Biochemical Pharmacology, Vol. 45, No. 4, pp. 819–825, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

RELAXATION OF RAT THORACIC AORTA BY *N*-NITROSO COMPOUNDS AND NITROPRUSSIDE AND THEIR MODIFICATIONS OF NUCLEIC ACID BASES THROUGH RELEASE OF NITRIC OXIDE

JIN-MEI WANG, SHOEI-YN LIN-SHIAU* and JEN-KUN LIN†
Institutes of Biochemistry and *Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

(*Received* 17 *September* 1992; *accepted* 7 *December* 1992)

Abstract—Nitroprusside and two newly synthesized direct-acting mutagens, *N*-nitroso-2-fluorenylactamide and *N*-nitroso-dinitroacetaminophen, were able to elicit the concentration-dependent relaxation of rat aorta. The vascular responses could be antagonized by hemoglobin that binds nitric oxide with high affinity and by methylene blue which inhibits soluble guanylate cyclase activity. We used four bases in DNA as the substrates to test the capacity to deaminate of the two compounds. Xanthine, hypoxanthine and uracil as well as some unusual bases such as 8-OH-adenine and 8-OH-guanine were found. These findings suggest that both the muscle relaxation and mutagenicity of these two compounds might be due to the release of nitric oxide in living cells.

N-Nitroso-2-fluorenylacetamide (N-NO-2-FAA‡) and *N*-nitroso-3,5-dinitro-acetaminophen (N-NO-DNAAP) were synthesized by reacting 2-fluorenylacetamide (2-FAA) and acetaminophen, respectively, with N_2O_3 in glacial acetic acid at 0°. N-NO-2-FAA was found to be a more powerful mutagen to *Salmonella typhimurium* TA 97, TA 98, TA 100, TA 1535 and TA 1538 than its parent compounds [1]. N-NO-DNAAP shows a significant inhibitory effect on survival of Chinese hamster ovary cell [2]. Moreover, these compounds can develop mutagenicity by spontaneous conversion to a diazonium ion which can attack macromolecules in living cells electrophilically [1].

Nitric oxide (NO) has been reported to be the common reactive intermediate for coronary artery relaxation elicited by two carcinogenic nitroso-compounds, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [3, 4] and *N*-nitroso-nornicotine [5], through activation of guanylate cyclase in tissue. This compound is a constituent of cigarette smoke [6], air pollutant [6] and recently discovered bioregulatory agent [7–9]. It can cause genomic alternation. *In vitro*, NO deaminates deoxynucleosides, deoxynucleotides and intact DNA at physiological pH [10, 11]. *In vivo*, NO has been shown to cause genetic mutation in *Salmonella typhimurium* TA 1535 using a modified Ames *Salmonella* reversion assay and cause chromosome aberration in primary lung cells [12, 13]. It is possible that genotoxicity of MNNG and *N*-nitrosonornicotine may be attributed to the release of nitric oxide.

In this study, we determined whether N-NO-2-FAA and N-NO-DNAAP can relax rat thoracic aorta through the same mechanism of MNNG and *N*-nitroso-nornicotine and compared their potencies with 2-FAA, dinitroacetaminophen (DNAAP), nitroprusside and MNNG. The deamination and hydroxylation abilities of N-NO-2-FAA and N-NO-DNAAP were then tested. The latter experiments can evaluate the possibility that the two compounds develop their genotoxicity by release of the NO free radical.

MATERIALS AND METHODS

Instrumentation. HPLC apparatus (Waters Associates, Milford, MA, U.S.A.) consisting of a Model 510 pump, a Model U6K universal injector, a Model 441-fixed wavelength detector operating at 254 nm and an automated gradient controller were used. The recorder, a Waters 745B data module and a Waters NOVA-PAK C18 column (3.9 × 150 mm), were employed. The gas chromatography–mass spectrometry (GC-MS) spectra were determined with a Hewlett–Packard Model 5690 Series 2 gas chromatography equipped with a 5971A mass selective detector. The injection port and detector were maintained at 250° and 280°, respectively. The separation was achieved on a fused-silica capillary column (12 m × 0.2 mm, Ultra-2 Hewlett–Packard) programmed after 3 min at 100°, from 100° to 250° at a rate of 8°/min and kept at 250° for 8 min. Helium was used as the carrier gas at an inlet pressure of 10 psi. The split ratio was 20:1 [14, 15].

The HPLC-electrochemical detector (ECD) profile was determined with a Waters Associates Model 590 HPLC pump using a Lichrospher 100 RP-18 column

† Corresponding author: Dr Jen-Kun Lin, Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei, Taiwan. Tel. (011) 886-2-356-2213; FAX (011) 886-2-391-8944.

‡ Abbreviations: N-NO-2-FAA, *N*-nitroso-2-fluorenylacetamide; N-NO-DNAAP, *N*-nitroso-3,5-dinitro-acetaminophen; 2-FAA, 2-fluorenylacetamide; GC-MS, gas chromatography–mass spectrometry; ECD, electrochemical detector; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Hb, oxyhemoglobin; MB, methylene blue; NE, norepinephrine; DMSO, dimethyl sulfoxide; DNAAP, dinitroacetaminophen; TMS, trimethylsilate.

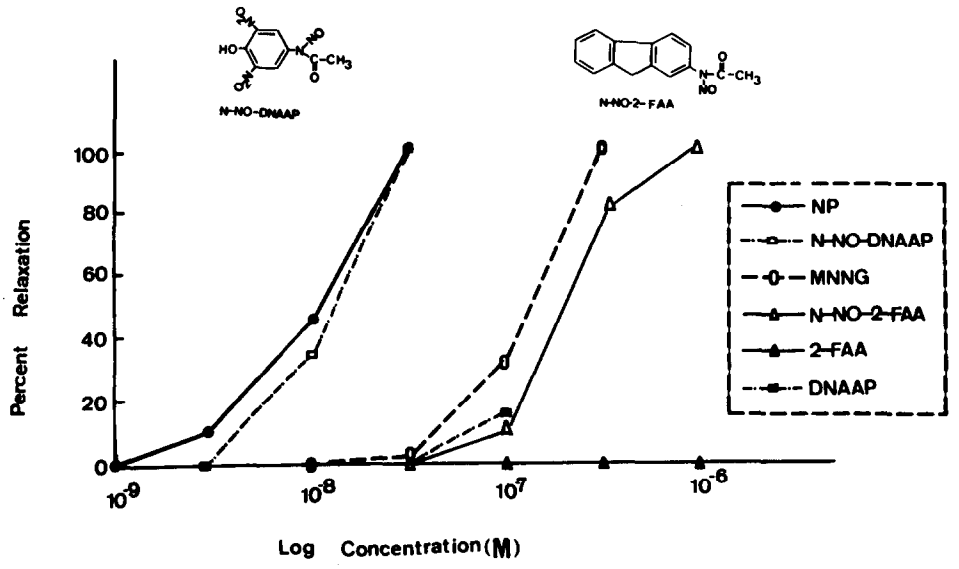

Fig. 1. Concentration–relaxation curves for nitroprusside (NP), N-NO-DNAAP, MNNG, N-NO-2-FAA, 2-FAA and DNAAP in rat aorta. Tone was induced by NE (10 μM).

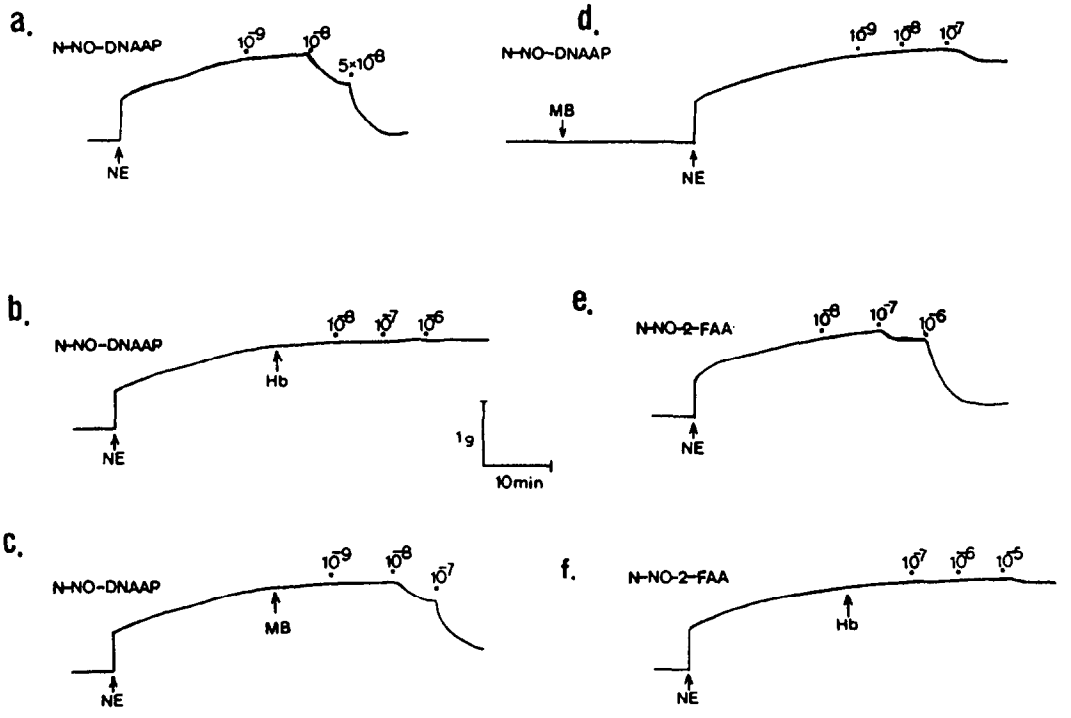

Fig. 2. Influence of Hb (10 μM) and MB on endothelium-independent relaxation induced by N-NO-2-FAA and N-NO-DNAAP in rings of rat aorta precontracted with NE (10 μM). (a) Concentration-dependent relaxation induced by N-NO-DNAAP in rat aorta. (b) Hb blocks the concentration-dependent relaxation induced by N-NO-DNAAP in rat aorta. (c) Pretreatment of MB (10 μM) for 5 min only slightly reduced the relaxation induced by N-NO-DNAAP. (d) Extending the pretreatment with MB (10 μM) for 30 min blocks the relaxation induced by N-NO-DNAAP. (e) Concentration-dependent relaxation induced by N-NO-2-FAA. (f) Hb blocks the relaxation induced by N-NO-2-FAA.

Table 1. Nitroso compound induced deamination of nucleic acids in DNA and its constituents at pH 7.4

Run	Nitroso compound (initial concentration 0.005 mM)	Substrate (initial concentration 0.1 mM)	Deamination product (final concentration, μM)
1	N-NO-DNAAP	Adenine	Hypoxanthine (3.26)
2	N-NO-DNAAP	Guanine	Xanthine (0.92)
3	N-NO-DNAAP	Cytosine	Uracil (0.3)
4	N-NO-2-FAA	Adenine	Hypoxanthine (1.23)
5	N-NO-2-FAA	Guanine	Xanthine (0.4)
6	N-NO-2-FAA	Cytosine	Undetectable

The four bases in DNA were determined by HPLC with UV detection [27]. The mobile phase contained 0.02 mol/L KH_2PO_4, pH 5.6.

(Merck, 5 μM, 0.4 × 50 mm) equilibrated with 10% methanol containing 12.5 mM citric acid, 30 mM sodium hydroxide, 25 mM sodium acetate and 10 μM acetic acid, pH 5.10, at a flow rate of 1 mL/min. Separation of bases was monitored at 254 nm in a Waters Model 441 absorbance detector on line with Waters Model 460 ECD using a glassy-carbon working electrode and an Ag/AgCl reference electrode. The molar ratio of 8-OH-deoxyguanosine to deoxyguanosine in each sample was determined based on the ECD at 0.8 V for 8-OH-deoxyguanosine and absorbance at 254 nm for deoxyguanosine [16]. Deoxyguanosine and 8-OH-deoxyguanosine were detected at 7.5 and 10.7 min at 25°, respectively, and identified by co-chromatography.

Chemicals. Nitroprusside, 2-FAA, AAP, MNNG, bovine hemoglobin type 1, methylene blue (MB), norepinephrine (NE), sodium dithionite, ibuprofen, deoxyguanosine, adenine, guanine, cytosine, thymine, xanthine, hypoxanthine and uracil were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium nitrate, citric acid, sodium hydroxide, sodium acetate, methanol, acetic acid, hydrochloric acid and dimethyl sulfoxide (DMSO) were purchased from E. Merck (Darmstadt, F.R.G.). For use as chromatographic standards, 8-OH-deoxyguanosine were prepared by the method of Kasai and Nishimura [17].

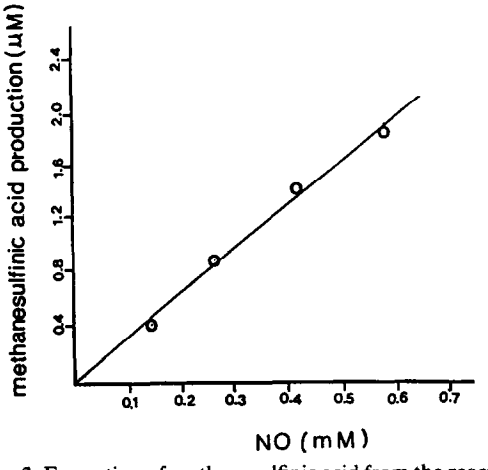

Fig. 3. Formation of methanesulfinic acid from the reaction of NO with DMSO. The reaction mixture contained DMSO (50 mM) and different concentration of NO (0.1–0.7 mM), in the presence of oxygen. The formation of hydroxyl radicals from NO solution was monitored by the production of the trapping product methanesulfinic acid which was estimated by colorimetric method with fast blue BB salt [20, 21].

Preparation of hemoglobin. Pure hemoglobin (oxyhemoglobin, Hb) was prepared by adding to a 1 mM solution of commercial hemoglobin (bovine hemoglobin type 1) in distilled water, a 10-fold molar excess of the reducing agent, sodium dithionite ($Na_2S_2O_4$). The sodium dithionite was then removed by dialysis against 100 volumes of distilled water at 4° for more than 2 hr. The purity of Hb was determined by spectrophotometry [18].

Bioassay studies. Wistar rats (150–200 g, either sex) were anesthetized with phenobarbital sodium (30 mg/kg) and killed. Thoracic aorta was quickly excised, cut into 4–5 mm rings, de-endothelialized and attached to a transducer (FTO3; Grass Instruments, Quincy, MA, U.S.A.) for recording of isometric tension with a Model 7 Polygraphy (Grass Instruments). The rings were maintained under 1 g of resting tension, superfused with Krebs buffer [3, 5] and saturated with 95% O_2/5% CO_2. The tissues were equilibrated for 60 min before contraction with NE (10 μM). Endothelial cells were removed from aortic rings by gently rubbing the intimal surface with a wooden stick for 30–60 sec. Successful removal of endothelial cells was demonstrated by the lack of relaxation in response to 10 μM acetylcholine. All experiments were performed in the presence of ibuprofen (10 μM), to prevent the synthesis of prostaglandins and other prostanoids.

Preparation of saturated NO solution. Distilled water was aerated with pure nitrogen for 2 hr before the preparation of NO solution which was kept sealed until used. NO gas (Linde Air Products Co.) was then bubbled into the nitrogen-treated distilled water for 30 min. The conversion of Hb to methemoglobin by a double-beam spectro-

photometer (Hitachi Model U-3210) was used to analyse the NO content of stock solution [19].

Colorimetric assay for hydroxyl radical. DMSO was used as a quantitative molecular probe for the generation of hydroxyl radicals (OH) in aqueous system. The procedures of colorimetric assay for hydroxyl radical were as described by Babbs and co-workers [20, 21].

Experimental conditions for obtaining hydroxylated and deaminated products. Adenine, guanine, cytosine and thymine were used to test the deamination and hydroxylation ability of N-NO-2-FAA or N-NO-DNAAP. Each base (0.1 mM) in a 1 M phosphate buffer reacted with 0.005 mM N-NO-DNAAP or N-NO-2-FAA at 37° and physiological pH for 12 hr. The half-life of N-NO-2-FAA and N-NO-DNAAP

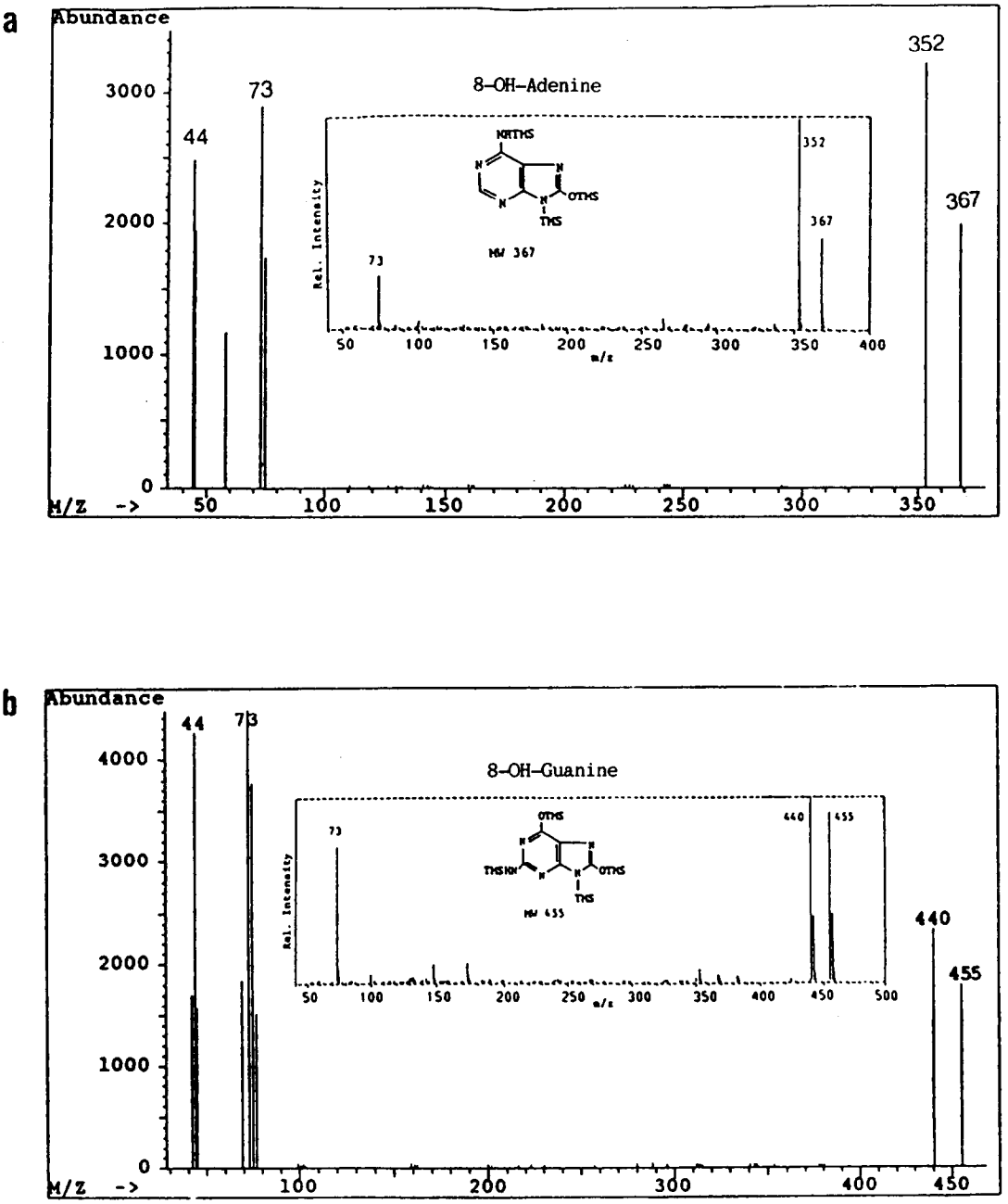

Fig. 4. (a) Mass spectrum of TMS derivative of 8-hydroxyadenine. (b) Mass spectrum of TMS derivative of 8-hydroxyguanine. The mass spectra of 8-OH-adenine and 8-OH-guanine in Ref. 14 were used as standard spectra which were shown in the inlets of (a) and (b), respectively.

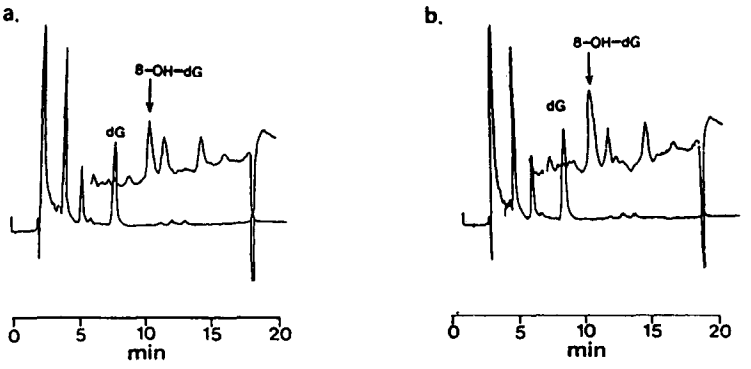

Fig. 5. (a) Representative HPLC ECD profile of deoxyguanosine (1 mM) treated with N-NO-DNAAP (0.05 mM) at 37° for 24 hr. (b) 8-OH-dG (0.3 pmol) co-chromatographic profile was present for further identification. N-NO-DNAAP and its metabolites were removed by 1 M H_2SO_4 saturated butanol. Top, electrochemical detector response; bottom, UV absorption at 254 nm. dG, deoxyguanosine; 8-OH-dG, 8-hydroxy-deoxyguanosine. For clarity, the electrochemical detector response prior to 6 min retention time is not shown.

in the pH 7.4 phosphate buffer at 37° were 32 and 18 min, respectively. The amount of the deaminated bases was then analysed by HPLC. Each of the lyophilized reaction products (2 mg) was trimethylsilylated in a Teflon-capped Hypo-vial (Pierce, Rockford, IL, U.S.A.) with a mixture of *N*-methyl-*N*-trimethylsilyltrifluoro-acetamide (Pierce) (0.1 mL) and acetonitrile (0.1 mL) by heating at 70° for 30 min. The presence of hydroxylated derivatives was then demonstrated by GC-MS analysis.

RESULTS

Effects of Hb and MB on the endothelium-independent relaxation induced by N-NO-2-FAA and N-NO-DNAAP

The concentration–relaxation curves of rat aorta by N-NO-2-FAA, N-NO-DNAAP, nitroprusside, 2-FAA, DNAAP and MNNG are shown in Fig. 1. The relaxation induced by 10^{-8}, 10^{-7} and 10^{-6} M N-NO-2-FAA was 0% ± 0%, 10% ± 2% and 86% ± 7.3%, respectively. The aorta was not relaxed by 2-FAA at any concentration tested. N-NO-DNAAP relaxed aorta in a manner similar but stronger than that with N-NO-2-FAA. The relaxation induced by 10^{-9}, 10^{-8} and 10^{-7} M N-NO-DNAAP was 0% ± 0%, 34% ± 5% and 100% ± 3%, respectively.

N-NO-2-FAA and N-NO-DNAAP were found to elicit a potent and marked relaxation of rat aorta. We used an antagonist to ascertain whether NO could account for the relaxant effect caused by these nitroso compounds. As shown in Fig. 2, pretreatment of aortic rings with Hb for 5–10 min at a concentration 10 μM abolished the endothelium-independent relaxation induced by N-NO-2-FAA or N-NO-DNAAP. On the other hand, pretreatment for 5–10 min with MB (10 μM) only partly reduced the relaxation effect induced by N-NO-2-FAA or N-NO-DNAAP. Increasing the concentration of MB to 100 μM or extending the period of pretreatment to 30 min intensified the blockade of induced relaxation.

The results of using adenine, guanine, cytosine and thymine to test the deamination ability of N-NO-2-FAA and N-NO-DNAAP are summarized in Table 1. It shows that guanine and adenine treated with *N*-nitroso compounds were readily deaminated. About 0.92 μM guanine converted to xanthine when 0.1 mM guanine was reacted with 0.005 mM N-NO-DNAAP, and 3.26 μM adenine converted to hypoxanthine under similar reaction conditions. Conversion from cytosine to uracil was rare and no conversion of thymine occurred. N-NO-2-FAA deaminated bases similarly but to a lesser extent than N-NO-DNAAP.

Oxidative damage of base and deoxynucleoside

As methanesulfinic acid, the primary product of the trapping reaction of DMSO with hydroxyl radicals, was formed when NO solution was added into a 50 mM aqueous DMSO solution, it seems that the levels of hydroxyl radicals produced are parallel to the concentration of NO in the reaction mixture (Fig. 3). We used GC-MS to analyse the hydroxylate reaction products of nitroso compounds and bases. The 8-OH-adenine and 8-OH-guanine were detected in the products of N-NO-2-FAA and N-NO-DNAAP. Their mass spectra are shown in Fig. 4. Figure 4a was assigned as the trimethylsilate (TMS) derivative of 8-OH-adenine. Intense M^+ and $(M\text{-}CH_3)^+$ ions were observed at m/z 367 and 352 (base peak), respectively. Ions at m/z 73 are commonly observed with TMS derivatives and serve no diagnostic purpose. Figure 4b was attributed to the TMS derivatives of 8-hydroxyguanine. Abundance M^+ and $(M\text{-}CH_3)^+$ ions at m/z 455 and 440 were observed in its mass spectrum.

HPLC-ECD was used for further quantitative analysis. As seen in Fig. 5, N-NO-DNAAP

caused a significant increase ($P < 0.005$) in 8-OH-deoxyguanosine content 8-OH-dGuo/dGuo= (12.77 ± 0.74)/1000. The effects of N-NO-2-FAA treatment on the formation of 8-OH-deoxyguanosine were less potent, giving the ratio of 8-OH-dGuo/dGuo= (3.32 ± 0.13)/1000.

DISCUSSION

It has been shown that the carcinogenic nitrosamide, MNNG and the *N*-nitroso-nornicotine from cigarette smoke relax bovine coronary artery through the common reactive intermediate, NO, and active guanylate cyclase [3–5]. Experiments reported in this communication demonstrated other *N*-nitroso compounds, N-NO-2-FAA and N-NO-DNAAP, which elicit a potent and marked relaxation of rat aorta, were also through NO release. These two nitroso compounds and NO similarly elicit dose-dependent relaxation of smooth muscle from rat aorta. The relaxation of rat aortas was not induced by the precursors of these two nitroso compounds, 2-FAA and DNAAP. Furthermore, the vascular responses were antagonized by Hb that bound NO with high affinity and by MB, the inhibitor of soluble guanylate cyclase.

NO, a fairly unstable free radical, was previously found to cause DNA damage *in vitro* and *in vivo*. In addition, the NO intermediate produced from immuno-stimulated macrophage could convert amines to nitrosoamines under physiological conditions [22]. Thus, one of the mutagenic mechanisms of N-NO-2-FAA and N-NO-DNAAP might be mediated by this NO factor. In order to prove this hypothesis, the four bases in DNA were used to test the deamination ability of N-NO-2-FAA and N-NO-DNAAP. Purines were more readily deaminated than pyrimidines. Moreover, some unexpected bases like 8-hydroxyadenine and 8-hydroxyguanine were found. These hydroxylated bases were identified by GC-MS and further quantitative analysis by HPLC-ECD. 8-Hydroxy derivatives, products of hydroxyl radical or single oxygen attack on one base of DNA [23–25], have been reported to cause misreading of DNA templates not only at that site but also at adjacent sites, suggesting that it may be involved in mutation and carcinogenesis. These findings prompted us to propose a mechanism of genotoxicity of N-NO-2-FAA and N-NO-DNAAP based on production of free radicals. Due to the low volatility of these bases, TMS derivatization is necessary to make them suitable for GC-MS analysis and easily interpretable mass spectra [26].

Acknowledgements—This study was supported by the National Taiwan University Research Grant, NTUH 80-001-A01 and NTUH 81-040-A13 and the National Science Council NSC 81-0412-B002-83, Taipei, Taiwan, ROC. We are grateful to Mr C. A. Shih for technical assistance with HPLC-ECD detection.

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