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No measurable increase in thymidine glycol or 8-hydroxydeoxyguanosine in liver DNA of rats treated with nafenopin or choline-devoid low-methionine diet

M.E. Hegi [1], D. Ulrich [2], P. Sagelsdorff [3], C. Richter [2] and W.K. Lutz [1]

[1] *Institute of Toxicology and* [2] *Laboratory of Biochemistry, Swiss Federal Institute of Technology, Zurich (Switzerland) and* [3] *Ciba Geigy AG, Basel (Switzerland)*

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Summary

Male rats were treated for 2 months with 1000 ppm nafenopin in the diet or for 4 or 7 days with a choline-devoid low-methionine diet. DNA was isolated from the livers and analyzed for the presence of *cis*-thymidine glycol-3′-phosphate (cis-dTGp) by ^{32}P-postlabeling and for the level of 8-hydroxy-deoxyguanosine (8-OH-dG) by electrochemical detection (ECD). In no DNA sample was the level of cis-dTGp above the limit of detection of 1 modified thymidine per 10^6 nucleotides. With 8-OH-dG, a background level of this modification of 20 8-OH-dG per 10^6 nucleosides was found in liver DNA of control rats, which was not affected by either treatment. It is postulated for thymidine glycol that a potential increase was below the limit of detection or was rapidly repaired in vivo and that the steady-state level of endogenous 8-hydroxydeoxyguanosine appears not to be influenced by the treatments chosen.

The formation of oxygen radicals is in part an endogenous process, which could result in DNA damage, mutagenesis, and 'spontaneous' carcinogenesis. Oxygen stress appears to be increased in a number of conditions ultimately leading to higher cancer incidence. For instance, the induction of liver tumors in rats by peroxisome proliferators (Rao and Reddy, 1987) and methyl donor-deficient diet (Mikol et al., 1983; Goshal and Farber, 1984) is often considered to be due to increased oxidative DNA damage.

Treatment of rats with the peroxisome proliferator nafenopin induces H_2O_2-generating enzymes of the peroxisomal β-oxidation chain up to 20-fold, while catalase is enhanced only by a factor 2 (Nemali et al., 1989). As cytosolic activities of glutathione transferase and peroxidase are markedly decreased upon nafenopin treatment, increased H_2O_2 leakage may lead to higher levels of Fenton-catalyzed hydroxyl radical production which in turn could be expected to result in increased DNA damage. Feeding rats a choline-devoid low-methionine diet induces, as a short-term reaction after 3 days of treatment, lipid peroxida-

Correspondence: Prof. Dr. W.K. Lutz, Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, CH-8603 Schwerzenbach (Switzerland).

Abbreviations: dNp, deoxyribonucleoside-3′-phosphate; cis-dTGp, *cis*-thymidine glycol-3′-phosphate; 8-OH-dG, 8-hydroxy-deoxyguanosine; ECD, electrochemical detection.

tion in the nuclear membrane of liver cells (Rushmore et al., 1987). Lipid peroxides may, in turn, induce radicals affecting DNA. DNA damage could be monitored after 3 days as alkali-labile lesions (Rushmore et al., 1986).

Thymine glycol and 8-hydroxyguanine are well-known DNA oxidation products (Frenkel et al., 1986; Floyd et al., 1986; Leadon, 1987; Kasai et al., 1986). While the former has never been detectable in DNA isolated from an intact animal (possibly because of efficient repair), increased levels of the latter have been shown after irradiation of mice (Kasai et al., 1986) or treatment of rats with high doses of the renal carcinogen potassium bromate (Kasai et al., 1987) ‡.

In this paper, these 2 markers were investigated, using highly sensitive detection methods, in the liver of rats under conditions which would have produced a high liver tumor incidence. A recently developed ^{32}P-postlabeling analysis was used for the determination of *cis*-thymidine glycol-3′-phosphate (cis-dTGp; Hegi et al., 1989) while 8-hydroxy-deoxyguanosine (8-OH-dG) was measured by electrochemical detection (ECD; Richter et al., 1988).

Materials and methods

Animals and treatments

(A) Three male rats (200 g; [TIF : RAI f]; Ciba Geigy Breeding Station, Sisseln, Switzerland) were treated for 2 months with 1000 ppm nafenopin (Ciba Geigy, Basel, Switzerland) in the diet. Three control rats were kept on laboratory chow no. 810 (NAFAG Futter, Gossau, Switzerland). (B) Male Fischer F-344 rats (180 g; [CDF(F-344)/CrlBR]; Charles River Wiga GmbH, Sulzfeld, F.R.G.) were fed either a choline-devoid low-methionine diet (methionine: 1.75 g/kg diet, NAFAG no. 9025 CMD) or a choline-supplemented diet (same diet supplemented with choline, 8 g/kg diet, NAFAG no. 9029 CS). Three rats on each diet were killed after 4 or 7 days. In both experiments food and water were available ad libitum.

DNA isolation and analysis

Rat liver was homogenized at 4 °C in 5 ml/g 250 mM mannitol, 70 mM sucrose, 5 mM HEPES, 0.1% BHT (butylated hydroxytoluene), pH 7.4, and centrifuged for 10 min at $800 \times g$ (4 °C). DNA was extracted from the nuclear pellet after treatments with proteinase K and RNase according to the procedure described by Gupta (1984). In order to prevent oxidative alterations of the DNA during workup all samples were kept under an atmosphere of nitrogen or freon. Cis-dTGp was measured by a method reported previously (Hegi et al., 1989). Briefly, an aliquot containing 1 mg DNA was hydrolyzed to the deoxyribonucleoside-3′-phosphates (dNp) and chromatographed twice on phenylboronate affinity columns to isolate and purify *cis*-glycols from natural dNp. Cis-dTGp was converted to [5′-^{32}P]*cis*-thymidine glycol-3′,5′-bisphosphate (cis-*pdTGp) by T4 polynucleotide kinase-catalyzed [^{32}P]phosphate (*p) transfer from [γ-^{32}P]ATP in the presence of 1 mM $BeCl_2$ at pH 7.5. The ^{32}P-labeled reaction products were resolved by 2-dimensional anion-exchange thin-layer chromatography (TLC) on polyethyleneimine cellulose and detected by autoradiography. Experimental details are given in the legend to Fig. 1. 8-OH-dG was determined by high-performance liquid chromatography (HPLC)/ECD according to Richter et al. (1988) with minor modifications. Briefly, DNA was hydrolyzed to deoxyribonucleosides and an aliquot of 100 μl (0.5–1 mg/ml) was chromatographed on RP_{18} (OD 5A Spheri 5, 0.46 × 25 cm, particle size 5 μm, Brownlee Laboratories, Santa Clara, CA) with 50 mM potassium phosphate, pH 5.5, containing 10% methanol. Flow rate was 1 ml/min. Detection of normal nucleosides was by spectrophotometry at 256 nm and of 8-OH-dG by ECD at +600 mV. Artifactual formation of 8-OH-dG during DNA workup has been ruled out previously (Richter et al., 1988).

‡ After submission of the original manuscript, Kasai et al. (1989) reported that treatment of rats with the peroxisome proliferator ciprofibrate at 0.025% in the diet for 40 weeks resulted in an increase of the control level of 8-OH-dG from 9 to 18 per 10^6 nucleotides. Whether this increase is causally related to the induction of liver tumors is doubtful. It is difficult to explain how the level of 8-OH-dG determined in the ciprofibrate-treated rats can be responsible for the induction of liver tumors while half that level in the controls cannot.

Results and discussion

The liver weight of the animals treated with nafenopin was doubled compared to controls. In

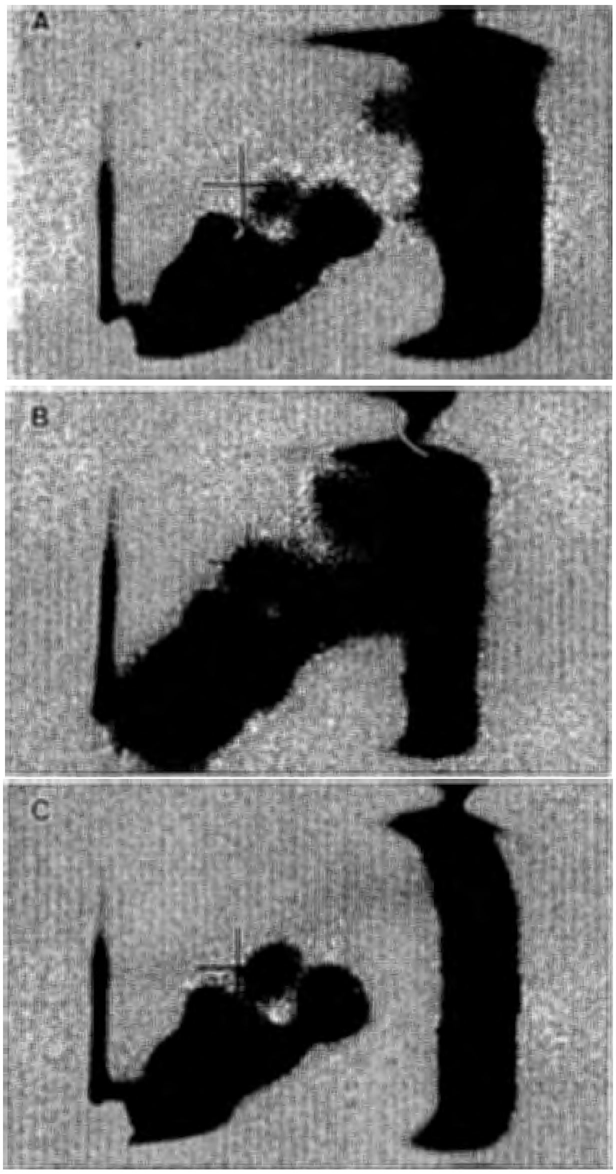

Fig. 1. Analysis of *cis*-thymidine glycol in liver DNA isolated from rats after treatment with nafenopin (A). (B) Analysis of the same DNA spiked with 5 pmole cis-dTGp before the phenylboronate affinity chromatography. (C) Control rat. The expected position of cis-pdTGp is indicated by a cross. Phosphorylation conditions: a 50-μl aliquot of the third fraction (0.5 ml) of the phenylboronate affinity chromatography was lyophilized and phosphorylated in the presence of 25 pmole (12 μCi; 444 kBq) [γ-^{32}P]ATP. Two-dimensional TLC for separating ^{32}P-labeled reaction products was performed in 0.12 M sodium phosphate buffer, pH 8.6, in the first dimension. The second dimension was, in addition, 0.5 M in boric acid.

the DNA, no formation of cis-dTGp could be detected, at a limit of detection of 1 cis-dTGp/10^6 dNp. Fig. 1 shows TLC chromatograms of the *cis*-thymidine glycol analyses. The position of thymidine glycol is shown in sample **B** which had been spiked with 5 pmole cis-dTGp before the phenylboronate affinity chromatography. The amount of 8-OH-dG in liver DNA was 0.060 $\pm$ 0.039 pmole/μg DNA in the treated rats (mean $\pm$ SD from 5 animals), not higher than in the controls (0.059 $\pm$ 0.025; n = 9). This latter value indicates a steady-state level in control liver of about 20 8-OH-dG molecules per 10^6 DNA nucleosides. This level is very close to the value determined recently by Kasai et al. (1989; see also footnote on p. 326) in rats treated with the peroxisome proliferator ciprofibrate, and about 40 times above the experimental limit of detection. The respective HPLC/ECD analyses are shown in Fig. 2.

Negative results were also obtained after short-term treatment with choline-devoid low-methionine diet. Neither treatment (4 days or 7 days) resulted in a detectable formation of thymidine glycol (TLC sheets not shown; same situation as depicted in Fig. 1) or an increase above control of the level of 8-OH-dG. The measured levels of 8-OH-dG after 4 days were 0.037 and 0.049 pmole/μg DNA for deficient vs. supplemented diet. The respective levels after 7 days were 0.039 and 0.041 pmole/μg DNA. The short duration of the treatment was based upon the report of Rushmore et al. (1987) who showed that lipid peroxidation in the nuclear membrane was a transient reaction with a maximum after a few days.

The 2 investigated markers have to be interpreted in a somewhat different way with respect to repair. Highly efficient pathways appear to have evolved for the repair of oxidation products of thymine (Teebor et al., 1988). The in vivo steady-state level might therefore be much below our limit of detection and a treatment-related increase could go unobserved. 8-OH-dG has also been shown to be repaired (Kasai et al., 1986) but the level of 20 8-OH-dG per 10^6 nucleosides in control animals could indicate that this lesion is not very dangerous.

The present negative data on in vivo DNA damage after treatment with peroxisome prolifera-

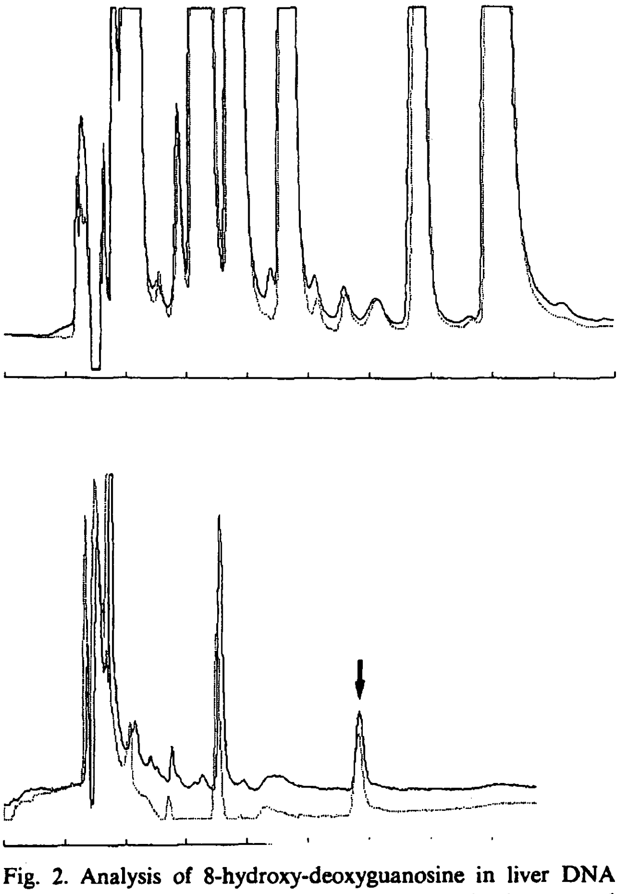

Fig. 2. Analysis of 8-hydroxy-deoxyguanosine in liver DNA isolated from rats after treatment with nafenopin (interrupted line) as compared to the control (full line)., The picture shows an overlay of the 2 chromatograms (total elution time: 20 min). Top chart: $A_{256\ nm}$; bottom chart: EC signal at +600 mV. 8-OH-dG is indicated by an arrow. The steady-state level of 8-OH-dG in the control corresponds to about 20 8-OH-dG/10^6 nucleosides.

tors are in line with most earlier studies where no DNA damage could be shown in vivo (Goel et al., 1985; Bentley et al., 1988). In only 1 report was nafenopin shown to slightly increase the rate of alkaline elution of hepatic nuclear DNA from polycarbonate filters (Bentley et al., 1987). However, when the intracellular H_2O_2 formation was expected to be stimulated to highest levels by enhancement of peroxisomal oxidation or by inhibiting catalase, the rate of DNA fragmentation could not be increased any further (Bentley et al., 1987).

It cannot be excluded that all methods used so far to detect DNA damage in vivo are not sensitive enough. However, other mechanistic possibilities are available to explain an effect in carcinogenesis. With the peroxisome proliferators, liver growth is an overt biological reaction. With choline-devoid low-methionine diet, an effect on the physiological methylation of DNA has been discussed (Rushmore et al., 1987) or an activation of protein kinase C by elevated levels of 1,2-*sn*-diacylglycerol (Blusztajn and Zeisel, 1989).

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References

Bentley, P., F. Bieri, F. Mitchell, F. Waechter and W. Stäubli (1987) Investigations on the mechanism of liver tumour induction by peroxisome proliferators, Arch. Toxicol., Suppl. 10, 157–161.

Bentley, P., F. Bieri, S. Muakkassah-Kelly, W. Stäubli and F. Waechter (1988) Mechanism of tumor induction by peroxisome proliferators, Arch. Toxicol., Suppl. 12, 240–247.

Blusztajn, J.K., and S.H. Zeisel (1989) 1,2-*sn*-Diacylglycerol accumulates in choline-deficient liver. A possible mechanism of hepatic carcinogenesis via alteration in protein kinase C activity?, FEBS Lett., 243, 267–270.

Floyd, R.A., J.J. Watson, J. Harris, M. West and P.K. Wong (1986) Formation of 8-hydroxydeoxyguanosine, hydroxyl free radical adduct of DNA in granulocytes exposed to the tumor promotor, tetradecanoylphorbolacetate, Biochem. Biophys. Res. Commun., 137, 841–846.

Frenkel, K., K. Chrzan, W. Troll, G.W. Teebor and J.J. Steinberg (1986) Radiation-like modification of bases in DNA exposed to tumor promoter-activated polymorphonuclear leukocytes, Cancer Res., 46, 5533–5540.

Ghoshal, A.K., and E. Farber (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens, Carcinogenesis, 5, 1367–1370.

Goel, S.K., N.D. Lalwani, W.E. Fahl and J.K. Reddy (1985) Lack of covalent binding of peroxisome proliferators nafenopin and WY-14 643 to DNA in vivo and in vitro, Toxicol. Lett., 24, 37–43.

Gupta, R.C. (1984) Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA in vivo, Proc. Natl. Acad. Sci. (U.S.A.), 81, 6943–6947.

Hegi, M.E., P. Sagelsdorff and W.K. Lutz (1989) Detection by ^{32}P-postlabeling of thymidine glycol in gamma-irradiated DNA, Carcinogenesis, 10, 43–47.

Kasai, H., P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama and H. Tanooka (1986) Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair, Carcinogenesis, 7, 1849–1851.

Kasai, H., S. Nishimura, Y. Kurokawa and Y. Hayashi (1987) Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA, Carcinogenesis, 8, 1959–1961.

Kasai, H., Y. Okada, S. Nishimura, M.S. Rao and J.K. Reddy (1989) Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long-term exposure to peroxisome proliferators, Cancer Res., 49, 2603–2605.

Leadon, S.A. (1987) Production of thymine glycols in DNA by radiation and chemical carcinogens as detected by a monoclonal antibody, Br. J. Cancer, 55, Suppl. VIII, 113–117.

Mikol, Y.B., K.L. Hoover, D. Creasia and L.A. Poirier (1983) Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets, Carcinogenesis, 4, 1619–1629.

Nemali, M.R., M.K. Reddy, N. Usuda, P.G. Reddy, L.D. Comeau, M.S Rao and J.K. Reddy (1989) Differential induction and regulation of peroxisomal enzymes: predictive value of peroxisome proliferation in identifying certain nonmutagenic carcinogens, Toxicol. Appl. Pharmacol., 97, 72–87.

Rao, M.S., and J.K. Reddy (1987) Peroxisome proliferation and hepatocarcinogenesis, Carcinogenesis, 8, 631–636.

Richter, C., J.-W. Park and B.N. Ames (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive, Proc. Natl. Acad. Sci. (U.S.A.), 85, 6465–6467.

Rushmore, T.H., E. Farber, A.K. Ghoshal, S. Parodi, M. Pala and M. Taningher (1986) A choline-devoid diet, carcinogenic in the rat, induces DNA damage and repair, Carcinogenesis, 7, 1677–1680.

Rushmore, T.H., D.M. Ghazarian, V. Subrahmanyan, E. Farber and A.K. Goshal (1987) Probable free radical effects on rat liver nuclei during early hepatocarcinogenesis with choline-devoid low methionine diet, Cancer Res., 47, 6731–6740.

Teebor, G.W., R.J. Boorstein and J. Cadet (1988) The repairability of oxidative free radical-mediated damage to DNA: a review, Int. J. Radiat. Biol., 54, 131–150.