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DNA methylation in rat tissues by a series of homologous aliphatic nitrosamines ranging from *N*-nitrosodimethylamine to *N*-nitrosomethyldodecylamine

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Aliphatic *N*-nitrosomethylalkylamines exhibit a remarkable organ specificity in rats, the principal targets for tumour induction being liver, oesophagus, urinary bladder and lung. We have determined the extent of DNA methylation in these tissues following a single oral dose (0.1 mmol/kg; 6 h survival) of each of 12 homologues, ranging from *N*-nitrosodimethylamine (C1) to *N*-nitrosomethyldodecylamine (C12). Methylpurines (7- and O^6-methylguanine) were determined by cation exchange HPLC with fluorescence detection. Highest levels of hepatic DNA methylation were found with *N*-nitrosodimethylamine (C1) and *N*-nitrosomethylethylamine (C2), the most potent hepatocarcinogens in this series. Concentrations of methylpurines in liver DNA decreased with increasing chain length for C1–C5. Administration of the higher homologues (C6–C12) caused levels of DNA methylation which by themselves were considered too low to account for their hepatocarcinogenicity. In rat oesophagus, DNA methylation closely paralleled carcinogenicity, the butyl and pentyl derivatives (C4, C5) being most effective. In rat lung, the extent of DNA methylation was generally lower and there was no apparent correlation with carcinogenicity. Methylation of kidney DNA also decreased with increasing chain length and was only detectable for C1–C5. In urinary bladder DNA, methylpurines were below or close to the limit of detection. It is concluded that the initiation of malignant transformation by DNA methylation alone (through hydroxylation at the methylene α-carbon) could be operative for C1 in kidney and lung, for C1 and C2 in liver, and C3–C5 in oesophagus. For the higher homologues, the extent of DNA methylation seems insufficient to explain the complex pattern of tissue specificity, suggesting that DNA modification other than, or in addition to, methylation may be responsible.

Introduction

Early investigations by Druckrey and co-workers (1) on the carcinogenicity of asymmetric nitrosamines in rats revealed a high degree of organ-specificity. Short-chain *N*-nitrosomethylalkylamines (C1, C2*) induced predominantly tumours of liver and lung whereas the higher asymmetric homologues (C3–C6) produced exclusively or preferentially oesophageal neoplasms.

*Abbreviations: *N*-Nitrosomethylalkylamines were designated according to the length of the alkyl chain opposite the methyl moiety, i.e. C1 (*N*-nitrosodimethylamine) to C12 (*N*-nitrosomethyldodecylamine).

Subsequent work by Lijinsky and co-workers on *N*-nitrosomethylalkylamines containing an alkyl group with more than five carbon atoms showed a striking alternating pattern, with nitrosamines containing an even-numbered carbon chain (C8, C10, C12) producing urinary bladder tumours and those with an odd-numbered carbon chain (C7, C9, C11) inducing liver and lung tumours (2,3). Investigations into the biological basis of this correlation between chemical structure and organ-specific carcinogenicity (Figure 1) have in the past focused on *N*-nitrosomethylbenzylamine (4), *N*-nitrosomethylethylamine (5) and *N*-nitrosomethylpentylamine (6), and have led to the assumption that the induction of oesophageal carcinomas is related to a preferential bioactivation of these nitrosamines by a P-450 isozyme in the oesophageal mucosa with high substrate specificity for asymmetric nitrosamines (4,5,7). In addition, there was evidence that the adverse biological effects of these agents is mediated by a methylation of cellular macromolecules. DNA modifications resulting from an initial hydroxylation of the methyl group were either not detectable (benzylation) or occurred at a considerably

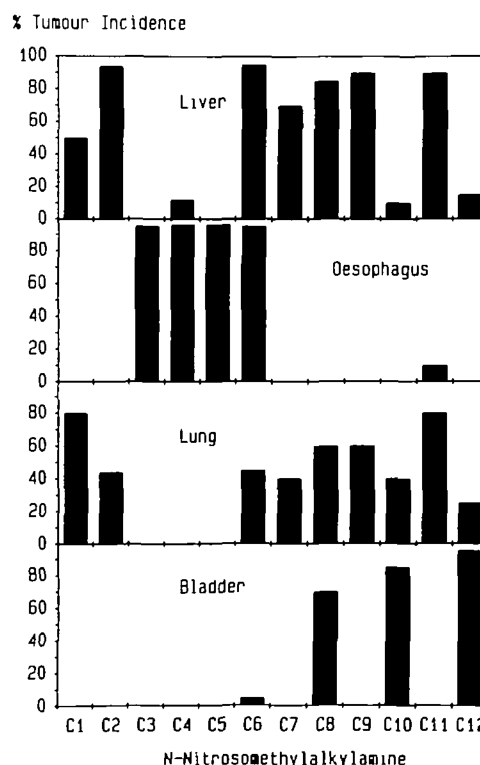

Fig. 1. Maximum reported tumour incidence in various tissues of male rats exposed to *N*-nitrosomethylalkylamines ranging from *N*-nitrosodimethylamine (C1) to *N*-nitrosomethyldodecylamine (C12). Carcinogens were given twice weekly by gavage at a dose of 0.014 (C3), 0.021 (C4), 0.025 (C1, C2), 0.04 (C6), 0.05 (C12) and 0.11 (C7-C11) mmol/kg. *N*-Nitrosomethylpropylamine (C3) and *N*-nitrosomethylpentylamine (C5) were administered in the drinking water at concentrations of 14 and 30 mg/l respectively. Compounds C1–C4 and C6–C12 were given to Fischer 344 rats, and C5 to rats of the Donryu strain. Data were compiled from refs (3,6,10,28).

lower extent (ethylation, hydroxyethylation) than did DNA methylation. This prompted us to determine the extent of DNA methylation in target and non target rat tissues produced by an equimolar dose of homologous *N*-nitrosomethylalkylamines, ranging from *N*-nitrosodimethylamine (C1) to *N*-nitrosomethyldodecylamine (C12). The study was designed to elucidate the structural requirements for bioactivation leading to DNA methylation and to correlate this with the results of long-term carcinogenicity studies.

Materials and methods

Chemicals

Nitrosamines were prepared as described previously (2,8–10). All nitrosamines were found to be pure and authentic as judged by n.m.r. spectroscopy. The samples were also checked by h.p.l.c. on RP-18 columns (Shandon, ODS Hypersil, 4.6 × 250 mm) with u.v.-detection at 230 nm. Using isocratic methanol/water eluents as mobile phase (10–85% methanol, depending on the hydrophobicity of the homologue), all samples eluted as a major peak followed by a smaller second peak representing the respective z-isomer.

Animals and treatment

Young male Fischer 344 rats (120–150 g body wt) were obtained from Charles River Wiga GmbH (FRG) and maintained on a standard laboratory diet with water *ad libitum*. Nitrosamines were dissolved in sterile-filtered tap water containing 0.25% Cremophor EL solubilizer (Sigma, St. Louis, MO) and subjected to mild bath sonication prior to use. Carcinogens were administered by gavage in a volume of 1 ml/100 g body wt.

The time course of formation and persistence of 7-methylguanine in hepatic DNA was determined in groups of two animals receiving a single dose of 0.1 mmol/kg of C3, C6 or C12. Survival times were 3, 6, 9 and 12 h. In a further experiment, groups of 10 animals received a single dose of 0.1 mmol/kg of each nitrosamine of the series from C1 to C12 and were killed 6 h later. Tissues were rapidly removed, frozen in liquid nitrogen and stored at −70°C until analysis.

DNA isolation and analysis of adducts

DNA was isolated by phenolic extraction and adsorption onto hydroxylapatite as previously described (5). Following mild acid hydrolysis (0.1 M HCl at 37°C for 20 h), the amounts of 7-methylguanine and O^6-methylguanine were determined by h.p.l.c. using a modification of the procedure of Swenberg and Bedell (11), as previously described (12). Briefly, purine bases were separated on a strong cation exchange column (Partisil SCX, 0.46 × 250 mm), eluted at 2 ml/min with 50 mM $NH_4H_2PO_4$, pH 2 (7-methylguanine), or with the same buffer containing 10% (vol/vol) methanol (O^6-methylguanine). Under these conditions, 7-methylguanine eluted at 9.5 min and O^6-methylguanine at 8.7 min. Quantification of methylpurines was carried out with a Shimadzu spectrofluorophotometer (RF-540), set at 295 nm for excitation and 370 nm for emission. Calibration of the fluorescence signal was performed by injecting radiolabelled methylpurines and determining both radioactivity and fluorescence.

Since the amount of DNA obtained from most tissues was too small to allow for individual analyses, organs from 10 animals were pooled before DNA isolation. Duplicate or triplicate analyses were carried out whenever possible and were usually found to differ by less than 10%.

Results

To estimate the most suitable survival time, i.e. when >90% of the nitrosamine would be metabolized, the time course of methylpurine formation in hepatic DNA was determined for some representative *N*-nitrosomethylalkylamines (C3, C6 and C12). Concentrations of 7-methylguanine produced in hepatic DNA by a single oral dose (0.1 mmol/kg) were highest between 3–6 h. At longer time intervals, up to 12 h, there was no significant change in the amout of 7-methylguanine (Figure 2). It was, therefore, decided that 6 h would be an appropriate time interval for determining the extent of DNA methylation by a large series of *N*-nitrosomethylalkylamines. The results of the latter experiment, in which groups of 10 rats received a single oral dose of 0.1 mmol/kg, are shown in Figure 3. The greatest extent of DNA methylation was observed in liver following administration of C1, with 7-methylguanine and O^6-methylguanine concentrations amounting to 3290 and 330 μmol/mol guanine respectively. Hepatic DNA methylation decreased with increasing length of the alkyl chain, the lowest value being observed for *N*-nitrosomethyldodecylamine (C12). The amount of 7-methylguanine produced by C12 comprised only 5% of that produced by an equimolar dose of *N*-nitrosodimethylamine (C1). Long-chain *N*-nitrosomethylalkylamines (C6–C12) all produced a low extent of hepatic DNA methylation (200–600 μmol 7-methylguanine/mol guanine).

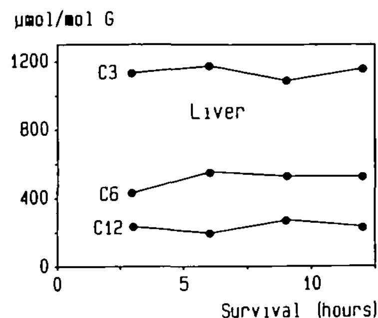

Fig. 2. Concentration of 7-methylguanine in hepatic DNA of male Fischer 344 rats at different time intervals following a single oral dose (0.1 mmol/kg) of *N*-nitrosomethylpropylamine (C3), *N*-nitrosomethylhexylamine (C6) and *N*-nitrosomethyldodecylamine (C12). Data from pooled livers of three animals for 3, 9 and 12 h and from 10 animals for 6 h. G, guanine.

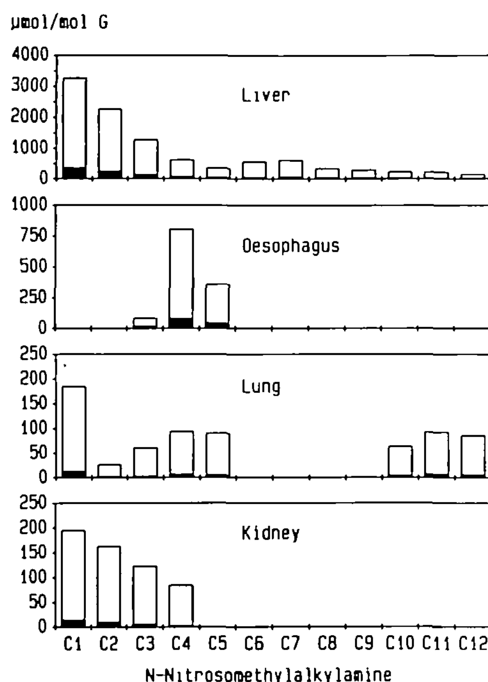

Fig. 3. DNA methylation in liver, oesophagus, lung and kidney of male F344 rats following a single oral dose (0.1 mmol/kg each) of homologous *N*-nitrosomethylalkylamines ranging from *N*-nitrosodimethylamine (C1) to *N*-nitrosomethyldodecylamine (C12). Concentrations of 7-methylguanine (open columns) and O^6-methylguanine (filled columns) are expressed as μmol/mol guanine.

In contrast, concentrations of 7-methylguanine in oesophageal DNA increased from values below the level of detection (C1 and C2) to 800 and 370 μmol 7-methylguanine/mol guanine for *N*-nitrosomethylbutyl- and -methylpentylamine, respectively. Only with C4 was the extent of methylation in the oesophagus higher than in liver. Following administration of long chain homologues

(C6 – C12), 7-methylguanine was not found in oesophagus. The detection limit for O^6-methylguanine was somewhat lower due to its strong fluorescence, and traces of this promutagenic base (<5 μmol/mol guanine) in oesophagus were detectable with all compounds with the exception of C2. The highest level of pulmonary DNA methylation (190 μmol 7-methylguanine/mol guanine) was found after administration of *N*-nitrosodimethylamine (C1), but this was still 15 times lower than in rat liver. 7-Methylguanine concentrations in lung ranging from 30 to 100 μmol/mol guanine were found with C2 – C5 and C10 – C12, whereas equimolar doses of C6 – C9 only produced trace amounts of O^6-methylguanine (<2 μmol/mol guanine). In kidney DNA, methylpurines were only detectable after administration of C1 – C4. As observed in rat liver, there was a tendency for a decrease in alkylation with increasing chain length. In the urinary bladder, trace amounts of O^6-methylguanine were detectable after administration of C6, C9, C10 (<2 μmol/mol guanine) and C12 (<5 μmol/mol guanine).

Discussion

Carcinogenic *N*-nitroso compounds are characterized by their capacity to selectively induce a high incidence of malignant tumours in a wide spectrum of target tissues (1,3,13). The biological basis of organ-specific carcinogenesis is not yet fully understood, but several factors have been implicated, including distribution of the parent carcinogen, tissue- and cell-specific bioactivation, cell turnover and DNA repair (for review see refs 14 – 20). The objective of the present study was to determine the extent to which aliphatic *N*-nitrosomethylalkylamines methylate cellular DNA in various rat tissues. Since carcinogenicity data are available for all of these compounds, it was possible to correlate organ specific tumour induction with levels of methylation in target and non target tissues. We realize that for each tissue, the extent of DNA modification resulting from chronic exposure may differ significantly from that found in single dose experiments, mainly due to different rates of cell turnover and DNA repair. It is also evident that enzymic metabolism of *N*-nitrosomethylalkylamines is very complex and generation of a methylating intermediate is only one of several possible endpoints of bioactivation. However, we feel that despite these limitations, the results allow some tentative conclusions and hypotheses which may be further tested in a more comprehensive study of DNA modifications resulting from exposure to long-chain asymmetric nitrosamines.

DNA methylation is most likely to result from a single hydroxylation step at the α-C position of the alkyl chain opposite the methyl moiety. Metabolism can occur at other carbon atoms prior to conversion to a methylating agent but the more polar intermediates so formed are stable and often more easily excreted than the parent nitrosamine. It is most probable that the pathway for the production of methyl adducts is initial alkyl α-C hydroxylation, since the resulting intermediate is very short-lived and will react rapidly with available nucleophiles. Following incubation of rat oesophagus with *N*-nitrosomethylpentylamine (C5) *in vitro*, Mirvish *et al.* (7) identified four hydroxy derivates, but only those resulting from attack at C-atoms 2 – 5. It was concluded that the α-hydroxy intermediate was too short-lived for chemical detection. This view is also supported by recent studies on *N*-nitrosomethylethylamine (C2) in our laboratory. Although there was evidence of extensive hydroxylation at the β-carbon of the ethyl group, this led to only a very small amount of DNA hydroxyethylation (5). Accordingly, one would expect a high extent of methylation only with those *N*-nitrosomethylalkylamines which are exclusively or predominantely bioactivated by initial α-C hydroxylation at the alkyl group opposite the methyl moiety. The results shown in Figure 3 suggest that this occurs for C1 and C2 in rat liver, and for C4 and C5 in the oesophagus.

For the homologous series from *N*-nitrosodimethylamine to *N*-nitrosomethylpentylamine (C1 – C5), DNA methylation in rat liver was found to decrease with increasing length of the alkyl chain (Figure 3). The generally low levels of methylation produced by C5 – C12 indicate that the liver does not possess P-450 isozymes capable of efficiently α-hydroxylating nitrosomethylalkylamines with chain lengths greater than four.

Comparing the present results with those of long-term carcinogenicity studies, we conclude that initiation of hepatocarcinogenesis by DNA methylation alone can only be assumed for nitrosodimethylamine (C1). With *N*-nitrosomethylethylamine (C2), methylation is by far the primary type of adduct formed (5,21), but ethylation has also been shown to occur, although at levels four times less than would be expected if this compound acted as a true hybrid between C1 and *N*-nitrosodiethylamine (12). The possibility must be considered that methylation may function to saturate the O^6-alkylguanine – DNA alkyltransferase and thereby potentiate the effects of more minor adducts. Both C1 and C2 are strong hepatocarcinogens and produced 7-methylguanine and O^6-methylguanine concentrations of more than 2000 and 200 μmol/mol guanine/0.1 mmol nitrosamine/kg respectively. *N*-Nitrosomethylpropylamine (C3) produced less than 100 μmol O^6-methylguanine/mol guanine at this dose and is not known to induce liver cancer in rats. All higher homologues (C4 – C12) caused substantially less DNA methylation than does C3, although several of these (C6 – C9 and C11) induce a high incidence of hepatic carcinomas following chronic oral administration. This would suggest that the initiation of liver carcinogenesis by these agents requires DNA modifications other than, or in addition to, methylation.

In their pioneering work on the structure – activity relationships of carcinogenic nitroso compounds, Druckrey and coworkers (1) established that the most powerful oesophageal carcinogens are asymmetric dialkylnitrosamines with a methyl group as one of the alkyl moieties. The present study on the complete series of 12 homologous aliphatic methylalkylnitrosamines offers closer insight into the structural requirements for oesophageal tumour induction. Methylation of oesophageal DNA by nitrosodimethylamine was below the level of quantitative detection and this corresponds with the observation that this agent has never produced tumours in this organ, irrespective of dose and route of administration (1,22). While the next higher homologue (C2) produced very low levels of alkylation in rat oesophagus, previous radiochromatographic analyses established that following a single i.p. or p.o. dose, concentrations of 7-methylguanine in rat liver are 100 times higher than in the oesophagus (5). Nevertheless, *N*-nitrosomethylethylamine does produce a low incidence of oesophageal carcinomas in addition to hepatic neoplasms when administered in the drinking water (9). The present data (Figure 3) show a considerable extent of oesophageal DNA methylation (7-methylguanine/guanine) by the next higher homologues, with oesophagus/liver ratios increasing from 0.069 (C3) to 1.28 (C4) and 0.98 (C5). These three compounds are powerful oesophageal carcinogens, producing highly malignant squamous carcinomas after an induction time of only 4 – 6 months (3,6,10). The other compounds studied produce neither oesophageal tumours nor extensive oesophageal DNA methylation, except for *N*-nitrosomethylhexylamine (C6) which is a fairly potent carcinogen in

this organ (10) even though its capacity to methylate target organ DNA was found to be low (Figure 3). In conclusion, the present study shows that (with the exception of C6) DNA methylation in rat oesophagus closely parallels carcinogenicity. In conjunction with earlier studies (4,7,23,24), this suggests that the oesophageal mucosa of rats contains a P-450 isozyme capable of metabolizing asymmetric nitrosamines at the α-methylene position, with highest substrate specificities for those with a chain length of 3–5 C-atoms. Our data also reveal that the amount of DNA methylation required to initiate liver carcinogenesis is considerably higher than that required to produce a similar incidence of oesophageal cancer. The results obtained suggest that any agent which in rat oesophagus produces ≥ 10 μmol O^6-methylguanine/mol guanine/0.1 mmol nitrosamine/kg is a strong and often selective oesophageal carcinogen, i.e. at levels 10–20 times lower than observed in liver DNA following administration of an equimolar dose of a potent hepatocarcinogen.

Lung tumours are induced by long-term exposure to *N*-nitrosomethylalkylamines at incidences ranging from 30 to 80% (Figure 1), with the exception of C3–C5. The lack of pulmonary response to the latter may be due to the fact that these agents very rapidly produce fatal oesophageal carcinomas. The extent of DNA methylation following a single exposure (Figure 3) was generally low and there was no correlation with long-term carcinogenicity. In particular, no methylpurines were detectable after administration of C6–C8, i.e. compounds known to induce a 40–60% incidence of pulmonary neoplasms after chronic exposure.

Kidney DNA, too, showed levels of DNA methylation ~15 times lower than in liver, but shares with the latter a tendency for decreasing levels of methylpurines with increasing chain length for C1–C4. All higher homologues (C5–C12) failed to produce detectable amounts of 7- and O^6-methylguanine, indicating a lack of P-450 enzymes capable of α-C hydroxylation if the number of C-atoms exceeds four. In carcinogenicity studies employing administration by gavage (C2 and C4) and in drinking water (C1–C5), kidney tumours were not reported. The reason for this discrepancy is not known but there seems to be a general tendency for rat kidney to undergo malignant transformation following pulsed doses rather than chronic exposure in drinking water (25).

The present data on DNA methylation do not explain the induction of liver and urinary bladder carcinomas by long-chain odd- and even-numbered *N*-nitrosomethylalkylamines respectively. We did detect small amounts (~5 μmol/mol guanine) of O^6-methylguanine in bladder DNA following administration of *N*-nitrosomethyldodecylamine (C12) and trace amounts after exposure to C6, C9 and C10. Since this does not correlate with the induction of bladder cancer by these agents, we assume that adducts other than methylpurines are responsible and/or the levels of methylation change after chronic administration. It is known, however, that methylation alone is sufficient to transform bladder epithelium of rats (26). We agree with the hypothesis put forward by Okada (27) that tissue specificity depending on even or odd chain length can best be explained by the Knoop type of β-oxidation which, in the case of even-numbered *N*-nitrosomethylalkylamines, would eventually yield *N*-nitrosomethylcarboxypropylamine, a potent bladder carcinogen in rats. If this were true, one would expect bladder tumours also after administration of *N*-nitrosomethylhexylamine and *N*-nitrosomethylbutylamine (C4), which should similarly be oxidized to form *N*-nitrosomethylcarboxypropylamine. The low incidence of bladder tumours produced by C6 (9) and the lack of a carcinogenic response of this organ to C4 is probably due to the very rapid and fatal induction of oesophageal carcinomas.

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