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LIPID PEROXIDATION INDUCED BY *N*-NITROSODIMETHYLAMINE (NDMA) IN RATS *IN VIVO* AND IN ISOLATED HEPATOCYTES

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To investigate the role of carcinogenic chemicals as a possible cause for oxidative damage, rats were treated with *N*-nitrosodimethylamine (NDMA) and various measures of lipid peroxidation were followed. As an indication of enhanced peroxidative processes *in vivo*, NMDA treatment produced rapidly an increase in the rate of ethane exhalation. A single i.p. or p.o. injection of 10 mg/kg b.w. elevated ethane exhalation by 13–14 fold; a single dose of 0.5 mg/kg of NDMA (the smallest dose tested) increased 5-fold the amount of ethane exhaled. Similarly, lipid peroxidation in the liver of NDMA-treated rats (measured by diene conjugation, chemiluminescence, the production of fluorescent and TBA-reactive material) was found to be increased rapidly showing a peak already 20 min after dosing. Simultaneously, NDMA-treatment slightly decreased antioxidant enzyme activities and GSH contents in the liver. In isolated rat hepatocytes the lucigenin-dependent chemiluminescence, as well as H_2O_2 release, were increased by micromolar concentrations of NDMA. Finally, it was shown that the rate of NADPH-stimulated ethane production by hepatic microsomes, prepared from untreated rats, was increased in the presence of NDMA. Thus, our results demonstrate that the alkylating NDMA can induce oxidative stress in rodents. Whether the same is true for other classes of carcinogens and processes known to affect tumor initiation/progression is presently under investigation.

KEY WORDS: lipid peroxidation, carcinogenesis, *N*-nitrosodimethylamine, short-term exposure.

INTRODUCTION

Since the increasing evidence suggests a link between oxidative damage and carcinogenesis,[1-4] we have initiated studies to establish whether chemical carcinogens directly cause oxidative stress. We selected the hepatocarcinogen *N*-nitrosodimethylamine (NDMA), already known to enhance microsomal lipid peroxidation,[5] and investigated its effects on lipid peroxidation *in vivo* and in isolated hepatocytes. In parallel, we have conducted long-term animal experiments to study the modifying role of lipid peroxidation in chemically induced tumors.[6]

The effect of NDMA on lipid peroxidation *in vivo* was determined by measuring the rate of ethane exhalation by NDMA-treated rats. In addition, liver tissue of NDMA-treated animals was used to measure lipid peroxidation (by diene conjugation, chemiluminescence, fluorescent and TBA-reactive products), the activities of antioxidant enzymes (superoxide dismutase, catalase, GSH-peroxidase and -transferase), as well

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as GSH contents. As NDMA is known to be metabolized rapidly, the analyses were performed shortly after single i.p. or p.o. exposures. Furthermore, we studied the effect of NDMA on lucigenin-dependent chemiluminescence and H_2O_2 released by isolated rat hepatocytes.

The results show that low doses of NDMA rapidly produce oxidative stress in rats, and also in isolated hepatocytes *in vitro* upon exposure to NDMA.

MATERIALS AND METHODS

Male Wistar rats (6–10 weeks old) were used in the study. NDMA, dissolved in water, was administered i.p. or p.o. to rats at doses indicated in the text. Ethane exhalation was measured by placing two rats in a gas-tight chamber with O_2 inflow and which had a valve for air sampling.[7]

Gas-chromatographic analysis of ethane was performed according to the method of Wendel and Dumelin.[7] For measurement of oxidative stress at tissue level, rats were killed by decapitation 10–60 min ot 24 h after dosing. The liver was removed and hepatic tissue fractions prepared by routine procedures immediately after killing the animals. Rat hepatocytes were isolated by the collagenase digestion technique.[8] The viability of isolated cells (tested by trypan blue exclusion) was always more than 85%. All analyses on lipid peroxidation were done using freshly prepared tissue fractions. Lipids from tissue specimens were extracted by the conventional chloroform–methanol (2:1) procedure. Extracted lipid fraction was used to estimate the levels of diene conjugation[9,10] and fluorescent products of lipid peroxidation.[11] The amount of TBA-reactive material was measured in the S9 fraction of the liver.[12] NADPH-Stimulated, lucigenin-dependent chemiluminescence in microsomal fractions and in isolated hepatocytes was measured by a Wallac 1251 Luminometer.[13,14] H_2O_2 release by isolated hepatocytes was determined by the chemical method using $Fe(SCN)_3$/KSCN.[15] The contents of reduced GSH was measured by the method of Saville.[16] The activities of superoxide dismutase (Cu/Zn-form),[17] catalase,[18] GSH-peroxidase[19] and GSH-transferase[10] were determined by the described methods.

RESULTS

The rate of ethane exhalation by rats increased rapidly after a NDMA dose (Fig. 1). A single i.p. or p.o. injection of 10 mg/kg b.w. elevated ethane exhalation 13–14-fold; the raate of ethane exhalation peaked 30 min after the i.p. dose, and 60 min after the p.o. dose. A single p.o. dose of 0.5 mg/kg was sufficient to produce a 5-fold increase in ethane exhalation (Fig. 1).

Similarly, when analyses were made from liver tissue of rats shortly after dosing, NDMA-treatment was found to enhance lipid peroxidation as measured by four different methods (Fig. 2). The biggest enhancement was seen in the amounts of diene conjugation (Fig. 2A) and fluorescent products (Fig. 2B). As in the case of ethane exhalation, the increases were rapid peaking 20 min after dosing. An exception, however, was the produciton of TBA-reactive material, which was highest 60 min after administration of the dose (Fig. 2C). In the case of fluorescent (Fig. 2B) and TBA-reactive (Fig. 2C) products, the values were clearly above control levels still 24 h after dosing.

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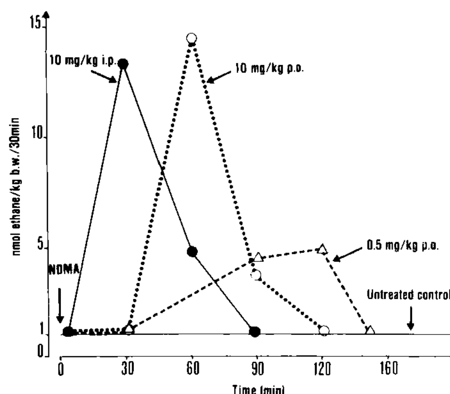

FIGURE 1 The rate of ethane exhalation by rats *in vivo* after a single dose of NDMA. Representative figures are given from experiments repeated 3–5 times, and expressed as nmole ethane/kg b.w./30 min.

Simultaneously with the increased lipid peroxidation, NDMA-treatment slightly decreased antioxidant enzyme activities and GSH contents in the liver (Table I). However, these changes were small when compared to those of the parameters of lipid peroxidation.

Enhancement of lipid peroxidation by NDMA was also demonstrated using isolated rat hepatocytes as the model system (Fig. 3A,B). The lucigenin-dependent chemiluminescene was increased by micromolar concentrations of NDMA (Fig. 3A). Strongest effect was obtained by 15 μM NDMA concentration. However, by NDMA concentrations higher than this no effects were seen. Moreover, the release of H_2O_2 from isolated hepatocytes increased in the presence of NDMA (Fig. 3B). The NDMA concentrations used in these experiments did not affect cell viability assessed by the trypan blue exclusion test.

Finally, when hepatic microsomes from nontreated animals were incubated with NADPH, the microsomal ethane production was increased after addition of NDMA. The highest increase was seen with 6 μM NDMA concentration (Fig. 3C).

DISCUSSION

The prooxidant states, defined as increased concentrations of active oxygen, organic peroxides and radicals,[1] are known to be involved in several pathological conditions, including cancer.[1–4] Some carcinogenic chemicals and processes have been shown to increase the prooxidant states, but systematical studies in this field are lacking. In general, the prooxidant states are generated *in vivo* by factors which increase the formation of free radicals, or alternatively by factors which impair the antioxidant defence system. The prooxidant states can be estimated indirectly by measuring the level of resulting lipid peroxidation.

In the present study we have shown that NDMA produces oxidative damage in the rat. This was demonstrated by increased lipid peroxidation *in vivo* in NDMA-treated animals, by increased chemiluminescence and H_2O_2 release of isolated hepatocytes upon exposure to NDMA, and also by increased ethane production by microsomes

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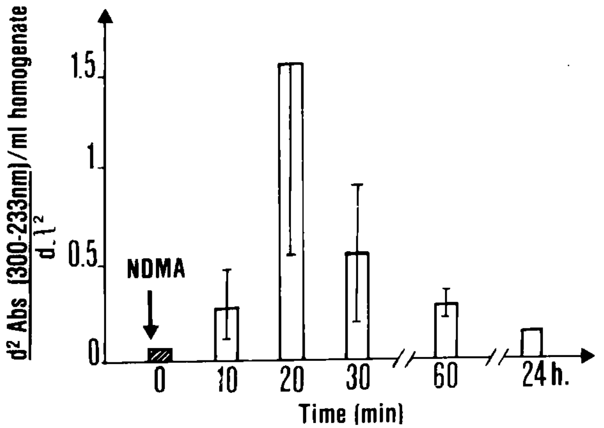

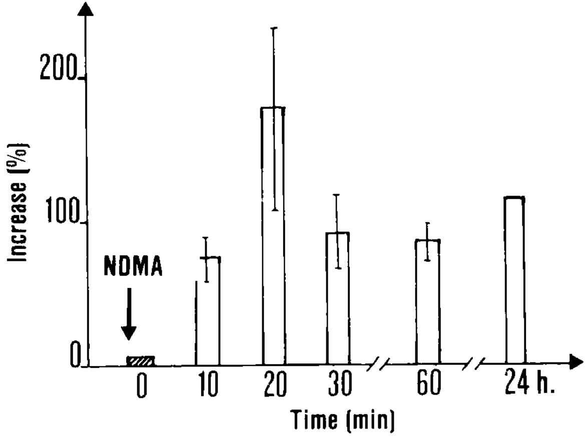

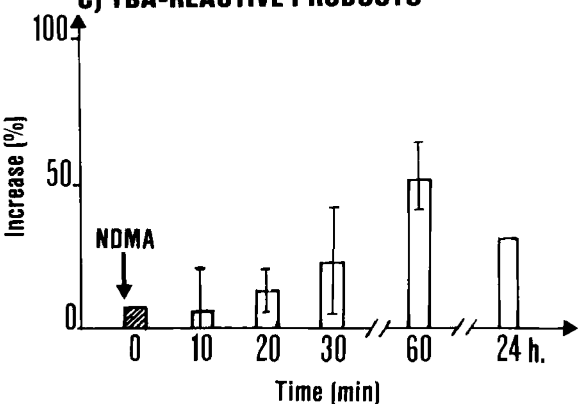

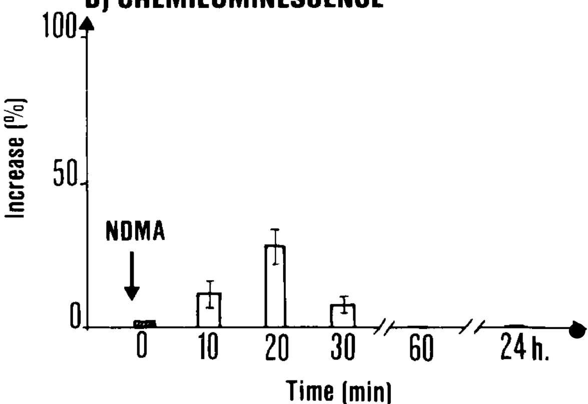

FIGURE 2 Hepatic lipid peroxidation of Wistár rats after a single i.p. dose (10 mg/kg b.w.) of NDMA. A. Diene conjugation, measured in the lipid extract of liver homogenate. Mean ± SEM from 4–10 animals; B. Fluorescent lipid peroxidation products measured in the lipid extract of S9 fraction. Expressed as % of increase over control value (100 arbitrary fluorescence units/g liver). Mean ± SEM from 4–10 animals; C. TBA-reactive products, measured in S9 fraction. Expressed as in (B). Control value was 4–5 μmol/mg prot D. NADPH-stimulated, lucigenin-dependent chemiluminescence, measured in the microsomal fraction. Expressed as in (B). Control value was 8,000–10,000 mV/mg prot.

TABLE I

Antioxidant enzyme activities and GSH contents in rat liver after a single i.p. dose (10 mg/kg) of NDMA

	% activity after NDMA treatment					
Assay for	0	10′	20′	30′	60′	24 h
Superoxide dismutase (SOD)	100	79*	79*	85*	85*	85
Catalase	100	86*	96	89	90	80
GSH peroxidase	100	91	94	103	85	106
GSH transferase	100	101	100	82*	81*	94
GSH content	100	87*	110	100	88	119

SOD was measured in liver homogenate and other variables in the cytosol (4–10 rats per time point); expressed as % activity compared to untreated animals.

*Significant level with $P < 0.01$. Control values were as follows: SOD, 4–6 μg SOD/mg prot.; Catalase, 600–700 units/mg prot.; GSH-peroxidase, 0.3 μmol/min. mg prot.; GSH-transferase, 1.7–2 μmol/min. mg prot.; GSH contents, 15–20 μg/mg prot.

incubated in the presence of NDMA. The results cannot, however, elucidate the mechanism by which NDMA produces oxidative damage. Possibly, metabolic activation of NDMA produces free radical species capable of initiating peroxidative processes in the cell. It has also been demonstrated that during the normal cytochrome P-450 mediated metabolism of xenobiotics, there is formation of superoxide anion and H_2O_2.[21]

Conspicuously, the effects produced by NDMA *in vivo* took place rapidly. The peak was reached within 20–60 min after NDMA administration, and in most cases the changes were no more seen 24 h after the dosing. The faster increase of ethane exhalation after i.p. than after p.o. administration is likely due to a difference in the pharmacokinetics of NDMA after administration by the different routes. The amount of TBA-reactive material increased slower than the other measures of lipid peroxidation. This is not surprising since by the TBA-method end products rather than early events of lipid peroxidation are measured.

It was recently reported that administration of several carcinogens to Fischer 344 rats had no effect on lipid peroxidation in the liver.[22] In that study lipid peroxidation was measured by the diene conjugation method in microsomal and nuclear cell fractions. Importantly, the animals were killed 18 h after administration of the carcinogen. Therefore, these results are not in contrast to ours, since we found that after the rapid increase diene conjugation was back on control level 24 h after administration of the NDMA. On the other hand, in a previous study we found that chronic (10 weeks) treatment of rats with *N*-nitrosodiethylamine (total dose 36 mg) elevated ethane exhalation, and this elevation was still seen months after cessation of the chronic treatment.[6]

The *in vitro* studies (Fig. 3) suggest that with increasing NDMA concentration the NDMA produced effects increase, but only up to a certain level; NDMA concentrations higher than this tend to decrease the effect. The reason for this phenomenon remains unclear.

In conclusion, our results clearly demonstrate that the alkylating carcinogen, NDMA, can induce prooxidant states in the rat. Whether the same is true for other classes of carcinogens and processess known to affect tumor initiation/progression is presently under investigation.

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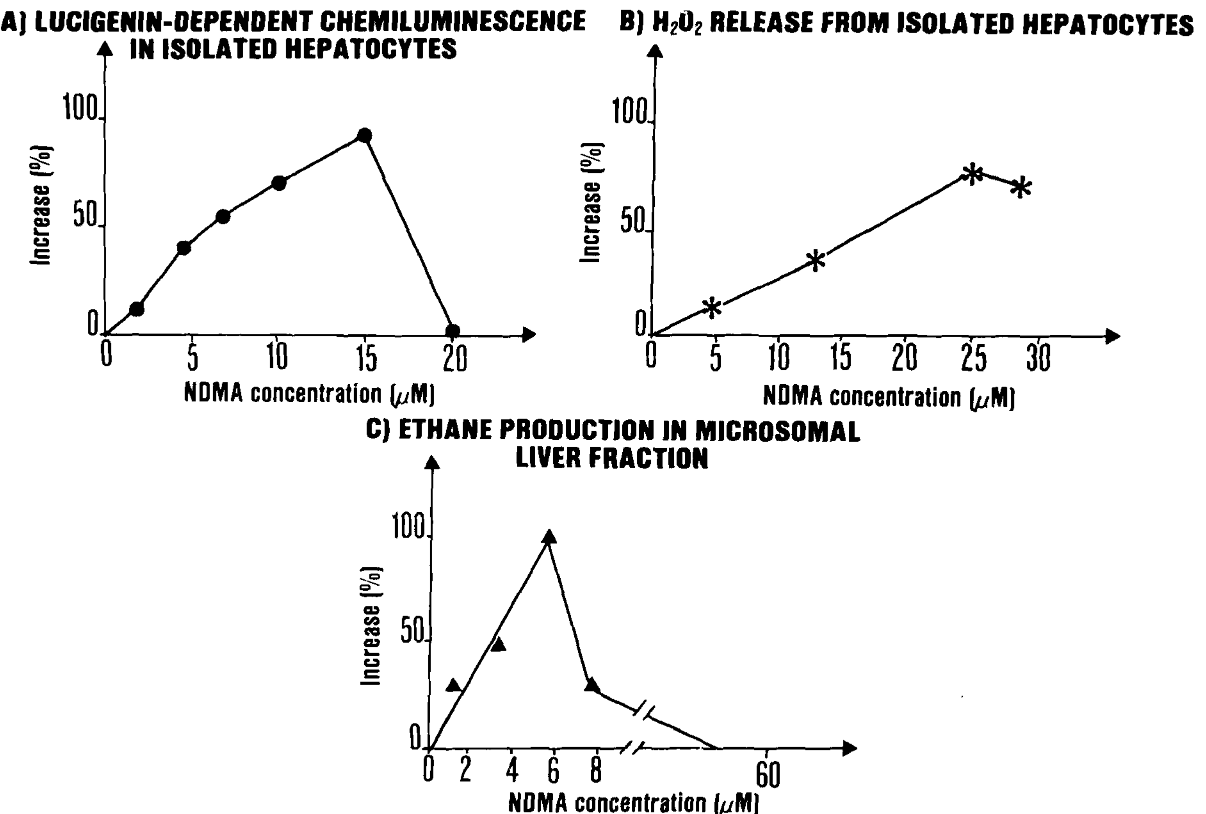

FIGURE 3 Production of reactive oxygen species and ethane by NDMA *in vitro*. Representative figures are given from experiments repeated 3–5 times. A. Lucigenin-dependent chemiluminescence measured in isolated rat hepatocytes (1–2 × 10^6 cells/ml), incubated with 2 mM NADPH and various NDMA concentrations. Expressed as % of increase compared to unexposed hepatocytes. Cell viability was 85% or more. Control value was 1000–2000 mv/10^6 cells; B. H_2O_2 release by isolated hepatocytes incubated with 2 mM NADPH and various NDMA concentrations. Expressed as in (A). Control value was 40–50 nmol/10^6 cells; C. Ethane production by hepatic microsomes measured in the gas phase of a 2 ml incubation mixture (2–4 mg microsomal protein; 2 mM NADPH; 2–10 μm NDMA; air, 30 min). Control value was 1 pmol/30 min × mg prot.

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