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NITRO-TYROSINE AS PROMOTER OF FREE RADICAL DAMAGE IN A DNA MODEL SYSTEM

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Nitro-tyrosine considerably promotes the degradation of DNA, when incubated with Cu^{2+} and ascorbate in oxygenated aqueous solution. This deleterious process requires oxygen and can be inhibited with catalase, indicating that H_2O_2 is involved, via the reduction of oxygen. Menadione and 2,4,6-trinitrobenzenesulfonate, known to catalyze particularly fast such reduction of oxygen, were only slightly more active than nitro-tyrosine. Degradation of DNA can be explained by a site-specific Fenton type reaction of H_2O_2 with the DNA-Cu^+ complex,

$$\text{DNA-Cu}^+ + H_2O_2 \longrightarrow \text{DNA}^{\cdot} \cdot\text{OH} + Cu^{2+} + OH^-$$

Copper-chelating agents (EDTA and penicillamine) prevent DNA degradation, whereas $^{\cdot}OH$-scavengers (t-butanol) are ineffective. The deleterious activity of nitro-tyrosine (and of other nitroaromatics) in the DNA model system may indicate important toxicological implications, since aromatic nitration is a significant mode of action of nitrogen dioxide.

Key words: Copper-catalyzed reactions; DNA, oxidative damage; hydrogen peroxide generation; menadione; nitro-tyrosine

INTRODUCTION

Nitroaromatic and electron affinic compounds with genotoxic and bactericidal activity are capable in many cases of mediating the production of activated oxygen species, $O_2^{\cdot -}$ and H_2O_2[1,2,3,4], e.g. with ascorbate (AH^-) as reducing agent[2],

$$\underset{\text{(1)}}{\overset{AH^-}{H^+ + A^{\cdot -}}} \quad \underset{\text{(2)}}{\overset{RNO_2}{RNO_2^{\cdot -}}} \quad \overset{O_2^{\cdot -}}{O_2} \xrightarrow[AH^-,\ H^+]{\text{(3)}} H_2O_2 \qquad (1\text{–}3)$$

The deleterious effects initiated by RNO_2 in biological systems may presumably also involve the transition metal ions Cu^{2+} and Fe^{3+}, since these can catalyze the transformation of $O_2^{\cdot -}$ and H_2O_2 into $^{\cdot}OH$[5,6,7,8], considered to be a most toxic entity,

$$O_2^{\cdot -} + Cu^{2+} \longrightarrow O_2 + Cu^+ \qquad (4)$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + OH^- + {}^{\cdot}OH \qquad (5)$$

Metal-catalyzed reactions of this type can particularly lead to site-specific damage[8,9,10] directly at target molecules which bind the reduced metal ion, for instance DNA in the case of copper[11],

$$\text{DNA-Cu}^{+} + \text{H}_2\text{O}_2 \longrightarrow \text{Cu}^{2+} + \text{OH}^{-} + \text{DNA}^{\cdot} \cdot \cdot \text{OH} \qquad (5A)$$

where Cu^+ complexation seems to occur at the N_7 position of guanine[12]. Appreciable amounts of iron and copper ions have in fact been detected in nucleic acids isolated from various tissues[13], hence it appears reasonable to assume that these contribute, e.g. by site-specific damage of DNA, to the genotoxic action of nitroaromatic compounds.

In a recent investigation of nitrogen dioxide reactions[14] we obtained evidence that tyrosine nitration is an important mechanism of action of $NO_2^{\cdot}$ in biological systems. In this context we now report that the degradation of DNA in model systems containing ascorbate and Cu^{2+} can be promoted by nitro-tyrosine as well as by other electron affinic compounds.

MATERIALS AND METHODS

Chemicals

DNA (type III, salmon testes) and menadione (vitamin K_3, 2-methyl-1,4-naphthoquinone) from Sigma Chemie, superoxide dismutase (SOD, bovine erythrocytes) from Boehringer Mannheim, L-ascorbic acid, catalase (bovine liver), ethidiumbromide (ETB), D-penicillamine, nitrofurazone (5-nitro-2-furaldehyde semicarbazone) and 3-nitro-L-tyrosine from Serva Feinbiochemica, ethylenediaminetetraacetic acid disodium salt·$2H_2O$ (EDTA), H_2O_2 (30%, stabilized with ammonium nitrate) and 2,4,6,-trinitrobenzene-sulfonic acid·$3H_2O$ from Fluka Feinchemikalien, and tert-butanol, $CuCl_2 \cdot 2H_2O$ and 4-nitroacetophenone from Merck were used as received without further purification.

Preparation and incubation of DNA solutions

Stock solutions were made up with redistilled water and adjusted to pH 7, the ascorbate and H_2O_2 solutions being prepared freshly for each experiment. The components of the model system were generally mixed in the following order, with typical concentrations during incubation of: 0.1 g/l DNA (~250 μM nucleotide) + 20 mM phosphate buffer (pH 6.8) + 50 μM $CuCl_2$ + additive (e.g. nitro-tyrosine) + 20 mM ascorbate. The addition of ascorbate defines the start of incubation, which was performed in a thermostat at 22°C throughout these experiments. Solutions were gently flushed with high purity O_2 (= aerobic conditions) or N_2 (= anaerobic conditions) before and during incubation. The incubation was terminated by addition of 20 mM EDTA (final concentration). After addition of EDTA the DNA was protected even in the order of days, due to copper chelation which prevents DNA degradation via reaction 5A.

Test of DNA degradation

Degradation of DNA was detected as previously[11,15] by adding ethidiumbromide (ETB) at a ratio of 1 ETB molecule per 5 DNA bases to the system. The characteristic

590 nm fluorescence of the DNA/ETB intercalation complex[16,17] was measured under excitation at 510 nm, using a Zeiss spectrofluorimeter. DNA degradation after various incubation times (t) was detected by the fluorescence loss, I(t)/I(0), where I(0) refers to the corresponding system protected by EDTA before addition of ascorbate. The relative fluorescence yield of unbound ETB (in absence of DNA) was $I_{ETB}/I(0) = 0.08$ under the condition applied. As previously[11,15], the DNA/ETB fluorescence was measured against a rhodamine B (0.5 μM) reference solution, thus I(0) provides a control of the double-stranded conformation of DNA for each experiment.

RESULTS

Nitro-tyrosine as promoter of DNA degradation

The progressive degradation of DNA, when incubated under standard condition (see above) with Cu^{2+} and ascorbate in absence and presence of nitro-tyrosine is shown in Figure 1; the following results were obtained:

A) Under aerobic conditions and in the absence of nitro-tyrosine, DNA degradation occurred over a time scale of several hours (Figure 1A); the process came to completion ($I(t)/I(0) \sim 0.1$) after about 6 hours. No DNA degradation was detectable in this time scale when the system was incubated anaerobically.

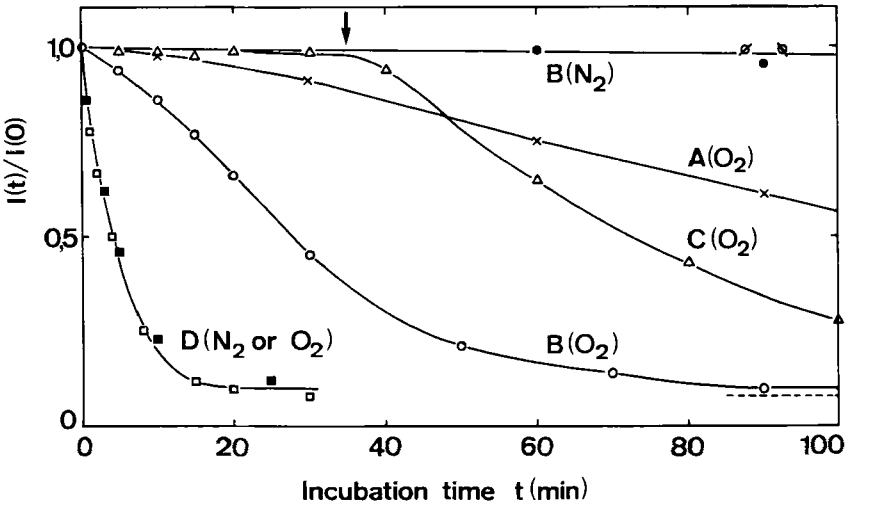

FIGURE 1 Degradation of DNA (0.1 g/l) under incubation with Cu^{2+} (50 μM) and ascorbate (20 mM) in phosphate buffered (20 mM, pH 6.8) aqueous solution at 22°C.

A(O_2): no additive, aerobic conditions.

B: in presence of 0.5 mM nitro-tyrosine, aerobic (O_2) or anaerobic (N_2) conditions.

C(O_2): in presence of 0.5 mM nitro-tyrosine and 25 mg/l catalase, aerobic condition, the arrow indicating the inhibition of catalase by addition of 2.5 mM NaN_3.

D: in presence of 5 mM H_2O_2, aerobic (□) or anaerobic (■) conditions.

The system **B**(O_2) was also incubated in the absence of either Cu^{2+} (ø) or ascorbate (ø). The dotted level indicates the relative fluorescence of unbound ETB, system **A** in absence of DNA.

B) In the presence of nitro-tyrosine the DNA degradation in the aerobic system was considerably speeded up, but again no effect was detectable under anaerobic conditions (Figure 1B, O_2 and N_2). The DNA was unaffected also when either Cu^{2+} (ø) or ascorbate (o) was deleted in the solution $B(O_2)$.

C) Catalase (25 mg/l) completely prevented DNA degradation in the aerobic system with (or without) nitro-tyrosine. The protective effect of catalase was abolished, however, after inhibition of the enzyme with azide (Figure 1C, arrow). Superoxid dismutase only slightly retarded the degradation in the system $B(O_2)$.

D) When H_2O_2 (5 mM) was added before incubating the system (A or B), rapid DNA degradation occurred both under aerobic and anaerobic conditions (Figure 1D, cf. also ref. 11).

The final degree of DNA damage in the aerobic systems A, B and C was similar in each case, indicating almost complete degradation of the DNA under these conditions. This can be seen from the relative DNA/ETB fluorescence, $I(\infty)/I(0) \sim 0.1$, which compares with that of unbound dye, $I_{ETB}/I(0) = 0.08$. When lowering the ascorbate concentration there was less DNA damage and some delay in its progression. A detailed analysis of the kinetics of the DNA degradation at various concentrations of the additives will be presented in a forthcomming paper.

Summing up, the above results reveal that efficient degradation of DNA occurs in aqueous solutions containing Cu^{2+} and ascorbate, and that this process is promoted by nitro-tyrosine. These effects are expressed only in presence of oxygen, unless H_2O_2 is added, and they are apparently due to the generation of H_2O_2, as shown by the protective effect of catalase.

Effect of additives, and comparison with other electron affinic compounds

In Table I the effects of t-butanol and penicillamine on the DNA degradation in the system containing nitro-tyrosine is shown. Furthermore, the promoting effect of nitro-tyrosine in these systems is compared with that of some electron affinic compounds, known to mediate the production of activated oxygen species by the reaction sequence (1-3)[2,3].

TABLE I

Promotion (inhibition) of DNA damage by various additives in model systems containing Cu^{2+} and ascorbate

Additive	$t_{1/2}$ min[a]	ER[b]
Control[c] (no additive)	110	1
nitro-tyrosine 0.5 mM	28	3.9
ibid + t-butanol 0.4 M	26	4.2
ibid + penicillamine 2 mM	300	0.4
menadione 0.5 mM + t-butanol 0.4 M	18	6.1
trinitrobenzenesulfonate 0.5 mM + t-butanol 0.4 M	22	5.0
nitrofurazone 0.5 mM + t-butanol 0.4 M	75	1.5
nitroacetophenone 0.5 mM + t-butanol 0.4 M	102	1.1

[a] Incubation time where $I(t)/I(0) = 0.5$.

[b] Enhancement ratio $ER = t_{1/2}(\text{control})/t_{1/2}$.

[c] 0.1 g/l DNA, 50 μM Cu^{2+}, 20 mM phosphate (pH 6.8), 20 mM ascorbate, O_2-saturated.

That t-butanol, an efficient ·OH-scavenger, is unable to protect the DNA against the action of nitro-tyrosine (Table I) is consistent with a site-specific mechanism[8,9,10,11,18], i.e. generation and reaction of ·OH radicals directly at or within the DNA molecule (reaction 5A). Efficient protection is achieved, on the other hand, when the copper-chelating agents EDTA (see Methods) or penicillamine (Table I) are introduced.

As shown in Table I, the enhancement ratio for DNA degradation under aerobic conditions in presence of menadione and trinitrobenzenesulfonate is only slightly higher than with nitro-tyrosine; nitrofurazone and nitroacetophenone are much less effective. Control experiments showed, as in the case of nitro-tyrosine (Figure 1), that O_2, Cu^{2+} and ascorbate are all prerequisite to degradation of DNA in presence of the electron affinic compounds tested.

DISCUSSION

The slow degradation of DNA in the presence of Cu^{2+} and ascorbate (Figure 1A) can be explained by the slow formation of H_2O_2 under autoxidation of ascorbate[19,20,21], i.e. the net reaction 6,

$$AH^- + H^+ + O_2 \longrightarrow A + H_2O_2 \qquad (6)$$

and simultaneous reduction of Cu^{2+} by ascorbate[12],

$$AH^- + Cu^{2+} \longrightarrow A^{\cdot -} + H^+ + Cu^+ \qquad (7)$$

These two reactions provide the precursors H_2O_2 and Cu^+, acting in the deleterious reaction 5A, which at the same time regenerates Cu^{2+}. The autoxidation of ascorbate is also catalyzed by copper[7,19,20], probably with intermediate formation of $O_2^{\cdot -}$ and involvement of reactions 7 and 8,

$$O_2^{\cdot -} + 2\,H^+ + Cu^+ \longrightarrow Cu^{2+} + H_2O_2 \qquad (8)$$

Nitro-tyrosine promoted the formation of H_2O_2, even though the reaction 6 is already catalyzed by copper; this is shown by the faster degradation of DNA due to the reaction 5A in the absence of catalase (Figure 1, $B(O_2)$). In this respect, nitro-tyrosine is almost as efficient as menadione and trinitrobenzenesulfonate (Table I), reported to be the most reactive ones in a series of compounds[2,3], with regard to propagation of the reaction sequence (1–2). The enhancement ratios given in Table I (ER = 4 to 6) appear to be much less dramatic, however, than those given for the rate of ascorbate oxidation (ER = 110 in the case of menadione)[2]. This is probably due to the lower substrate concentrations and the effect of copper on ascorbate autoxidation in our system. Furthermore, the DNA degradation is delayed due to the relatively slow reaction 5A; a rate constant of $k_{5A} \sim 1\ M^{-1}\ s^{-1}$ can be roughly estimated from Figure 1D. The total amount of reduced oxygen, i.e. H_2O_2, formed in the above model system via the reactions 1–3 is certainly very much lower than the initial ascorbate concentration since ascorbate is continuously consumed also by the reaction 7. It should be noted also that ascorbate is seemingly inefficient in protecting the DNA by chelation of copper or by antioxidative reactions, i.e. radical scavenging and chemical repair.

Many nitroaromatic compounds, including the last three shown in Table I, are also

known to be hypoxic cell radiosensitizers[22,23], probably due to interactions with target radicals[22] (e.g. DNA$^{\cdot}$ $^{\cdot}$ $^{\cdot}$OH),

$$DNA^{\cdot}\ {}^{\cdot}\ {}^{\cdot}OH + RNO_2 \longrightarrow DNA(^{\cdot}OH)RNO_2 \longrightarrow \text{enhanced target damage} \quad (9)$$

It is unlikely, however, that reaction 9 contributes to the DNA degradation observed in the above model system, since DNA$^{\cdot}$ $^{\cdot}$ $^{\cdot}$OH was generated in oxygenated solutions where oxygen enhancement (analogous to reaction 9) should prevail. In view of the experiment D (Figure 1) it also appears questionable whether oxygen enhancement is detectable at all with the ETB method.

In conclusion, the above results reveal that nitro-tyrosine resembles some electron affinic compounds in that it catalyzes the reduction of oxygen to $O_2^{\cdot -}$ and H_2O_2. If propagation of this process (reactions (1-3)) is intrinsic to the genotoxicity of these compounds, than nitro-tyrosine can be expected to be similarly active. Our results also illustrate that the catalytic and site directing effect of copper ions may be involved in the propagation of biological damage by nitroaromatic and other cytotoxic agents, and that DNA is a possible target. Metabolic reductive activation of nitro compounds can be achieved, as in this study, with ascorbate[2], but also enzymically e.g. with nitro-reductases[1,4]. Increasingly it is recognized that aromatic nitration is a significant mode of action of nitrogen dioxide[11,24,25], and particularly in this context we hope that the present model system study can provide some evidence for possible toxicological implications.

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