR using a KapaSybr®FastqPCR Kit (KapaBiosystems, USA) on the MiniOpticon system (Bio-Rad). The Real-Time PCR conditions and the primer sequences for *Dnmt1* and β-*actin* were described previously [26].

Primers for *p53* were as follows: p53 F-5'TCTGTTTCAAAAAGCAAAAAGATGAC-3', p53 R-5' ATAGCAAGGAAAGTCATGAACTGCCA-3' (GenBank No NM_030989.3).

Western blot analysis of p53 and DNMT1 proteins expression

Western blotting was performed as previously described [26]. The following antibodies were used: p53 (dilution 1:5000), DNMT1 (1:2000), β-actin (1:5000), peroxidase-conjugated goat antimouse IgM antibody and donkey anti-goat IgM antibodies (1:5000 dilution) (Santa CruzBiotechnology, USA). Proteins were resolved by 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Bands were quantified by the Image Quant software (Molecular Dynamics, version 5.2) and normalised relative to β-actin. Fold changes were expressed by normalising the corresponding value of the vehicle-treatment animals to one of the internal controls.

Statistical analysis

Protein levels were analysed by the *Student's t*-test. Statistical analyses were performed using Statistica software (version 6). REST software Randomization tests (Pair Wise Fixed Reallocation Randomization TEST) was used to assess the statistical significance of demonstrated differences in mRNA levels [18]. A probability level less than 0.05 was used as a criterion for significance.

RESULTS

Effect of PB on p53 mRNA and protein expression

Using Real-Time PCR, gene-specific mRNA expression was quantified in the liver from rats treated with PB and results were expressed relative to the number of β-*actin* transcripts. The mRNA level of *p53* from the control group was set at 1.00 and mRNA expression of experimental groups was evaluated by its relative ratio. As shown in Figure 1A, repeated treatment with PB (3 days of dosing), decreased *p53* mRNA in the liver of *Wistar* rats in comparison to their respective vehicle-treated animals. The transcript level of *p53* gene was decreased to approximately 60% of the normalised control level.

Given that PB treatment decreased *p53* gene transcription, we then questioned whether the protein level of this gene was also affected by PB. The densitometric scanning of western blot results showed that protein levels in the liver of rats exposed to PB were also decreased compared to controls. Figure 1B, shows that the protein level was reduced by up to 34% as a result of three PB doses.

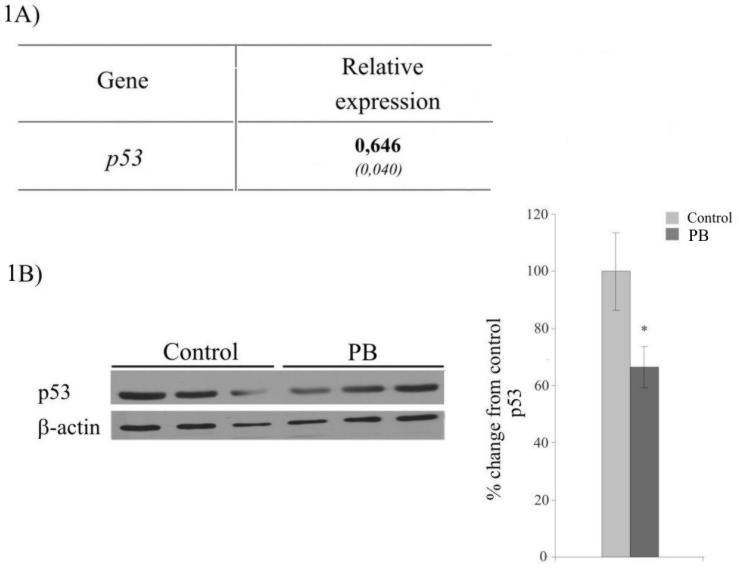
Effect of PB on Dnmt1mRNA and protein expression

A comparison of results from methylation analysis with the results from quantifying the expression of epigenetic modulating enzymes, like DNMTs, can provide a valuable insight into the causes of epigenetic alterations [10].

Since DNMT1 is considered to be primarily responsible for the maintenance of DNA methylation and that it copies the pre-existing methylation pattern onto the daughter strand after DNA replication [13], we have therefore examined DNMT1 expression in PB--treated rats. The effect of PB on hepatic *Dnmt1* mRNA and protein expression is shown in Figure 2.

Figure 2A demonstates that, relative *Dnmt1* mRNA levels were changed significantly as compared to the control animals. The treatment with a tumor-promoting dose of PB for 3 days resulted in up-regulation of *Dnmt1* transcription. A statistically significant difference was evident when comparing the data of the exposed group vs. those of the vehicle control group. We have shown a statistically significant (p=0.04) increase in *Dnmt1* mRNA by over 50%.

An analysis of DNMT1 expression was then performed and increased DNMT1 was detected in response to PB (Figure 2B). DNMT1 protein in rat liver was found to be significantly increased by 22% as compared with controls.



1A) Real-Time PCR analysis was used to determine mRNA levels of p53 gene. The data were normalized to an endogenous reference,β-actin and expressed as relative to control value; p-values are given in brackets; and statistical analyses were carried out using randomization test.

1B) Representative bands obtained by Western immunoblotting analysis of p53. The graphs on the right represent the mean ± S.E.M. from group of five rats (n=5), normalized to β-actin. The mean value of protein, indicated in percentage, relative to controls, considered 100%.

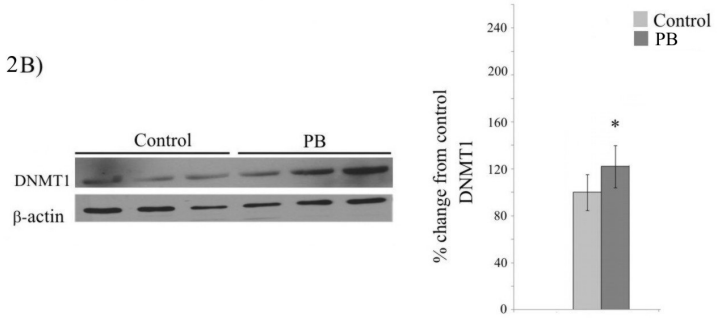
Asterisks (*) indicate statistical difference from the corresponding control group.

Figure 1. The effects of PB on *p53* gene expression (at mRNA and protein level) in the liver of male *Wistar* rats.

2A)

Gene	Relative expression
Dnmt1	**1,575** *(0,040)*

2B)



2A) Real-Time PCR analysis was used to determine mRNA levels of Dnmt1 gene. The data were normalized to an endogenous reference, β-actin and expressed as relative to control value; p-values are given in brackets; and statistical analyses were carried out using randomization test.

2B) Representative bands obtained by Western immunoblotting analysis of DNMT1. The graphs on the right represent the mean ± S.E.M. from group of five rats (n=5), normalized to β-actin. The mean value of protein, indicated in percentage, relative to controls, considered 100%.
Asterisks (*) indicate statistical difference from the corresponding control group.

Figure 2. The effects of PB on DNA methyltranferases 1 (DNMT1) expression in the liver of male *Wistar* rats.

DISCUSSION

The *p53* is one of the most frequently altered tumor suppressor genes in cancer [3]. It is also well known that mutations in *p53* result in loss-of-function. In addition to this genetic inactivation, epigenetic mechanisms also contribute to the inactivation or down-regulation of tumor suppressor genes, including *p53*[21]. The encoded p53 protein, which is ubiquitously expressed in tissue, keeps genome stability under stress, and is involved in multiple cellular activities. This protein plays a key role in the regulation of the cell cycle, DNA repair and apoptosis [22, 23]; these biological processes being critical for the initiation of carcinogenesis [4].

DNA methylation is one of several epigenetic mechanisms that cells use to control gene expression and DNA hypermethylation acts as an alternative mechanism for inactivating tumor suppressor genes [10].

Previously, we had examined methylation status of *p53* in cytosine residues located at nt: -450, -261, and -179 and that phenobarbital (PB) was found to stimulate *p53* promoter hypermethylation [14]. Because it is known that DNA methylation and gene expression are closely linked [11], in the way that methylation can lead to inappropriate gene silencing [9, 24], a Real-Time PCR and western blot was undertaken to respectively measure levels of the *p53* transcript and protein. Our results indicate that expression of *p53* was down-regulated after short-term exposure (3 days) of the animals to PB. A concomitant decrease in expression of p53 protein was also observed, confirming that the decreased RNA expression translated into a decrease in protein expression. This suggests that an epigenetic component such as promoter methylation might play an important role in regulating *p53* expression.

Some studies have documented a correlation between aberrant *p53* gene promoter methylation with low levels of mRNA production. This relationship was reported in human primary hepatocellular carcinoma [20] and in lymphoblastic leukemia [1]. In contrast, some studies [15, 25] suggest that other epigenetic modifications might be involved in regulating the *p53* gene. On the other hand, there is evidence on the importance of hypermethylation in the non-CpG island-containing promoter coding region in gene inactivation [19]. Within this context, it should be noted that the *p53* promoter region does not contain a CpG island and therefore, may be more sensitive to site-specific methylation [12, 20].

We have also demonstrated a PB-mediated significant increase in *Dnmt1* mRNA and protein levels. Increased expression of DNMT1 has been reported in development of hepatocellular [8] and pancreatic carcinomas [17]. Furthermore, it has been reported that DNMT1 induces hypermethylation of tumor suppressor genes to epigenetically mediate their repression [7]. This leads to the assumption that PB affects hypermethylation of *p53* through its positive regulation of DNMT1.

In conclusion, suppression of *p53* mRNA and protein expression, (which is probably a result of epigenetic

changes and, particulary aberrant *p53* promoter hypermethylation), can be associated with tumor promoting activity of phenobarbital.

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Conflict of interest
The authors declare no conflict of interest.

REFERENCES

1. *Agirre X., Novo F.J., Calasanz M.J., Larráyoz M.J., Lahortiga I., Valgañón M., García-Delgado M., Vizmanos J.L.*:TP53 is frequently altered by methylation, mutation, and/or deletion in acute lymphoblastic leukaemia. Mol-Carcinog 2003;38:201-208.
2. *Bachman A.N., Phillips J.M., Goodman J.I.*:Phenobarbital induces progressive patterns of GC-rich and gene-specific altered DNA methylation in the liver of tumor-prone B6C3F1 mice. Toxicol Sci 2006;91, 393-405.
3. *Bai L., Zhu W.G.*: p53: Structure, Function and Therapeutic Applications. Journal of Cancer Molecules 2006;2:141-153
4. *Baylin S.B., Ohm J.E.*: Epigenetic gene silencing in cancer: a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 2006;6:107–116
5. *Bollati V., Bacciarelli A.*: Environmental epigenetics. Heredity 2010;105: 105-112.
6. *Bombail V., Moggs J.G., Orphanides G.*: Perturbation of epigenetic status by toxicants. Toxicol Lett 2004;149, 51-58.
7. *Choi J.H., Min N.Y., Park J., Kim J.H., Park S.H., Ko Y.J., Kang Y., Moon Y.J., Rhee S., Ham S.W., Park A.J., Lee K.H.*: TSA-induced DNMT1 down-regulation represses hTERT expression via recruiting CTCF into demethylated core promoter region of hTERT in HCT116. BiochemBiophys Res Communications 2010;391:449-454.
8. *Choi M.S., Shim Y.H., Hwa J.Y., Lee S.K., Yu E.*: Expression of DNA methyltransferases in multistep hepatocarcinogenesis. Hum Pathol 2003;34:11–17.
9. *Das P.M., Singal R.*: DNA methylation and cancer. J ClinOncol 2004;22: 4632-4642.
10. *DeAngelis T.J., Farrington W.J., Tollefsbol T.O.*:An Overview of Epigenetic Assays. Mol Biotechnol 2008;38: 179–183.
11. *Esteller M.*; Epigenetic in cancer. N. Eng. J. Med., 2008;358: 1148-1159.
12. *Gao S., Skeldal S., Krogdahl A., Sørensen J.A., Andreasen P.A.*:CpG methylation of the PAI-1 gene 5'-flanking region is inversely correlated with PAI-1 mRNA levels in human cell lines. Thromb Haemost 2005;94: 651–660.
13. *Goyal R., Reinhardt R., and Jeltsch A.*: Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. Nucleic Acids Research 2006; 34: 1182–1188.
14. *Kostka G., Urbanek-Olejnik K., Wiadrowska B., Bańkowski R.*: Indukowana fenobarbitalem hipermetylacja rejonu promotorowego genu p53 w wątrobie szczura szczepu Wistar. Rocz Panstw Zakl Hig 2008;59: 455-465 (in Polish).
15. *Kumar M., Lu Z., Tkwi A.A., Chen W., Callander N.S., Ramos K.S., Young K.H., Li Y.*: Negative regulation of the tumor suppressor p53 gene by microRNAs. Oncogene 2011;30: 843-853.
16. *Liu W., Liu J., Ao L., Zhou Z., Zhou Y., Cui Z., Yang H., Cao J.*: Dynamic changes in DNA methylation during multistep rat lung carcinogenesis induced by 3-methylcholantrene and diethylnitrosamine. ToxicolLett 2009;189: 5-13.
17. *Peng D.F, Kanai Y., Sawada M., Ushijima S., Hiraoka N., Kitazawa S., Hirohashi S.*: DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. Carcinogenesis 2006;27: 1160-1168.
18. *Pfaffl M.W., Horgan G.W., Dempfle L.*: Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30: e36.
19. *Pogribny I.P., Rusyn I.*: Role of epigenetic aberrations in the development and progression of human Hepatocellular carcinoma. Cancer Lett 2012;342: 223-230.
20. *Pogribny L.P., James S.J.*: Reduction of p53 expression in human primary hepatocellular carcinoma is associated with promoter region methylation without coding region mutation. Cancer Lett 2002;176: 169-174.
21. *Saldaña-Meyer R.,and Recillas-Targa F.*: Transcriptional and epigenetic regulation of the p53 tumor suppressor gene. Epigenetics 2011;6:1068-1077.
22. *Schuler M., Green D.R.*: Mechanisms of p53-dependent apoptosis. Biochem Soc Trans. 2001;29:684-688.
23. *Scoumanne A., and Chen X.*: Protein methylation: a new regulator of the p53 tumor suppressor. Histol Histopathol 2008;23: 1143–1149.
24. *Sharma S, Kelly TK, and Jones PA.*: Epigenetics in cancer. Carcinogenesis 2010; 31: 27- 36.
25. *Tian S., Huang S., Wu S., Guo W., Li J., He X.*: MicroRNA-1285 inhibits the expression of p53 by directly targeting its 3 ˙untranslated region. Biochem Biophys Research Commun 2010; 396: 435–439
26. *Urbanek-Olejnik K., Liszewska M., Winczura A., Kostka G.*: Changes of c-Myc and Dnmt1 mRNA and protein levels in the rat livers induced by dibuthyl phthalate treatment. Toxicol Ind Health, first published on December 5, 2013 as doi:10.1177/0748233713512363

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