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Significant Increase of 8-Hydroxydeoxyguanosine in Liver DNA of Rats Following Short-term Exposure to the Peroxisome Proliferators Di(2-ethylhexyl)phthalate and Di(2-ethylhexyl)adipate

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8-Hydroxydeoxyguanosine (8-OH-dG) levels were examined in liver and kidney DNA after di(2-ethylhexyl)phthalate (DEHP), di(2-ethylhexyl)adipate (DEHA) and phthalic anhydride administration to male 6-week-old F-344 rats in the diet at concentrations of 1.2, 2.5 and 1.5%, respectively. Significant increases in 8-OH-dG levels were observed only in the liver (target organ of DEHP and DEHA carcinogenesis) DNA after 1 and 2 weeks of treatment with DEHP and DEHA, respectively. The results suggest the involvement of oxidative DNA damage in hepatocarcinogenesis by peroxisome proliferators.

Key words: 8-Hydroxydeoxyguanosine — Peroxisome proliferator — Di(2-ethylhexyl)phthalate — Di(2-ethylhexyl)adipate — DNA damage

Peroxisome proliferators are considered to be a novel class of non-mutagenic carcinogens, which induce hepatomegaly and peroxisome proliferation in the livers of rodents.[1] Thus, hepatocellular tumors develop after long-term oral administration of hypolipidemic drugs such as clofibrate, nafenopin and ciprofibrate.[2] DEHP and DEHA are also well known peroxisome proliferators,[3] DEHP being the plasticizer most commonly used for vinyl products. DEHA has also found application as a plasticizer, particularly in food product containers. There is some evidence that these 2-ethylhexyl compounds exhibit hepatocarcinogenic potential in rodents, while PAn, a precursor in the manufacture of phthalate esters, did not increase the occurrence of tumors.[4] Since peroxisome proliferators exert no direct mutagenicity, and do not interact with DNA or cause DNA damage,[5,6] it was suggested that the carcinogenicity of these agents might be mediated by oxidative DNA damage resulting from an increase of the hydrogen peroxide-generating peroxisomal fatty acid β-oxidation enzyme system in the liver.[7–9] This hypothesis was supported by the finding of increased hepatic lipofuscin in rats chronically treated with peroxisome proliferators and the fact that peroxisome proliferator-induced hepatocarcinogenesis can be inhibited by antioxidants.[10,11]

Recently, formation of 8-OH-dG by hydroxylation at the C-8 position of deoxyguanosine residues in DNA[12] was reported to be a reliable marker of oxidative DNA damage.[13] The present study was undertaken to examine the effects of the plasticizer DEHP and related compounds on 8-OH-dG levels in liver and kidney DNA in an attempt to relate peroxisome proliferation to carcinogenic activity.

A total of 40 male F-344 rats (6-week-old, specific pathogen-free, Charles River Inc., Kanagawa) were divided into 4 groups, each consisting of 10 animals. DEHP, DEHA and PAn, purchased from Wako Pure Chemical Ind. Ltd., Osaka, were each mixed in powder diet CRF1 (Charles River Inc.) at concentrations of 1.2, 2.5 and 1.5% and the diets thus prepared were fed to the animals *ad libitum*. Control rats were similarly fed powder diet (CRF1) not containing the compounds. The body weights and food consumption were recorded twice a week. Five rats of each group were killed at week 1 and the remaining 5 rats were killed at week 2 by exsanguination under ether anesthesia. The livers and kidneys from all animals were quickly removed and weighed. The liver and kidney DNAs were isolated immediately by the method of Kasai *et al.*[13] and stored in a dried state under argon at $-80^{\circ}C$ until required for 8-OH-dG analysis. DNA was digested to deoxynucleosides by treatment with nuclease P1 (Yamasa Shoyu Co. Ltd., Tokyo) and alkaline phosphatase (Sigma Chemical Co., USA) and the resulting deoxynucleoside mixture was injected into a high-performance liquid chromatography system coupled with an electrochemical detector: apparatus, Kontron HPLC Pump 420; column, Beckman Ultrasphere ODS (0.46×25 cm); UV detector, Shimadzu SPD-6A (290 nm); EC detector, ESA Coulochem 5100A (0.35 V); eluent, 8% aqueous methanol containing 10 m*M*

Abbreviations used: DEHP, di(2-ethylhexyl)phthalate; DEHA, di(2-ethylhexyl)adipate; PAn, phthalic anhydride; 8-OH-dG, 8-hydroxydeoxyguanosine.

Table I. Effects of DEHP, DEHA or PAn Administration on 8-OH-dG Levels and Organ Weights in Liver and Kidney of Male F-344 Rats

Organ	Experimental weeks	Control (Dose) (0%)	DEHP (1.2%)	DEHA (2.5%)	Pan (1.5%)
		8-Hydroxydeoxyguanosine/10^5 deoxyguanosine			
Liver	1	1.42±0.15	2.04±0.21[b]	2.21±0.46[a]	1.39±0.13
	2	1.49±0.08	2.44±0.60[a]	2.13±0.22[b]	1.49±0.15
Kidney	1	1.18±0.24	1.10±0.24	1.17±0.31	0.99±0.34
	2	1.25±0.19	1.44±0.19	2.07±0.75	1.23±0.23
		Relative organ weights (g/100 g body weight)			
Liver	1	4.33±0.36	6.58±0.40[b]	5.91±0.32[b]	4.10±0.15
	2	3.79±0.11	6.60±0.23[b]	5.93±0.31[b]	3.93±0.19
Kidney	1	0.79±0.06	0.78±0.03	0.82±0.02	0.72±0.03
	2	0.70±0.03	0.80±0.03[b]	0.86±0.01[b]	0.70±0.04

Values represent the mean±SD for data from 5 rats. Significantly different from the respective control value: *a*) $P<0.05$, *b*) $P<0.01$.

NaH_2PO_4.[13] The data were statistically analyzed by applying the Cochran *t* test.

The results are shown in Table I. Significantly reduced body weight gain as well as food consumption were observed in the DEHA group (data not shown). The absolute and relative liver weights at weeks 1 and 2 in the DEHP and DEHA groups were significantly higher than in the controls. The absolute and relative kidney weights in the DEHA group and relative kidney weights in the DEHP group at week 2 were also significantly higher than in the controls. Significant increases of 8-OH-dG levels (8-OH-dG/10^5 deoxyguanosine) in liver DNA were found at weeks 1 and 2 compared to the controls in both the DEHP and DEHA groups. In the DEHA group, at week 2, the 8-OH-dG level in kidney DNA was increased, but the change was not statistically significant. In contrast, no increase in 8-OH-dG level in either liver or kidney DNA was observed for the PAn group.

Since 8-OH-dG is an adduct that results from the damage to DNA caused by active oxygen radicals (most probably by hydroxyl radicals), it is a useful indicator of oxidant-induced DNA damage. It has been found that 8-OH-dG in DNA results in misreading during *in vitro* DNA synthesis[14] and a close relation between the formation of 8-OH-dG in DNA and organ specificity of carcinogenesis by potassium bromate (kidney) and some hypolipidemic peroxisome proliferators (liver), such as ciprofibrate, aluminum clofibrate and simfibrate, was reported.[13, 15, 16] After daily intragastric administration of DEHP (7.5 g/kg body weight/day) for 8 days, a significant increase in liver DNA 8-OH-dG was earlier observed in rats.[16] In this study, after administration of DEHP, DEHA and PAn to rats for 1 or 2 weeks, significant increases of 8-OH-dG level in liver DNA together with hepatomegaly were observed in the DEHP and DEHA groups, but not in the PAn (non carcinogen) group. These results thus suggest that the formation of 8-OH-dG in liver DNA is closely related to the hepatocarcinogenic activity of 2-ethylhexyl compounds. These effects are presumably directly linked to the hepatomegaly via increased peroxisome formation.

It was earlier reported that whereas chronic exposure of rats to ciprofibrate results in increased 8-OH-dG levels in liver DNA, administration of a single dose of the same compound has no such effect.[15] However, in the present study, we could detect an increase of liver DNA 8-OH-dG within one week, suggesting relatively rapid action of 2-ethylhexyl esters. Although it was not statistically significant, the increasing tendency of 8-OH-dG levels in the kidney DNA after 2 weeks of DEHA administration seems interesting. This might be related to the promoting effect of the phthalic ester as found in our two-stage rat renal carcinogenesis study.[17]

Recently, it was reported that the increase of 8-OH-dG in mitochondrial DNA caused by reactive oxygen species is more severe than that in nuclear DNA.[18] It therefore appears of importance that 8-OH-dG levels in both nuclear and mitochondrial DNA be examined after peroxisome proliferator treatment. In conclusion, we found that 8-OH-dG levels were significantly increased in the liver, a target organ for DEHP and DEHA carcinogenesis in a very short time compared to that required for carcinogenicity testing, and so the present results indicate 8-OH-dG levels to offer a very effective endpoint for prediction of carcinogenicity based on the underlying mechanisms of non-mutagenic agent action.

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REFERENCES

1) Rao, M. S. and Reddy, J. K. Peroxisome proliferation and hepatocarcinogenesis. *Carcinogenesis,* **8,** 631–636 (1987).
2) Stott, W. T. Chemically induced proliferation of peroxisomes: implications for risk assessment. *Regul. Toxicol. Pharmacol.,* **8,** 125–159 (1988).
3) Moody, D. E. and Reddy, J. K. Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. *Toxicol. Appl. Pharmacol.,* **45,** 497–504 (1978).
4) Kluwe, W. M. Carcinogenic potential of phthalic acid esters and related compounds: structure-activity relationships. *Environ. Health Perspect.,* **65,** 271–278 (1986).
5) Warren, J. R., Simmon, V. F. and Reddy, J. K. Properties of hypolipidemic peroxisome proliferators in the lymphocyte [^{3}H]thymidine and *Salmonella* mutagenesis assays. *Cancer Res.,* **40,** 35–41 (1980).
6) Gupta, R. C., Goel, S. K., Earley, K., Singh, B. and Reddy, J. K. ^{32}P-post-labeling analysis of peroxisome proliferator-DNA adduct formation in rat liver *in vivo* and hepatocytes *in vitro. Carcinogenesis,* **6,** 933–936 (1985).
7) Tomaszewski, K. E., Agarwal, D. K. and Melnick, R. L. *In vitro* steady-state levels of hydrogen peroxide after exposure of male F-344 rats and B6C3F1 mice to hepatic peroxisome proliferators. *Carcinogenesis,* **7,** 1871–1876 (1986).
8) Fahl, W. E., Lalwani, N. D., Watanabe, T., Goel, S. K. and Reddy, J. K. DNA damage related to increased hydrogen peroxide generation by hypolipidemic drug-induced liver peroxisomes. *Proc. Natl. Acad. Sci. USA,* **81,** 7827–7830 (1984).
9) Elliott, B. M., Dodd, N. J. F. and Elcombe, C. R. Increased hydroxy radical production in liver peroxisomal fractions from rats treated with peroxisome proliferators. *Carcinogenesis,* **7,** 795–799 (1986).
10) Reddy, J. K., Lalwani, N. D., Reddy, M. K. and Qureshi, S. A. Excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by methyl clofenapate and other hypolipidemic peroxisome proliferators. *Cancer Res.,* **42,** 259–266 (1982).
11) Rao, M. S., Lalwani, N. D., Watanabe, T. K. and Reddy, J. K. Inhibitory effect of antioxidants ethoxyquin and 2(3)-*tert*-butyl-4-hydroxyanisole on hepatic tumorigenesis in rats fed ciprofibrate, a peroxisome proliferator. *Cancer Res.,* **44,** 1072–1076 (1984).
12) Kasai, H. and Nishimura, S. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res.,* **12,** 2137–2145 (1984).
13) Kasai, H., Nishimura, S., Kurokawa, Y. and Hayashi, Y. Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA. *Carcinogenesis,* **8,** 1959–1961 (1987).
14) Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E. and Nishimura, S. DNA templates containing 8-hydroxydeoxyguanosine are misread both at the modified base and at adjacent residues. *Nature,* **327,** 77–79 (1987).
15) Kasai, H., Okada, Y., Nishimura, S. Rao, M. S. and Reddy, J. K. Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long-time exposure to a peroxisome proliferator. *Cancer Res.,* **49,** 2603–2605 (1989).
16) Takagi, A., Sai, K., Umemura, T. Hasegawa, R., Kurokawa, Y. and Kasai, H. Production of 8-hydroxydeoxyguanosine in rat liver DNA by oral administration of peroxisome proliferators. *Igaku no Ayumi,* **149,** 65–66 (1989) (in Japanese).
17) Kurokawa, Y., Takamura, N., Matsushima, Y, Imazawa, T. and Hayashi, Y. Promoting effect of peroxisome proliferators in two-stage rat renal tumorigenesis. *Cancer Lett.,* **43,** 145–149 (1988).
18) Richter, C., Park, J. W. and Ames, B. N. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA,* **85,** 6465–6467 (1989).