Free Rad. Res. Comms., Vol. 9, No. 1, pp. 39–47
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DNA SINGLE STRAND BREAKAGE BY H_2O_2 AND FERRIC OR CUPRIC IONS: ITS MODULATION BY HISTIDINE

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(Received July 24, 1989; in revised form October 9, 1989)

The role of histidine on DNA breakage induced by hydrogen peroxide (H_2O_2) and ferric ions or by H_2O_2 and cupric ions was studied on purified DNA. L-histidine slightly reduced DNA breakage by H_2O_2 and Fe^{3+} but greatly inhibited DNA breakage by H_2O_2 and Cu^{2+}. However, only when histidine was present, the addition of EDTA to H_2O_2 and Fe^{3+} exhibited a bimodal dose response curve depending on the chelator metal ratio. The enhancing effect of histidine on the rate of DNA degradation by H_2O_2 was maximal at a chelator metal ratio between 0.2 and 0.5, and was specific for iron. When D-histidine replaced L-histidine, the same pattern of EDTA dose response curve was observed. Superoxide dismutase greatly inhibited the rate of DNA degradation induced by H_2O_2, Fe^{3+}, EDTA and L-histidine involving the superoxide radical.

These studies suggest that the enhancing effect of histidine on the rate of DNA degradation by H_2O_2 and Fe^{3+} is mediated by an oxidant which could be a ferrous-dioxygen-ferric chelate complex or a chelate-ferryl ion.

KEY WORDS: Hydrogen peroxide, iron, copper, histidine, circular DNA.

INTRODUCTION

We recently reported that histidine increased the rate of DNA single-strand breakage by hydrogen peroxide (H_2O_2) on purified DNA and greatly enhanced the cytotoxic and clastogenic effects of H_2O_2 on chinese hamster lung fibroblast.[1] Others also showed that histidine increased the frequency of chromosomal aberrations and the cytotoxicity induced by the hypoxanthine-xanthine oxidase system[2] or by hydrogen peroxide.[3] However conflicting results exist concerning the effect of histidine. Low concentrations of histidine provided a marked decrease in paraquat induced cellular killing[4] and inhibited DNA single-strand breaks induced by the xanthine-xanthine oxidase system.[5] Furthermore, histidine and its derivatives such as carnosine or homocarnosine showed antioxidant activity.[6] The protective effect of histidine may be due to a combination of several factors: histidine is an effective hydroxyl radical scavenger[7] and an efficient chelator for copper and iron.[8,9]

In this study, the effects of ferric (Fe^{3+}) and cupric (Cu^{2+}) ions on the DNA single-strand breakage by hydrogen peroxide and histidine were investigated using the replicative form of the phage fd. The data show that the protective or the enhancing effect of histidine on DNA single-strand breakage induced by H_2O_2 depend on the

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metal used and on the metal chelator ratio. The ferric chelate in the presence of H_2O_2 and histidine could form a ferrous dioxygen ferric chelate complex or a chelate-ferryl ion leading to DNA single-strand breakage.

MATERIALS AND METHODS

Materials

Superoxide dismutase (SOD, EC 1.15.1.1) 3000 U/mg was obtained from Sigma Chemical Co, Saint Louis MO. L- and D-histidine were from Fluka. Ethylenediamine tetraacetic acid disodium salt (EDTA), hydrogen peroxide, $CuSO_4$, $FeCl_3$ and other salts were analytical reagent grade. The DNA used was the replicative form of the phage fd (fd RF DNA) purified through isopycnic centrifugation in cesium chloride ethidium bromide as described.[10] After several precipitations in ethanol and 0.3 M NaCl, DNA was resuspended in 10 mM *Tris*-HCl pH = 7.9, 10 mM NaCl and stored at −20°C. DNA preparations typically contained 60–80% covalently closed circular (ccc) supercoiled molecules, 20–40% open relaxed circle molecules and virtually no linear molecules.

Detection of DNA nicking

DNA single-strand breaks were assayed by measuring the conversion of the covalently circular double-stranded supercoiled DNA (Form I) to a relaxed circular double-stranded DNA. Although the accumulation of linear DNA (Form III) was indicative of double-stranded cuts in the Form I, its accumulation could also be derived from breakage of nicked form II. The sequence of electrophoretic mobility was: relaxed form (Form II) < linear form (Form III) < supercoiled form (Form I). The number of nicks introduced per molecule can be calculated from the fraction of DNA molecules undergoing the transition from supercoiled to relaxed circle assuming a Poisson distribution of the nicks among the molecules.

The fd RF DNA (150 ng) was incubated in 10 mM *Tris*-HCl pH = 7.9, 10 mM NaCl with various agents in microfuge tubes (Eppendorf). Final concentrations of Fe^{3+} and Cu^{2+} were 50 μM and 1 mM for L- and D-histidine. The reaction was started by the addition of hydrogen peroxide in such a way that its final concentration was 3 mM. The final volume was 20 μL and the incubation mixture was kept at 37°C for varying periods of time. The reaction was stopped by the addition of 10 μl of electrophoresis sample buffer (4 M urea, 50% sucrose, 50 mM EDTA and 0.1% bromophenol blue).

The samples were loaded on an horizontal 1% agarose slab gel and the electrophoresis was conducted in a mini apparatus (Bethesda Research Laboratories, Bethesda, MD) for 16 h at 25 V, at room temperature. The agarose electrophoresis running buffer was 40 mM *Tris* acetate pH = 8.4, 10 mM EDTA. Gels were stained for 2 h in running buffer containing 0.5 μg/ml ethidium bromide and then destained for several h. DNA bands were visualized by illuminating the gel with UV light and photographs were taken with a Polaroid Camera equipped with a yellow filter using a black and white Polaroid film type 665.

Quantification of bands was achieved by measuring areas of densitometer tracings. Under the conditions of staining used in these experiments, it was found that the

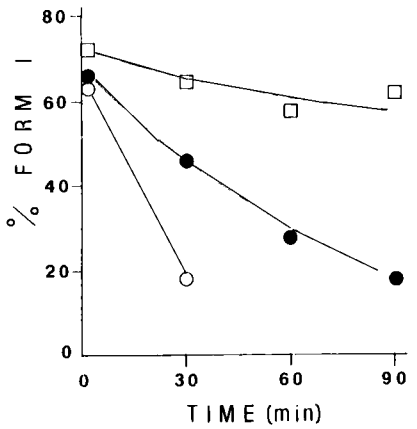

FIGURE 1 Kinetics of nicking supercoiled DNA (Form I) by H_2O_2 and histidine in the presence or absence of metal. The percent of DNA remaining supercoiled is plotted against time. 150 ng of fd RF DNA in 10 mM Tris-HCl, 10 mM NaCl, pH = 7.5 was incubated with 3 mM H_2O_2 and 1 mM L-histidine at 37°C: □ no metal added, ○ 50 μM Fe^{3+}, ● 50 μM Cu^{2+}.

decrease in percent of supercoiled DNA was matched with an increase of relaxed DNA. Experiments were done at least twice, on different days. The results shown are typical of those obtained.

RESULTS

The incubation of supercoiled covalently closed circular DNA (Form I) at 37°C with 3 mM H_2O_2 and 1 mM L-histidine did not lead to DNA single-strand breakage in the time studied (Figure 1). The addition of 50 μM Fe^{3+} or 50 μM Cu^{2+} to the mixture containing 3 mM H_2O_2 and 1 mM L-histidine induced the formation of DNA single-strand breaks. The time taken to introduce one nick per molecule was about 30–60 min in the presence of 50 μM Fe^{3+} and 90 min in the presence of 50 μM Cu^{2+}. The comparison of the kinetics of degradation of the DNA supercoiled (time to introduce

TABLE I

Comparison of the rate of DNA nicking by H_2O_2 in the presence or absence of histidine. The time to introduce one nick per molecule can be calculated from the fraction of DNA molecules undergoing the transition from supercoiled to relaxed circle, assuming Poisson distribution of the nicks among the molecules.

Conditions	Time to introduce one nick per molecule (min) L-histidine	
	None[a]	1 mM
3 mM H_2O_2	50	ND[b]
3 mM H_2O_2 + 50 μM Fe^{3+}	15	30–60
3 mM H_2O_2 + 50 μM Cu^{2+}	0.5	90

a = data previously reported,[13] obtained in the same experimental conditions: 150 ng fd RF DNA, 10 mM Tris-HCl, 10 mM NaCl pH = 7.5.

b = No DNA single strand breakage was observed after an incubation period of 90 min.

one nick per molecule), by H_2O_2 with and without L-histidine is shown in Table I. It appeared that L-histidine slightly decreased DNA single-strand breakage by 3 mM H_2O_2 and 50 μM Fe^{3+} whereas DNA single-strand breakage by 3 mM H_2O_2 and 50 μM Cu^{2+} was strikingly inhibited.

Because histidine is known as an iron and copper chelating agent, the competitive effect of an increasing EDTA concentration on the DNA nicking by H_2O_2 and Fe^{3+} or by H_2O_2 and Cu^{2+} with or without L-histidine was investigated.

Ferric ions

When fd RF DNA was incubated for 30 min at 37°C in 10 mM *Tris*-HCl, 10 mM NaCl containing 3 mM H_2O_2 and 50 μM Fe^{3+} without L-histidine, the protective effect of EDTA increased linearly with the logarithm of its concentration up to 50 μM, corresponding to an EDTA:Fe^{3+} molar ratio 1, (Figure 2A). At an EDTA concentration above 50 μM the protective effect of EDTA reached a plateau and remained incomplete ($\simeq$ 50%). Furthermore, the amount of the linear form (Form III) decreased with an increase of EDTA concentration (Figure 2B).

In the presence of 1 mM L-histidine, the EDTA dose-response curve was bimodal (Figure 2A). The rate of supercoiled DNA degradation increased with the concentration of EDTA up to 10 μM – 25 μM (or EDTA:Fe^{3+} ratio 0.2–0.5). It also appeared that in the range of EDTA concentration 1 to 10 μM, there is a relationship between the appearance of the linear form (Form III) and the increase of EDTA concentration (Figure 2B). However, at an EDTA concentration above 25 μM, the increase of EDTA concentration yielded an increase of the DNA remaining supercoiled (Figure 2A) as well as a decrease in Form III at 50 μM EDTA (Figure 2B).

Cupric ions

As above, the fd RF DNA was incubated for 30 min at 37°C in 10 mM *Tris*-HCl,

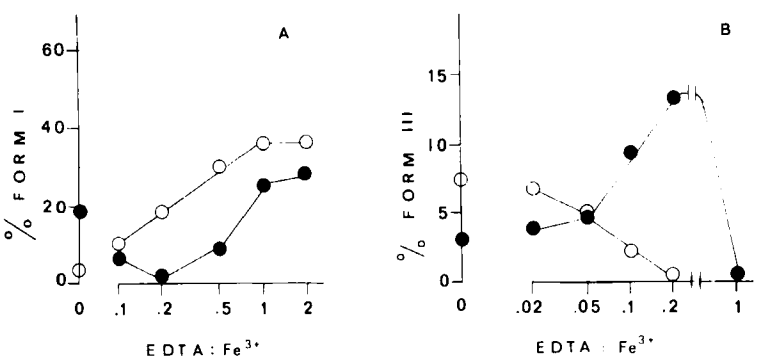

FIGURE 2 Dose effect of EDTA on the nicking of DNA by H_2O_2 and Fe^{3+} in the presence or absence of histidine. 150 ng fd RF DNA in 10 mM *Tris*-HCl, 10 mM NaCl pH = 7.5 was incubated with 3 mM H_2O_2, 50 μM Fe^{3+} and various concentrations of EDTA (0, 5, 10, 25, 50 and 100 μM) without ○ or with 1 mM L-histidine ●. Incubation proceeded at 37°C for 30 min. In the absence of treatment, DNA preparation contained 60% Form I and virtually no linear molecules (Form III). *Panel A*: The percent of DNA remaining supercoiled (Form I) is plotted against EDTA: Fe^{3+} ratio. *Panel B*: The percent of linear DNA molecules (Form III) is plotted against EDTA: Fe^{3+} ratio.

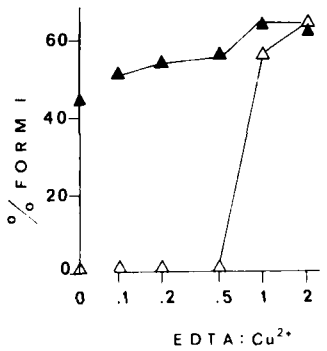

FIGURE 3 Dose effect of EDTA on the nicking of supercoiled DNA (Form I) by H_2O_2 and Cu^{2+} in the presence or absence of histidine. 150 ng fd RF DNA in 10 mM *Tris*-HCl, 10 mM NaCl pH = 7.5 was incubated with 3 mM H_2O_2, 50 μM Cu^{2+} and various concentrations of EDTA (0, 5, 10, 25, 50 and 100 μM) without △ or with 1 mM L-histidine ▲. Incubation proceeded at 37°C for 30 min. In the absence of treatment, DNA preparation contained 60% Form I and virtually no linear molecules.

10 mM NaCl containing 3 mM H_2O_2 and 40 μM Cu^{2+} with or without L-histidine. In the absence of L-histidine, no DNA supercoiled molecules were detectable until the EDTA concentration reached 50 μM. At this EDTA concentration, the recovery of Form I was almost complete (Figure 3). The addition of 1 mM L-histidine greatly inhibited the degradation of DNA supercoiled by H_2O_2 and Cu^{2+}. The increase of EDTA concentration provided an additional effect which was complete at an equimolar EDTA Cu^{2+} ratio.

In the presence of Fe^{3+} and H_2O_2, D- and L-histidine both showed the same EDTA dose response curve (Figure 4), indicating that there was no stereospecificity of histidine in the modulation of DNA single-strand cleavage by H_2O_2, Fe^{3+} and EDTA. To gain insight into the mechanism of the enhancing effect of histidine on single-

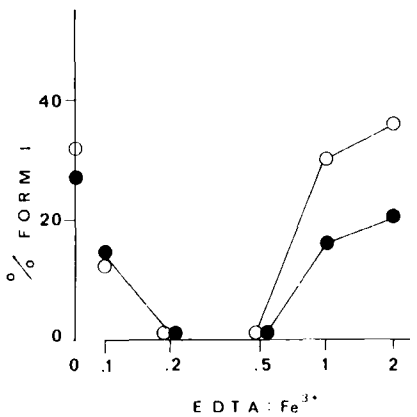

FIGURE 4 Comparison of the dose effect of EDTA on the nicking of supercoiled DNA (Form I) by H_2O_2 and Fe^{3+} either with 1 mM L-histidine ● or with 1 mM D-histidine ○. The experimental conditions are as in Figure 2. In the absence of treatment, DNA preparation contained about 70% Form I and virtually no linear molecules.

TABLE II

Effects of the increase of the ionic strength and superoxide dismutase (SOD) on the DNA single-strand breakage by H_2O_2, iron EDTA with or without histidine. 150 ng fd RF DNA was incubated in 10 mM Tris-HCl, 10 mM NaCl (except when indicated differently) pH = 7.5, with 3 mM H_2O_2, 50 μM Fe^{3+}, 25 μM EDTA without or with 1 mM L-histidine for 15 min. The temperature was 37°C.

	Form I (%) L-histidine	
Conditions	None	1 mM
None	81	80
H_2O_2	46 (0)[a]	39 (0)
H_2O_2 (100 mM NaCl)	58.5 (36)	35 (−10)
H_2O_2 + SOD (0.1 μg/ml)	57 (31)	61 (56)
H_2O_2 + SOD (10 μg/ml)	66.5 (59)	72 (85)
H_2O_2 + SOD boiled (10 μg/ml)	15 (−89)	47.5 (22)

(a): The percentage of inhibition on the disappearance of plasmid Form I is mentioned in brackets.

strand breakage by H_2O_2, the responses to an increase of the ionic strength and superoxide dismutase were investigated at an EDTA:Fe^{3+} molar ratio 0.5. The fd RF DNA was incubated for 15 min at 37°C in 10 mM *Tris*-HCl, 10 mM NaCl containing 3 mM H_2O_2 50 μM Fe^{3+}, 25 μM EDTA with or without 1 mM L-histidine. An increase of the ionic strength provided a decrease of the rate of DNA degradation, in the absence of histidine, whereas no change in the rate of DNA degradation was observed when 1 mM L-histidine was added (Table 2). Superoxide dismutase, unlike heat-inactivated superoxide dismutase, dose-dependently inhibited DNA single-strand breaks induced by Fe^{3+}, EDTA and H_2O_2 with and without L-histidine (Table 2). However, superoxide dismutase inhibited the rate of DNA degradation induced in the presence of histidine more efficiently than in its absence. Boiled superoxide dismutase enhanced DNA strand scission only when histidine was not added.

DISCUSSION

We recently reported that histidine accelerated the formation of single-strand breaks induced by H_2O_2,[1] potentially due to the presence of trace amounts of iron, histidine or one of its oxidation products. In this study, it is shown that histidine, in the presence of EDTA and Fe^{3+} enhances DNA breakage induced by H_2O_2. In contrast, when Cu^{2+} replaces Re^{3+}, such an enhancing effect is not observed.

H_2O_2 can react with reduced metals such as Fe^{2+} or Cu^{1+} bound to the DNA[11] generating through the Fenton reaction an extremely powerful oxidant, the hydroxyl radical. This latter reactive oxygen species generated at the DNA metal binding site may attack DNA at either the sugar or the base site[12] ultimately leading to DNA single-strand breakage. In a recent work using the same experimental conditions employed in this study, it has been shown that H_2O_2 could react with metal bound to DNA following the sequence of reactions (1a, 2a) and (1b, 2b) leading to DNA single-strand breaks.[13]

$$\text{DNA} - Fe^{3+} + H_2O_2 \rightarrow \text{DNA} - Fe^{2+} + O_2^{\cdot-} + 2H^+ \quad (1a)$$

$$\text{DNA} - Cu^{2+} + H_2O_2 \rightarrow \text{DNA} - Cu^{1+} + O_2^{\cdot-} + 2H^+ \quad (1b)$$

$$DNA - Fe^{2+} + H_2O_2 \rightarrow DNA - Fe^{3+} - OH^{\cdot} + OH^{-} \quad (2a)$$

$$DNA - Cu^{1+} + H_2O_2 \rightarrow DNA - Cu^{2+} - OH^{\cdot} + OH^{-} \quad (2b)$$

The addition of histidine slowed down the rate of DNA single-strand breakage induced by H_2O_2 and Fe^{3+} or by H_2O_2 and Cu^{2+}. The protective effect of histidine could arise from its ability to chelate copper and iron. Indeed, when iron and copper were removed from DNA, H_2O_2 could not react with them in a site specific manner. In the same way, the addition of EDTA removed metal from DNA inhibiting reactions 1a, 1b and 2a, 2b. However, the protective effect afforded by EDTA at an EDTA metal ratio above unity was incomplete with Fe^{3+} (50%) and complete with Cu^{2+}. Indeed, Fe^{3+} – EDTA in solution included the seventh coordination site which was freed or occupied by an easily dissociable ligand such as water[14] and so was readily reduced by hydrogen peroxide generating the ferryl ion complex FeO^{2+} – EDTA.[15]

$$Fe^{3+} - EDTA + H_2O_2 \rightarrow Fe^{2+} - EDTA + O_2^{\dot{-}} + 2H^{+} \quad (3)$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow FeO^{2+} - EDTA + H_2O \quad (4)$$

Such a ferryl ion could be involved in DNA single-strand breakage.[16] The presence in the Cu^{2+} – EDTA complex of an available coordination site able to react with H_2O_2 was not documented but the inability of Cu^{2+} – EDTA to catalyse the dismutation of the superoxide anion radical[17] suggested that all coordination sites were hindered in such a complex. The capacity of H_2O_2 to reduce Fe^{3+} — EDTA unlike Cu^{2+} – EDTA, generating the superoxide radical via reaction 3, seemed to be crucial to elucidate the role played by histidine. As a matter of fact the bimodal aspect of EDTA dose response was only observed with Fe^{3+}. The enhancing effect of histidine on DNA single-strand breakage induced by H_2O_2 appeared to be maximum at an EDTA Fe^{3+} molar ratio between 0.2 and 0.5. Thus, metal ions in excess of EDTA would be distributed to form complexes with DNA and with histidine. However at an EDTA Fe^{3+} molar ratio of 0.5, the increase of the ionic strength did not affect the rate of DNA degradation promoted by H_2O_2 and histidine, indicating that the iron remaining bound to DNA and able to react with H_2O_2 was negligible. However, in the absence of histidine, at the same EDTA Fe^{3+} molar ratio, iron in excess was bound to DNA and can react with H_2O_2 in a site specific manner as suggested by the decrease of the rate of DNA degradation with the increase of the ionic strength. When the concentration of EDTA increased, the iron complexed with histidine decreased and so the rate of DNA single strand breakage was lowered. It appears that both EDTA-iron and histidine-iron were involved in the enhancement of DNA single-strand breakage by H_2O_2 and histidine. Thus, the possibility that EDTA-iron and histidine-iron could form a ferrous-dioxygen-ferric complex may be considered.

$$H_2O_2 + EDTA - Fe^{3+} \rightarrow EDTA - Fe^{2+} + O_2^{\dot{-}} + 2H^{+} \quad (3)$$

$$H_2O_2 + Histidine - Fe^{3+} \rightarrow Histidine - Fe^{2+} + O_2^{\dot{-}} + 2H^{+} \quad (5)$$

$$O_2^{\dot{-}} + EDTA - Fe^{3+} \rightarrow EDTA - Fe^{2+} + O_2 \quad (3a)$$

$$O_2^{\dot{-}} + Histidine - Fe^{3+} \rightarrow Histidine - Fe^{2+} + O_2 \quad (5a)$$

$$EDTA - Fe^{2+} + Histidine - Fe^{3+} + O_2 \rightarrow EDTA - Fe^{2+} - O_2 - Fe^{3+} - Histidine \quad (6)$$

$$\downarrow$$

$$DNA\ damage$$

$$\text{EDTA} - Fe^{3+} + \text{Histidine} - Fe^{2+} + O_2 \rightarrow \text{EDTA} - Fe^{3+} - O_2 - Fe^{2+} - \text{His tidine} \downarrow \text{DNA damage} \quad (7)$$

If the hypotheses exposed above are correct, it is expected that superoxide dismutase will reduce the formation of such a complex by lowering the rate of reactions (3a) and/or (5a). Results in Table 2 show that this is the case.

This ferrous-dioxygen-ferric complex could be effective because the oxidation and reduction of iron may occur within the same complex eliminating dissociation and diffusion processes. The involvement of a ferric-dioxygen-ferrous chelate complex has been suggested elsewhere to be involved in the initiation of lipid peroxidation.[18]

The increase of DNA degradation by boiled SOD in the absence of histidine (Table 2) could be due to the loss of the copper of superoxide dismutase leading to an increase of copper able to bind DNA and to react with H_2O_2.

An alternative scheme may be envisaged: If reactions (5) and/or (5a) are faster than reactions (3) and (3a), we can postulate iron exchange according to:

$$\text{Histidine} - Fe^{2+} + \text{EDTA} - Fe^{3+} \rightarrow \text{Histidine} - Fe^{3+} + \text{EDTA} - Fe^{2+} \quad (8)$$

Thus, the effect of histidine could be due to an enhanced rate of formation of the damaging *EDTA*-ferryl via reactions (5) and/or (5a) followed by reactions (8) and (4). In this scheme, it is also expected that superoxide dismutase decreases DNA degradation by lowering the rate of reaction (5a).

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Accepted by Prof. H. Sies