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Nafenopin-induced rat liver peroxisome proliferation reduces DNA methylation by N-nitrosodimethylamine *in vivo*

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The hypolipidaemic drug nafenopin (NAF) has been shown to enhance the hepatocarcinogenic effect of N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine in rats. We have investigated whether the NAF-induced peroxisome proliferation in hepatocytes interferes with NDMA's metabolism and interaction with DNA. Adult male Wistar rats received a single i.p. injection of [^{14}C]NDMA (2 mg/kg) and were killed 4 h later. DNA was isolated from liver and kidney, hydrolysed in 0.1 N HCl and analysed by Sephasorb chromatography. In rats pre-treated with NAF (0.2% in the diet over a period of 3 weeks), the concentration of N7-methylguanine in hepatic DNA (μmol/mol guanine) was 46% below control values. This is probably due to the greater amount of target DNA, as NAF caused a marked hepatomegaly with a 50% increase in total liver DNA content. Concentrations of N7-methylguanine in kidney DNA were twice as high in NAF-pre-treated animals when compared to control rats. This is unlikely to result from a shift in the metabolism of NDMA from liver to other rat tissues since the time course and extent of the conversion of [^{14}C]NDMA to $^{14}CO_2$ and ^{14}C-labelled urinary metabolites were identical in NAF-treated and control animals. There was no indication that NAF inhibits the activity of the hepatic O^6-alkylguanine-DNA alkyltransferase.

Introduction

Nafenopin and related hypolipidaemic agents exert profound effects on the liver of experimental animals including mice, rats, hamsters, cats, dogs and monkeys (1,2). These effects include a stimulation of hepatic DNA synthesis, a striking hepatocyte peroxisome proliferation (3,4), and the induction of hepatomegaly. An increase in peroxisomal β-oxidation of fatty acids is responsible for the hypolipidaemia caused by these compounds (5–7). It has been suggested that the biological effects of nafenopin and clofibrate are mediated by a cytosolic protein receptor in liver and kidney (8).

In 1976, Reddy *et al.* (9) reported a high incidence of hepatocellular carcinomas in acatalasemic mice after treatment with nafenopin. These findings have subsequently been extended to other strands of mice and rats, and it has been shown that a number of different peroxisome-proliferating agents, including the industrial plastizicer di(2-ethylhexyl)phthalate, are similarly effective (10–14). From these observations Reddy and coworkers concluded that nafenopin and related peroxisome stimulators may represent a novel class of hepatocarcinogens. The mechanism of tumour induction by these drugs is unknown. They are not mutagenic in the *Salmonella*-microsome assay and apparently do not interact with DNA *in vivo* (15,16). The hypothesis that increased intracellular production of clastogenic H_2O_2 and oxygen radicals ($OH\cdot, O_2^-$) by the stimulated β-oxidation system may be responsible for the neoplastic transformation has not yet been confirmed (14,17,18). On the other hand, some investigations indicate that nafenopin and clofibrate may act as hepatic tumour promoters. Hypolipidemic drugs with peroxisome-proliferating activity are inducers of hepatic ornithine decarboxylase (19). Low doses of clofibrate, WY-14,643, or nafenopin given simultaneously with, or after, N-nitrosodiethylamine significantly increase the number of hepatic tumours in rats (20–23). Reports from other laboratories seem to indicate that peroxisome proliferations have no promoting effect on hepatic tumour induction by N-2-fluorenylacetamide (24,25) and N-nitrosodiethylamine (26). These contradictory observations are difficult to interpret since it is not known whether or to what extent nafenopin and related compounds interfere with the bioactivation of the respective initiating agents. To investigate these questions, we studied the metabolism of N-nitrosodimethylamine (NDMA)* in nafenopin-pre-treated rats. The results of our experiments show that nafenopin reduces the interaction of NDMA with hepatic DNA *in vivo*.

Materials and methods

Animals

Adult male Wistar rats (body wt. 150–200 g) were purchased from Ivanovas, Kisslegg, FRG, and kept under standard conditions.

Chemicals

N-nitroso-[^{14}C]dimethylamine ([^{14}C]NDMA) (sp. act. 53.4 mCi/mmol) was obtained from New England Nuclear, Boston, MA, USA. Unlabelled NDMA (Schuchardt, Munich, FRG) was added to lower the sp. act. The radiochemical purity was checked by h.p.l.c. and proved to be >97%. [Methyl-^{3}H]Thymidine (sp. act. 71.7 Ci/mmol) was from New England Nuclear, Boston, MA, USA. Immediately before i.p. injection, NaCl was added to give a 0.9% (w/v) solution. Nafenopin [2-methyl-2-*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxypropionic acid] was generously provided by Drs. W.Stäubli and R.Hess, Ciba Geigy AG (Basel, Switzerland). Sephasorb HP Ultrafine was from Deutsche Pharmacia (Freiburg, FRG), Lumagel scintillation cocktail from LKB (Karlsruhe, FRG). For dipping autoradiography, an Ilford Nuclear Research Emulsion (K5) was used. The remaining chemicals were from Sigma (Taufkirchen, FRG) and Merck (Darmstadt, FRG).

Nafenopin pre-treatment

Adult male Wistar rats were fed on a standard laboratory diet (Altromin^R) enriched with 0.2% nafenopin (w/w) for a period of 3 weeks. Control animals received the standard diet and water *ad libitum*.

Electron microscopy

Control and nafenopin-pre-treated animals were perfused via the aorta with 2.5% glutardialdehyde under thiopental anaesthesia. Livers were removed, embedded in araldite, cut in semi-thin and ultra-thin sections and processed as described elsewhere (27). The ultra-thin sections were examined in a Zeiss OM2 electron microscope.

*Abbreviations: NDMA, N-nitrosodimethylamine, [^{14}C]NDMA, N-nitroso-[^{14}C]dimethylamine; [^{3}H]dT, (methyl-[^{3}H])thymidine; 7-meG, N7-methylguanine; [O^6]meG, [O^6]methylguanine.

Determination of liver DNA, RNA, and protein

Control and nafenopin pre-treated animals were killed under light ether anaesthesia. Livers were rapidly removed and stored at −70°C. DNA and RNA were extracted following the procedure of Schmitt and Thannhauser (28). DNA concentrations were determined using the Burton Method (29), and RNA concentrations using the orcin reaction (30). Proteins were isolated by TCA extraction as described earlier (31) and protein concentrations determined with a modified Bradford assay (32).

Hepatic DNA synthesis in vivo

Control and nafenopin pre-treated animals received a single i.p. injection of [methyl-^{3}H]thymidine ([^{3}H]dT) (2 μCi/g) and were killed 30 min later. The livers were removed, frozen in liquid nitrogen and stored at −70°C. DNA was isolated following the Schmitt-Thannhauser procedure (28). Thymidine incorporation was determined by liquid scintillation counting using Lumagel cocktail. In addition, liver segments were fixed in buffered formaldehyde (5%, v/v), embedded in paraffin and cut in 2-μm sections. These sections were used for dipping autoradiography with Ilford-K5 emulsion.

Metabolism of NDMA in vivo

Control and nafenopin-pre-treated animals (2 each) received a single i.p. injection of [^{14}C]NDMA (2 mg/kg; 0.5 mCi/mmol) and were placed in a metabolic cage (Jencons Metabowl, Hemel Hempstead, UK). Expired $^{14}CO_2$ was absorbed by two serially connected Nilox columns, each containing 600 ml of 1 N NaOH, and urine was collected for quantification of excreted ^{14}C-metabolites. Samples (0.5-ml) were analysed at various time intervals. For liquid scintillation counting, NaOH and urine samples were diluted with distilled water (1:4, v/v) and subsequently mixed with 8 ml Lumagel. During the experiment, control rats were fed on a standard laboratory diet and water *ad libitum*, whereas pre-treated animals received the same diet containing 0.2% (w/w) nafenopin.

DNA alkylation in vivo

Control and nafenopin pre-treated animals received a single i.p. injection of [^{14}C]NDMA (2 mg/kg; 10 mCi/mmol) and were killed 4 h later. DNA was isolated from liver and kidney by phenolic extraction as described earlier (33), hydrolysed in 0.1 N HCl (37°C, 20 h), neutralised and analysed on a Sephasorb column using 10 mM NaH_2PO_4 (pH 5.5) as mobile phase (flow rate 1.5 ml/min). Absorbance at 260 nm was measured with a Perkin Elmer spectrophotometer and radioactivity determined after addition of Lumagel (counting efficiency, 85%). Amounts of methylated DNA purines were expressed as fraction of the parent base guanine assuming that the specific activity of the methyl adducts was half of that of the injected [^{14}C]NDMA.

Results

Nafenopin treatment induced a striking peroxisome proliferation in hepatocytes. These proliferating organelles possessed a finely granular matrix without dense core structures and were distributed throughout the cytoplasm. They were of highly variable size and shape, in contrast to hepatic peroxisomes of control animals. The cytoplasmic accumulation of peroxisomes led to a marked hypertrophy of hepatocytes.

The above changes were accompanied by a marked hepatomegaly. The liver mass increased by a factor of 2.5 compared to control animals (Table I). The liver enlargement was accompanied by a 3-fold increase in the hepatic protein content. DNA and RNA concentrations were reduced by 40 and 25%, respectively, whereas the protein concentration was increased. Total liver DNA increased by a factor of 1.5 compared to the livers of control animals (Table I). [^{3}H]dT incorporation into hepatic DNA was, however, identical in control animals and after 3 weeks of dietary nafenopin exposure, as revealed by biochemical studies (Table I) and by dipping autoradiography of liver slices (data not shown).

The metabolic degradation of NDMA was studied in a metabolic cage experiment with 2 nafenopin-pre-treated and 2 control animals (Figure 1). After a single i.p. injection of [^{14}C]NDMA, time course and extent of metabolism were identical in both groups. During the 48 h observation period, the animals exhaled 63% of the ^{14}C-radioactivity as $^{14}CO_2$ and excreted 10% as ^{14}C-labelled urinary metabolites. The experiment with nafenopin-pre-treated rats was repeated, confirming this result.

Table I. Effect of nafenopin on liver weight, DNA, RNA and protein

	Control animals	Nafenopin pre-treated animals	
Liver weight (g)	6.97 ± 0.37	17.31 ± 2.31	(248%)[a]
DNA (mg/g)	2.97 ± 0.12	1.69 ± 0.07	(57%)
Total liver DNA (mg DNA/liver)	19.4 ± 0.8	29.2 ± 1.2	(151%)
[^{3}H]dT incorporation into DNA (d.p.m. x 10^{-3}/mg DNA)	169 ± 45	169 ± 41	(100%)
RNA (mg/g)	10.18 ± 0.29	7.76 ± 0.3	(76%)
Protein (mg/g)	148.87 ± 1.60	226.62 ± 22.10	(152%)
Total liver protein (g)	1.29 ± 0.01	3.92 ± 0.38	(304%)

Nafenopin pre-treated animals received a 0.2% diet over a period of 3 weeks. Nucleic acids and proteins were extracted from liver and quantified as described in the text. For [^{3}H]dT incorporation, animals received a single i.p. injection of [methyl-^{3}H]thymidine [^{3}H-dT]; 2 μCi/g) and were killed 30 min later. The data shown are mean values of 4 rats ± S.D.
[a]Data in parentheses indicate nafenopin values as a percentage of controls.

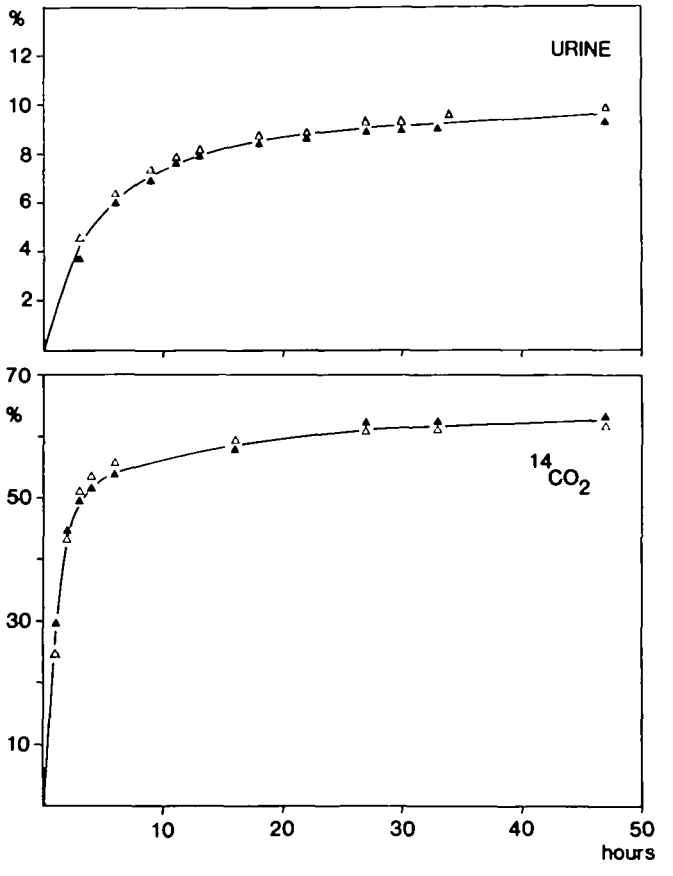

Fig. 1. Metabolism of N-nitroso[^{14}C]dimethylamine *in vivo*. Nafenopin-pre-treated (▲) and control animals (△) received a single i.p. injection of [^{14}C]NDMA (2 mg/kg; 0.5 mCi/mmol) and were placed in a metabolic cage. Urinary excretion of [^{14}C]metabolites (upper) and [^{14}C]exhalation (lower) were monitored over 48 h. The data are plotted as cumultative percentages of the total radioactivity administered.

To assess the extent of DNA alkylation *in vivo*, animals received a single i.p. injection of [^{14}C]NDMA (2 mg/kg; 10 mCi/mmol) and were killed after 4 h. Nafenopin pre-treatment reduced the concentrations of N7-methylguanine (7-meG) and [O^6]methylguanine ([O^6]meG) in hepatic DNA by ~50% (Figure 2 and Table II). In contrast, the levels of 7-meG in kidney DNA of nafenopin-treated rats were twice as high as in control animals.

In this organ, the pro-mutagenic base [O⁶]meG was only detectable after nafenopin pre-treatment.

Discussion

The present study corroborates several hepatotrophic effects of nafenopin in Wistar rats which have been reported previously for various experimental animals (1 – 4), cultured rat hepatocytes (34), and human hepatocytes heterotransplanted to athymic nude mice (35). Dietary nafenopin caused a 2.5-fold increase of the liver weight (Table I). The hepatomegaly was mainly due to a striking proliferation of cytoplasmic peroxisomes and represented a combination of organ hypertrophy and hyperplasia, as revealed both electron microscopically and by the increase in total liver DNA (11,36). The stimulation of hepatic DNA synthesis must have occurred during the initial period of nafenopin administration, as at the end of the 3-week feeding period, [³H]dT incorporation into hepatic DNA was identical in both nafenopin-exposed and control animals (Table I). Furthermore, the biochemical data revealed a stimulation of hepatic RNA synthesis and a 3-fold increase in total liver protein in nafenopin-fed animals. These results are consistent with the findings of Chatterjee *et al.* (37) who described a reversible alteration of hepatic mRNA species of peroxisomal and non-peroxisomal proteins induced by the hypolipidemic drug WY-14,643. Lazarow *et al.* (7) reported that among hepatic proteins a subset of peroxisomal polypeptides including the β-oxidation enzymes are preferentially induced by hypolipidaemic drugs. The marked increase in hepatic protein content (Table I) is associated with a considerable loss of glycogen (38).

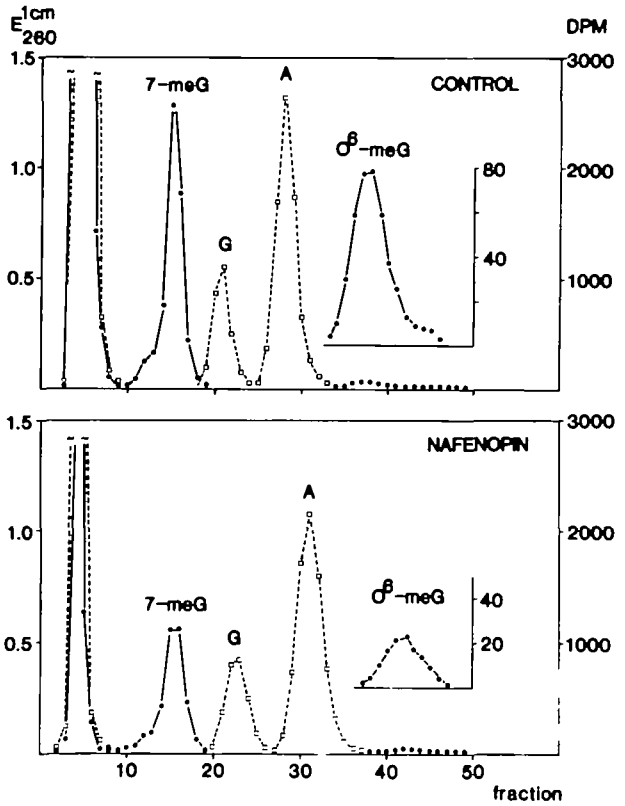

Fig. 2. Chromatographic profiles of liver DNA hydrolysates. Nafenopin-pre-treated and control animals received a single i.p. injection of N-nitroso [¹⁴C]dimethylamine (2 mg/kg; 10 mCi/mmol) and were killed 4 h later. DNA was isolated by phenolic extraction, hydrolysed in 0.1 N HCl, neutralised and analysed on a Sephasorb column. Fraction volume was 3.9 (upper) and 3.7 ml (lower). A, adenine; G, guanine; 7-meG, N7-methylguanine; [O⁶]meG, [O⁶]methylguanine; $E^{1\,cm}_{260}$ ■——■; d.p.m. ●——●.

NDMA and related dialkylnitrosamines are enzymically bioactivated by isoenzymes of the microsomal cytochrome P450 system (39). This bioactivation proceeds via α-C-hydroxylation and finally yields electrophilic alkyl intermediates which react with cellular macromolecules including DNA (40 – 42). It has been demonstrated in long-term carcinogenicity studies, that for numerous carcinogenic N-nitroso compounds, the initial extent of DNA akylation is closely correlated with the incidence and location of tumours (43). We determined two methylated DNA purines, 7-meG, the major methyl adduct, and [O⁶]meG, which is believed to be a promutagenic lesion involved in the initiation of malignant transformation (44,45).

Surprisingly, nafenopin pre-treatment reduced the concentrations of methylated liver DNA purines after a single i.p. dose of [¹⁴C]NDMA by 50%, whereas in kidney the extent of DNA alkylation was twice as high as in control animals (Table II). Several factors may be responsible for these changes. The decrease in hepatic DNA methylation could be due to the higher content of DNA, i.e., a same number of methyl diazonium ions react with twice the amount of hepatic DNA. Alternatively, nafenopin could inhibit the metabolism of NDMA in liver; this would also explain the increase in kidney DNA methylation. The latter mechanism has been demonstrated to be operative in rats kept on a protein-free diet (46,47) and was associated with a significant increase in the incidence of NDMA-induced kidney tumours. A similar inter-organ shift of the metabolism of NDMA and related nitrosamines has been observed after administration of ethanol (48,49,50) and disulfiram (51,52). It is unlikely, however, that this mechanism also applies to the present experiments since both the extent and time course of the conversion of [¹⁴C]NDMA to $^{14}CO_2$ and ¹⁴C-labelled urinary metabolites were identical in nafenopin-treated and control animals (Figure 1). We have also considered the possibility that the marked changes in the size and structure of nafenopin-adapted hepatocytes

Table II. DNA methylation in liver and kidney

Organ	Control animals			Nafenopin pre-treated animals		
	7-meG	[O⁶]meG	[O⁶]meG/7-meG	7-meG	[O⁶]meG	[O⁶]meG/7-meG
Liver	720 ± 26	50.5 ± 8.4	0.069 ± 0.0067	388 ± 50	12.7 ± 1.3	0.033 ± 0.0057
Kidney	38 ± 6	n.d.	n.d.	83 ± 4.5	5.5 ± 1.9	0.065 ± 0.017

Nafenopin pre-treated (0.2% diet; 3 weeks) and control animals were given a single i.p. dose of [¹⁴C]NDMA (2 mg/kg; 10 mCi/mmol). After 4 h survival, DNA was isolated and analysed as described in the text.
7-meG, N7-methylguanine; [O⁶]meG, [O⁶]methylguanine; n.d., not detectable; values for methylated purines are expressed as μmol/mol guanine. Liver data are mean values of 3 animals (controls) or 5 animals (nafenopin) ± S.D. Kidney data are means of triplicate determinations of pooled tissues from 4 (controls) and 5 animals (nafenopin).

may lead to a greater fraction of N-nitroso-methyl(hydroxymethyl)amine, the proximate carcinogen NDMA being excreted into the systemic circulation. In this case, a higher extent of DNA alkylation would be expected not only in kidney, but also in lung and even in tissues (e.g., intestine) with minimal capacity for NDMA bioactivation. However, we found no increase in pulmonary DNA methylation, and methylated purines were not detectable in colonic DNA from either control or nafenopin-treated rats (data not shown). Therefore, we also consider the possibility that the increase in kidney DNA alkylation may be due to a direct effect of nafenopin. The pro-mutagenic lesion [O^6]meG can be removed by a cellular DNA repair enzyme, [O^6]alkylguanine-DNA alkyltransferase (53). The alkyltransferase has been demonstrated in *Escherichia coli* (54) and in numerous animal and human tissues (44); it has been shown that the hepatic enzyme system in rats is inducible by partial hepatectomy, hepatotoxic agents, and X-irradiation (55,56,57) i.e., under conditions of liver regeneration. Dietary nafenopin led to a significantly lower [O^6]meG/7-meG ratio in hepatic DNA (Table II). This could indicate an increased repair capacity, but is more likely that this change is due to the lower extent of [O^6]meG formation. The rate of repair of this promutagenic base is dose-dependent, as the alkyltransferase is inactivated during the repair process. Assessment of the [O^6]alkylguanine repair system using an *in vitro* assay (58) also provided no evidence of an induction of the hepatic alkyltransferase; these data were difficult to interpret due to the nafenopin-induced changes in total liver protein content. The present study was stimulated by the observation that nafenopin enhances hepatic tumour formation by symmetric dialkylnitrosamines (20 – 23). More recent studies seem to indicate that under different experimental conditions nafenopin may have no effect on, or even depress, the development on enzyme-altered foci (26) and hepatic neoplasms (24,25). These controversial findings are at present difficult to explain. Nafenopin and related agents cause profound metabolic and structural alterations which may modulate hepatic carcinogenesis in different ways. Our own results demonstrate, however, that nafenopin pre-treatment of Wistar rats reduces the interaction of the hepatocarcinogenic nitrosamine NDMA with hepatic DNA *in vivo*. If the same effect occurs during chronic concomitant administration of both agents, one would expect a lower incidence of hepatic tumours.

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