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DIFFERENTIAL EFFECTS OF HISTIDINE ON HYDROGEN PEROXIDE-INDUCED BACTERIAL KILLING AND DNA NICKING IN VITRO

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The hydrogen peroxide dose-response curves for *Escherichia coli* killing and DNA nicking *in vitro* display remarkably similar bimodal patterns. The concentrations of the oxidant resulting in maximum mode one killing, however, exceeds by two orders of magnitude those resulting in the mode one DNA nicking response. Addition of histidine differentially affects the experimental curves describing the dose-dependency for bacterial killing and DNA damage *in vitro*. Indeed, the lethal effect elicited by the oxidant in the presence of the amino acid is strictly concentration-dependent and thus the inactivation curve loses its bimodal character. In marked contrast, histidine abolishes DNA damage generated by low concentrations of hydrogen peroxide ($< 100\ \mu M$) in the *in vitro* system (the mode one DNA nicking response) but greatly increases DNA damage produced by concentrations of the oxidant higher than 1 mM (the mode two DNA nicking response).

Experimental results also suggest that treatment of covalently closed circular double-stranded supercoiled DNA with hydrogen peroxide, in the presence of both histidine and iron, may result in the formation of DNA double strand breakage, a type of lesion which is not efficiently produced by the oxidant in the absence of the amino acid.

Taken together, the above results indicate that histidine differentially affects the *in vitro* DNA cleavage and *E. coli* lethality induced by hydrogen peroxide and suggest that different molecular events mediate mode one DNA nicking *in vitro* and mode one killing of bacterial cells.

KEY WORDS: *E. coli* killing, DNA nicking, histidine, hydrogen peroxide.

INTRODUCTION

The comparison of the cytotoxic response of bacterial and mammalian cells to challenge with H_2O_2 points out marked differences, particularly as far as the pattern of the dose-response curves, and the concentrations of H_2O_2 producing maximal cytotoxic effects, are concerned. In mammalian cells, the cytotoxicity of the

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oxidant varies in different cell types and is markedly cell density-dependent. It becomes measurable at micromolar concentrations and increases in a concentration-dependent fashion[1-3]. In *E. coli*, the inactivation curve is characterized by a bimodal pattern and two modes of killing can be distinguished[4,5]. Mode one killing occurs below 5 mM H_2O_2, the maximum rate of bacterial killing occurring at about 2.5 mM. Mode two killing requires concentrations of H_2O_2 higher than 10 mM, and partial resistance can be observed between 5 and 12.5 mM H_2O_2.

In vitro the H_2O_2-concentration dependency for DNA damage, measured by phage inactivation or nicking of purified DNA, displays qualitatively the same bimodal pattern[6-8]. Recently, reports from various laboratories have demonstrated that histidine enhances the toxicity of hydrogen peroxide in *E. coli* cells[9,10] (although only the effects on cell turbidity and those on viability after treatment with an oxidant concentration resulting in mode one killing were tested in the two studies, respectively), as well as in mammalian cells[10-13]. It exacerbates the clastogenicity of H_2O_2 in mammalian cells as measured by DNA single strand breakage[13], formation of micronuclei[11], and chromosomal aberrations[14].

The aim of the present study is to investigate and characterize the effects of histidine on H_2O_2-induced killing of *E. coli* and nicking of purified DNA.

MATERIALS AND METHODS

Materials

Histidine was from Fluka (Zürich, Switzerland), sodium chloride and ferrous sulfate were from Merck (Darmstadt, Germany). Other chemicals and reagent grade biochemicals were from Sigma Chemical Co (St Louis, MO, U.S.A.) or Flow Laboratories (MC.Lean, VA, U.S.A.). Hydrogen peroxide was purchased as a 30% solution from Prolabo (Paris, France).

Bacterial Strain and Growth

The *E. coli* strain used, AB 1157, was routinely grown in our laboratory. Bacteria were grown overnight (16–18 h) at 37°C in K medium (1% glucose, 1% casamino acids, 25 μg/ml $MgSO_4$ 7 H_2O, 2 μg/ml $CaCl_2$ and 1 μg/ml thiamine hydrochloride in M9 salts). Samples were diluted 50-fold in fresh K medium and grown at 37°C to about 10^8 cells/ml under aerobic conditions and then treated for the experiments.

Survival Curves

Cells grown to about 10^8 cells/ml were harvested by centrifugation at room temperature, washed once with M9 salts and resuspended in an appropriate medium. Unless otherwise stated, experiments were performed at a density of 10^8 bacteria/ml and treatment with H_2O_2 (15–30 min) was in 3 ml of cell suspension placed in a 20 ml scintillation counting vial with 200 rpm shaking, at 37°C. The challenge was terminated by dilution in M9 salts, the cells were immediately plated in quadruplicate in LB agar plates and incubated at 37°C for 24 hours to allow colony formation.

DNA

The DNA used was the plasmid pBR 322 purchased from Boehringer (Manheim, Germany). It was ethanol precipitated once, centrifuged, decanted and dried overnight. DNA was resuspended in 40 mM NaCl and stored at 4°C. The concentration of DNA was estimated spectrophotometrically. DNA preparations typically contained about 80–85% double stranded covalently - closed circular (ccc) supercoiled molecules, 15–20% nicked circular molecules and no linear duplexes.

Detection of DNA Nicking

DNA single-strand breaks were assayed by measuring the conversion of supercoiled DNA (form I) to nicked DNA (form II). The number (r) of nicks introduced per molecule can be calculated from the fraction of DNA molecules remaining supercoiled (P_0) assuming a Poisson distribution of the nicks among the molecules:

$$P_0(r) = e^{-r}$$

The experiments were performed in 1.5 ml Eppendorf polypropylene tubes. 0.2 μg of plasmid pBR 322 was incubated in 150 mM NaCl. The pH of the solutions was adjusted to 7.0 using NaOH 0.1 N. Hydrogen peroxide was added just before the addition of a solution containing L-histidine and ferrous iron. The final volume was 20 μl and the incubation mixture was kept 45 minutes at 37°C. The reaction was stopped by addition of 10 μl electrophoresis sample buffer (4M urea, 50% sucrose, 50 mM EDTA and 0.1% bromophenol blue). The electrophoresis, the visualisation of DNA by ethidium bromide staining and the quantification of the bands were performed as described previously[15].

RESULTS

H_2O_2-Mediated Bacterial Killing

The responses of *E. coli* cells to treatment with hydrogen peroxide for 15 min in complete medium (K medium), minimal glucose medium (M9 salts plus glucose) or minimal glucose medium plus histidine are illustrated in Figure 1.

As previously reported[4,5], two modes of killing are observed following exposure of *E. coli* to increasing concentrations of H_2O_2 in K medium. Maximum mode one killing occurs at 2.5 mM H_2O_2 (about 70% reduction in the ability to form colonies) and mode two killing becomes apparent at concentrations above 10–15 mM. An intermediate zone of partial resistance is detectable between these two regions of toxicity. When treatments are performed in minimal glucose medium, mode one killing becomes less pronounced and the maximal effect (about 50% reduction in the ability to form colonies) is produced at 5 mM H_2O_2. The intermediate zone of partial resistance is apparent also under these experimental conditions and the mode two killing response occurs at concentrations above 10–15 mM H_2O_2.

The behaviour in K medium could be the result of the enhancing or protecting effects of histidine and the casaminoacids on the effect of H_2O_2 as previously described[10]. As a matter of fact we have demonstrated that histidine significantly increases the toxicity elicited by a concentration of the oxidant resulting in the mode one killing response under conditions of treatment in a minimal glucose medium[10].

We have therefore investigated the effect of the amino acid on the toxicity curve

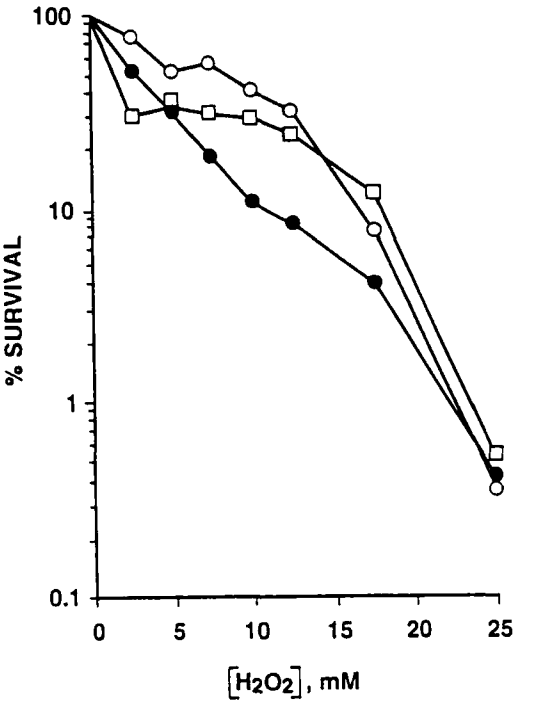

FIGURE 1 Survival of *E.coli* cells exposed for 15 minutes to increasing concentrations of hydrogen peroxide in K medium (□), minimal glucose medium (○) or minimal glucose medium containing 870 μM L-histidine (●). Treatments were performed at a density of 10^8 cells/ml. Results are the mean of three to four separate experiments. Standard errors were less than 10%.

elicited by increasing levels of hydrogen peroxide in minimal glucose medium. Experimental data illustrated in Figure 1 clearly demonstrate that 870 μM histidine (a concentration corresponding to that of the K medium) produces remarkable changes in the shape of the dose-response curve resulting from treatment with the oxidant in the absence of the amino acid.

Firstly, histidine increases the killing elicited by low concentration of H_2O_2. At 5 mM H_2O_2 the percentages of survival are about 30% or 50%, in the presence or absence of the amino acid, respectively (Figure 1). The effect of histidine on the killing induced by concentrations of H_2O_2 in the range 150–600 μM is reported in Figure 2. For these experiments, we took advantage of the fact that the toxicity of hydrogen peroxide is cell density dependent, and therefore significant killing can be observed by lowering the cell density as well as the level of the peroxide. As shown in figure 2, treatments of 5×10^5 *E. coli* cells/ml of minimal glucose medium with 150, 300 and 600 μM H_2O_2 results in about 85%, 75% and 70% survival, respectively. Addition of histidine decreases these values to 57%, 40% and 25%.

Secondly, but most importantly, histidine suppresses the intermediate zone of partial resistance and therefore a linear dose-response inactivation curve can be obtained under the latter experimental conditions (Figure 1). At 25 mM H_2O_2, the presence of the amino acid does not affect the toxicity of H_2O_2.

The effect of increasing concentrations of the amino acid on the cytotoxic

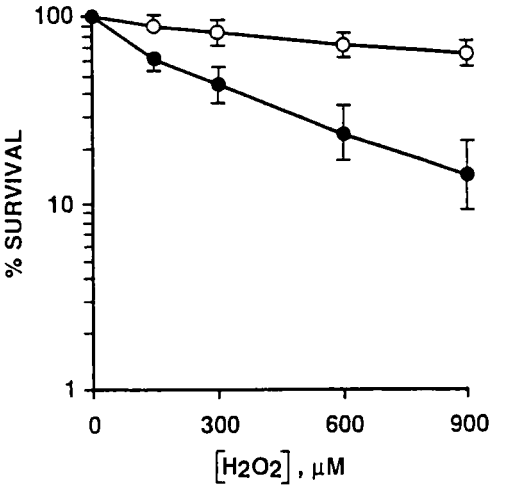

FIGURE 2 Survival of *E.coli* cells exposed for 30 minutes to increasing concentrations of hydrogen peroxide in minimal medium in the absence (○) or presence (●) of 870 μM histidine. Treatments were performed at a density of 5.10^5 cells/ml. Results represent the mean ± S.E.M. calculated from at least three separate experiments.

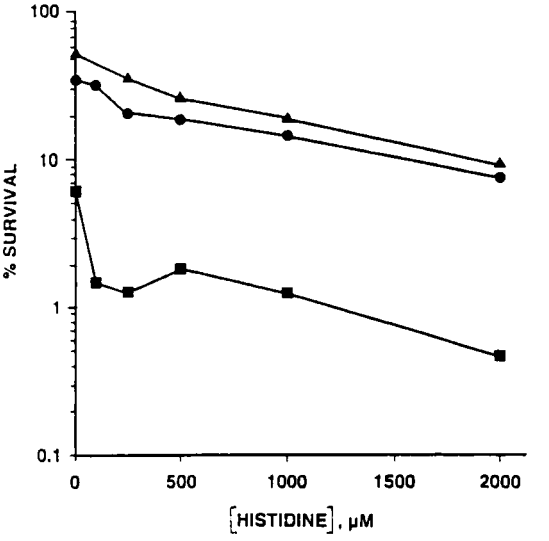

FIGURE 3 Survival of *E. coli* cells exposed for 15 minutes to 2.5 (▲), 10 (●) or 17.5 (■) mM in minimal medium as a function of increasing concentrations of histidine. Treatments were performed as described in the legend to Figure 1. Results are the mean of four or six separate experiments.

response elicited by 2.5 mM H_2O_2 (representative of mode one killing), 10 mM H_2O_2 (intermediate zone of partial resistance) and 17.5 mM H_2O_2 (mode two killing) was also investigated. Figure 3 indicates that the effect of the amino acid becomes apparent at concentrations as low as 100 μM and that the response increases up to 2 mM, at all oxidant concentrations.

H_2O_2-Mediated DNA Nicking

The ability of hydrogen peroxide to induce DNA single strand breaks can be assessed in an *in vitro* system by using covalently closed circular double-stranded supercoiled DNA (ccc DNA or form I).

Supercoiled ccc DNA (form I) was incubated in 150 mM NaCl, pH 7.0, in the absence or presence of 1 or 10 μM ferrous iron and increasing concentrations of hydrogen peroxide (45 min at 37°C). DNA nicking was assayed by gel electrophoresis. The results shown in Figures 4A and 5A indicate that the amount of form I DNA decreases at 10 μM H_2O_2 (mode one nicking response). Increasing the oxidant concentration above 10 μM up to 3 mM leads to a paradoxical reduction in the level of DNA fragmentation (intermediate zone of resistance). At concentrations higher than 3 mM a progressive decrease in the fraction of DNA remaining supercoiled was apparent (mode two nicking response). Thus, the plot of the DNA strand breaks induced by increasing concentrations of hydrogen peroxide in an *in vitro* system displays a pattern qualitatively similar to the dose-response inactivation curve of *E. coli* challenged with the oxidant. Similar results were obtained in other studies[7,8]. The data reported in Figure 4A were obtained in the presence of 1 μM Fe(II) whereas those illustrated in Figure 5A were obtained with 10 μM Fe(II). At the highest iron concentration, hydrogen peroxide more effectively induces the mode one nicking response, whereas the extent of mode two DNA nicking is similar at both iron concentrations.

The addition of histidine (0.3 or 1 mM) produces remarkable changes in the shapes of these dose-response curves (Figures 4A and 5A). Indeed, the amino acid, at both concentrations, diminishes the mode one DNA nicking elicited by hydrogen peroxide. Similar effects are observed in the presence of both 1 μM (Figure 4A) and 10 μM (Figure 5A) divalent iron.

The enhancing effect of histidine is apparent with 0.3 and 1 mM histidine at the lowest iron concentration (Figure 4A) whereas is detectable only at 1 mM histidine when treatments are performed in the presence of 10 μM Fe (II) (Figure 5A).

Concentrations of hydrogen peroxide higher than 1 mM, in the presence of 10 μM Fe (II), also result in the formation of a low level of form III DNA, thus suggesting the formation of either two single strand breaks in close vicinity in the opposite strands of the DNA or DNA double strand breaks (Figure 5B). In the presence of histidine (0.3 or 1 mM) this type of damage becomes more apparent at 1 μM Fe (II) (Figure 4B). Interestingly, the appearance of form III DNA is actually higher and the effect of the amino acid is concentration dependent at the lowest iron concentration (Figure 4B). At 10 μM iron (II) 1 mM, unlike 0.3 mM histidine increases the formation of form III (Figure 5B).

Results reported in Table I further illustrate the differential enhancing effect elicited by histidine on DNA nicking by H_2O_2 at 1 or 10 μM Fe(II). Indeed, whereas form I totally disappears when histidine (both at 0.3 and 1 mM) is added along with 10 mM H_2O_2 and 1 μM ferrous iron, a significant fraction of DNA remains supercoiled when divalent iron is 10 μM (41% and less than 5% DNA form I is observed in the presence of 0.3 and 1 mM histidine, respectively).

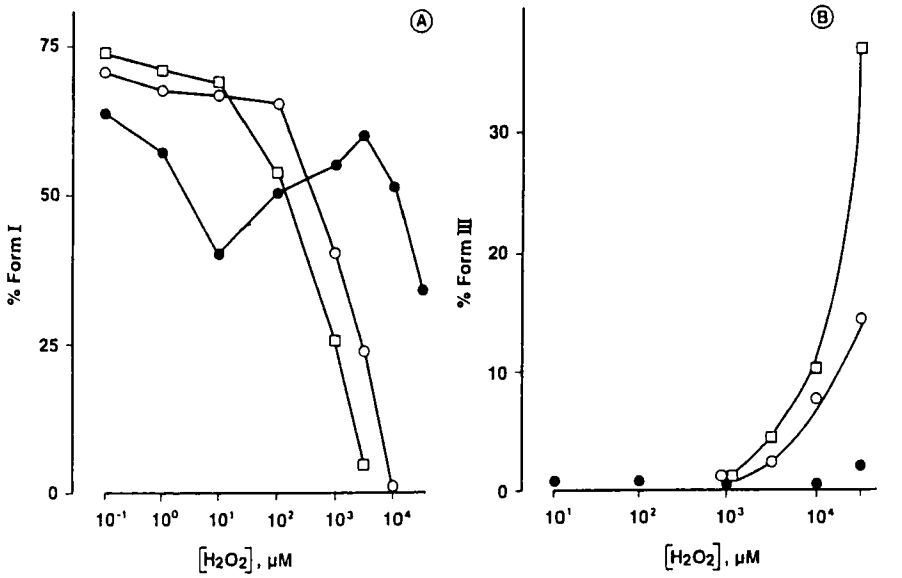

FIGURE 4 Effect of L-histidine on DNA nicking induced by increasing H_2O_2 concentrations (10^{-1} to 10^{4} μM) and 1 μM Fe II. pBR 322 (0.2 μg) was incubated for 45 minutes at 37°C in 150 mM NaCl, pH = 7 without (●) or with 0.3 mM (○) or 1 mM (□) L-histidine.
Panel A: The percentage of DNA remaining supercoiled (form I) is plotted against H_2O_2 concentrations.
Panel B: The percentage of linear molecules (form III) is plotted against H_2O_2 concentration.

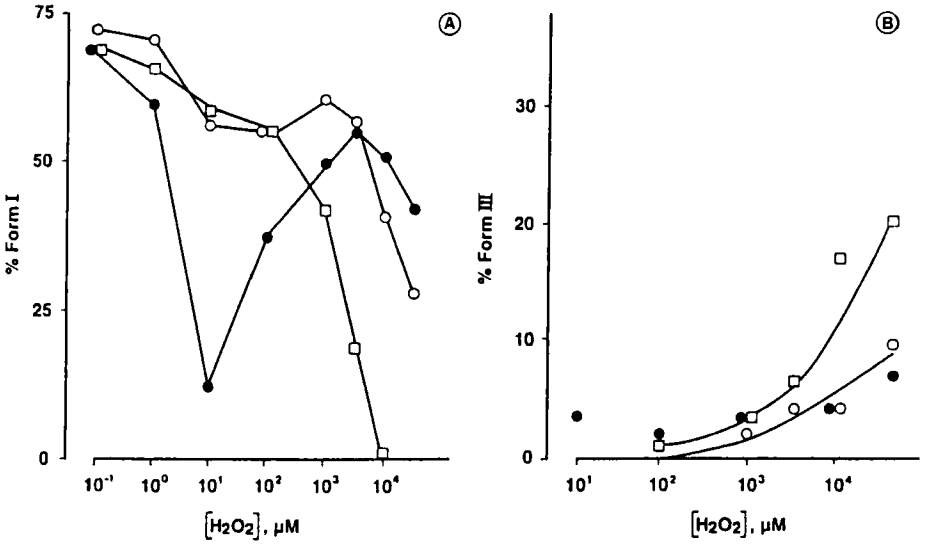

FIGURE 5 Effect of L-histidine on DNA nicking induced by increasing H_2O_2 concentrations (10^{-1} to 10^{4} μM) and 10 μM Fe II. The experimental conditions and the symbols are the same as in Figure 4. Panel A and panel B respectively represent the percentages of DNA remaining supercoiled (form I) and of linear DNA molecules (form III) as a function of H_2O_2 concentration.

TABLE 1
Effect of L-histidine on DNA nicking induced by hydrogen peroxide (10 mM) and various concentrations of ferrous iron. pBR 322 DNA (0.2 μg) was incubated in 150 mM NaCl (pH = 7) for 45 minutes at 37°C. In the absence of treatment, DNA preparations contained about 80% of form I.

[L-histidine]	DNA remaining supercoiled (%) Fe II (μM)		
	0	1	10
None	64.5	51.5	51.2
0.3 mM	64	0	41.2
1 mM	63.6	0	<5

DISCUSSION

Two modes of killing can be distinguished in the inactivation of *E. coli* challenged with hydrogen peroxide[4]. The first mode of killing occurs at low concentrations of H_2O_2 and is maximal at 2.5 mM whereas mode two killing becomes apparent at very high oxidant concentrations (>10 mM). The protective effect afforded by metal chelators dypiridyl, o-phenanthroline or desferrioxamine[6], and the great sensitivity of mutant strains defective in recombinational or excision repair[16] to low concentrations of H_2O_2, suggest that DNA is a relevant target for mode one killing and that DNA damage is mediated by transition metals. The dose-response curve of DNA damage by H_2O_2, as measured in *in vitro* systems[6,7], is also biphasic and shares common features with the inactivation curves observed in bacteria.

The results presented in this paper show that a substantial difference makes not superimposable the dose-response curves describing the H_2O_2 mediated inactivation of *E. coli* or the nicking of ccc DNA. Indeed, the maximum level of DNA nicking is obtained at 10 μM H_2O_2 i.e. two orders of magnitude below the concentration responsible for the induction of the maximum rate of bacterial killing. One explanation for this discrepancy could be that enzymatic and non enzymatic scavenging systems may lower the effective H_2O_2 concentration in close proximity to the *E. coli* DNA molecule. It should be noted, however, that exposure of *E. coli* mutants lacking catalase (Kat E Kat G mutants) does not result in a leftward but, rather, in a downward shift of the dose-response curve and thus the concentrations of H_2O_2 producing mode one killing remain unchanged with respect to the wild type strain[16].

In the same frame of analysis, it has to be recalled that mutants lacking DNA repair activity (exonuclease III-deficient) display similar behaviour when incubated with H_2O_2. Thus, the H_2O_2 concentration resulting in mode one killing appears to be independent of the bacterial defense mechanisms.

This paper also shows that histidine differentially affects the H_2O_2-mediated inactivation of *E. coli* cells and the nicking of ccc DNA. On the one hand histidine hinders mode one nicking and on the other hand it enhances mode one killing. This could arise from the concentrations of H_2O_2 respectively involved in the two systems. The protective effect of histidine on DNA nicking induced by low concentrations of the oxidant (<100 μM) in the presence of ferrous iron has been investigated in our laboratories[17]. ESR experiments suggest that histidine may compete with DMPO for the hydroxyl radical in keeping with its well known

scavenging properties[18,19] and with its protective effect against γ-irradiation which has been reported for histidine above 10 mM[20]. In addition, work by Imlay *et al.*[6] has suggested a role for a putative ferryl radical in the production of DNA damage by H_2O_2, and histidine may prevent the formation of DNA lesions by chelating the metal portion of this species. Our experiments utilizing low density bacterial cultures, however, demonstrate that at slightly higher H_2O_2 concentrations (150–300 μM) the amino acid, once again, is an enhancer of the cytotoxic response elicited by the oxidant. Consistently, previous results also indicate that histidine markedly augments the cytotoxicity as well as DNA single strand breakage caused by H_2O_2 in the range 25–100 μM in mammalian cells[13,21].

These results emphasize the existence of a remarkable difference between the effects produced by histidine on hydrogen peroxide-induced mode one killing of bacteria and mode one DNA nicking *in vitro*, and suggest that different reactive oxygen species mediate these two events.

Results in this paper also suggest that histidine affects the quality/quantity of oxyradicals being formed during challenge of bacteria with increasing concentrations of H_2O_2. This inference is the consequence of the observed effects of histidine on the so called intermediate zone of partial resistance, the inactivation curve losing the biphasic character in its presence. It should be noted, however, that alternative explanations could be considered. For example, histidine might somehow impair or abolish defensive mechanisms triggered by the oxidant in the range 5–10 mM. Because of the observed effects on mode one killing of *E. coli* cells and on DNA nicking, we favor the first of these two hypotheses.

H_2O_2-induced mode two killing does not seem to be significantly affected by histidine, unlike mode two DNA nicking which is significantly enhanced in the presence of the amino acid. The effect of histidine on DNA damage by H_2O_2 *in vitro* is concentration-dependent and also depends on the concentration of iron (II). Surprisingly, at 10 μM Fe (II) the enhancing effect is only observed at 1 mM histidine whereas no effect is detectable at 300 μM. On the other hand, the histidine-mediated enhancement of H_2O_2-induced DNA damage *in vitro* occurs at both concentrations, when it is in the presence of only 1 μM iron (II). Although these and the above results do not allow a straightforward conclusion, it can be speculated that the effect of the amino acid is highly dependent on the stoichiometry ferrous iron/histidine/hydrogen peroxide.

Finally, experimental results reported in this paper suggest that hydrogen peroxide and histidine cause DNA double strand breakage, a lesion that is not proficiently repaired by the cells and thus highly cytotoxic[22]. In addition, this type of lesion is not efficiently produced by the oxidant alone. We previously reported that DNA double strand breaks are produced by hydrogen peroxide in mammalian cells only when H_2O_2 is added along with histidine and that the mechanism whereby the amino acid enhances the toxicity elicited by the oxidant does indeed involve the formation of DNA double strand breakage[13,20]. Whether or not this mechanism is operative also in bacteria remains to be established.

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