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THE L-HISTIDINE-MEDIATED ENHANCEMENT OF HYDROGEN PEROXIDE-INDUCED DNA DOUBLE STRAND BREAKAGE AND CYTOTOXICITY DOES NOT INVOLVE METABOLIC PROCESSES

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Abstract—The cytotoxic response of Chinese hamster ovary (CHO) cells to challenge with hydrogen peroxide was highly dependent upon the temperature of exposure, being markedly higher at 37° than at 4°C. Increasing intracellular levels of L-histidine prior to challenge with hydrogen peroxide increased the toxicity elicited by the oxidant at both physiologic and ice-bath temperatures. The effect of the amino acid, however, was more pronounced under conditions at 4°C, as compared to 37°C. Indeed, at 4°C the oxidant was nontoxic at submillimolar levels and pre-exposure to L-histidine restored cytotoxicity to levels slightly higher than those observed after treatment at 37°C (in the micromolar range).

Pre-exposure to the amino acid increased the production of DNA double-strand breaks (DSBs) elicited by treatment with the oxidant both at 37° and 4°C. A remarkable correlation was found when the level of this lesion was plotted against the cytotoxic response observed using different concentrations of L-histidine or hydrogen peroxide, or treating the cells with the oxidant either at 37° or 4°C, thus suggesting the existence of a cause-effect relationship. The overlapping correlation curves obtained with cells challenged with the oxidant at 4° or 37°C also suggest that similar molecular mechanisms mediate the formation of DNA DSBs under both experimental conditions. Two lines of evidence provide experimental support for this inference: (1) the kinetics of repair of DNA DSBs generated at 37° or 4°C were virtually superimposable; this would suggest that the same repair pathway(s) is/are responsible for the removal of DNA DSBs generated at the two temperatures; and (2) the size distribution of double-stranded DNA fragments produced under the two treatment conditions, resulting in a similar cytotoxic response, was basically identical. This is indicative of remarkable similarities in the topology of chromosomal domains where DSBs are generated.

Overall, the results presented in this paper provide further experimental evidence supporting the notion that DNA DSBs are responsible for the L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity, and demonstrate that the mechanism whereby the amino acid enhances the ability of hydrogen peroxide to produce DNA double strand breakage and cell killing does not depend on cellular metabolism and/or energy-dependent reactions.

Key words: hydrogen peroxide; L-histidine; DNA double-strand breaks; cytotoxicity; pulsed field gel electrophoresis

Previous studies have demonstrated that L-histidine increases both the cyto- and genotoxic effects of hydrogen peroxide in cultured mammalian cells [1–11]. Among the genotoxic effects enhanced by the amino acid is the formation of SSBs† [4–7] as well as the production of micronuclei [1, 2, 10], sister-chromatid exchanges [1, 2, 10], and chromosomal aberrations [11]. Recent work from our laboratory has indicated that, in the presence of L-histidine, H_2O_2 also produces DNA DSBs [5–8], a potentially lethal lesion. The experimental evidence collected indicates that the intracellular fraction of the amino acid was responsible for the enhanced formation of DNA DSBs and cytotoxicity and that these two events were always associated, suggesting a cause-effect relationship [6–8]. In marked contrast, the extracellular fraction of the amino acid seemed to mediate the increased formation of DNA SSBs, an event that appeared unrelated to cytotoxicity [6–8].

The mechanism of the L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity is still unsolved. Other laboratories have suggested a number of hypotheses, although none fully explains the molecular basis of such effects (see Discussion section). In a previous study [5], we investigated the effect of L-histidine on hydrogen peroxide-induced cytotoxicity at 4°C using a co-incubation protocol. We found that although the level of DNA SSB-induction was significantly augmented, no effect was produced in terms of increase in the extent of DNA double-strand breakage and toxicity. These results, once again, emphasize a dissociation between SSBs and cytotoxicity and, further, suggest an association between the formation of DNA DSBs and the increased cytotoxic response. It is important to point out, however, that at 4°C the amino acid cannot be taken up by the cells. Therefore, it is not surprising that the latter parameters were not influenced under these experimental conditions because, as mentioned above, the intracellular fraction of the amino acid is responsible for the L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity and DNA DSB formation [6–8].

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† Abbreviations: SSB, DNA single-strand breaks; DSB, DNA double-strand breaks; CHO, Chinese hamster ovary; PFGE, pulsed field gel electrophoresis; CHEF, contour clamped homogenous electric field.

The question, thus, arises as to whether L-histidine, once it has been taken up by the cells, can increase the ability of hydrogen peroxide to generate DNA double-strand breakage and induce cytotoxicity under conditions at ice-temperature.

The reason for studying the effect of L-histidine in cells injured with the oxidant at 4°C is related to the fact that this temperature is not permissive for enzyme and/or energy-dependent processes. Comparing the effects of the oxidant at the two temperatures in cells preloaded with the amino acid can, therefore, help us to understand whether the enhancing effects of the amino acid require active cellular metabolism. The use of this experimental system is validated by previous investigations [12–16].

MATERIALS AND METHODS

Cell culture and treatments

CHO (strain AA8) cells were grown in monolayer culture in McCoy's 5A medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Seralab, Sussex, U.K.), penicillin (50 units/mL), and streptomycin (50 μg/mL), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) gassed with an atmosphere of 95% air-5% CO_2.

Stock solutions of hydrogen peroxide and L-histidine were freshly prepared in Saline A. All treatments with these agents were carried out in Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose). Cells were first incubated with L-histidine at 37°C for 20 min and then treated with hydrogen peroxide for 30 min at either 37° or 4°C. The cells were washed at each change of solution with prewarmed or prechilled Saline A, depending on the protocol rationale. These solution changes were performed very gently to minimize detachment of cells from the culture dish.

Because the cytotoxicity of hydrogen peroxide toward CHO cells is dependent on cell density [12], a constant cell density was used at the hydrogen peroxide treatment stage. This was achieved by preplating 2.5×10^5 cells into 60-mm tissue culture dishes and incubating them for 14 hr at 37°C, during which time their number increased to between 4 and 5×10^5. The treatment volume was also kept constant at 2 mL. After treatment, the cells were washed twice with ice-cold Saline A and harvested by exposure to 4 mL of 1% trypsin for 5 min on ice. Trypsin activity was terminated by the addition of 2 mL of ice-cold complete growth medium and the cells were transferred to a test tube. The number of cells was determined using a Model ZM Coulter counter.

Cytotoxicity assays

Cytotoxicity was determined by conventional clonogenic survival assays. After treatment, appropriate dilutions of cells (selected to give approximately 100 viable cells per dish) were plated in triplicate into 60-mm tissue culture dishes and incubated at 37°C for 8 days. The resulting colonies were stained with 5% gentian violet-ethanol and counted visually. Surviving cells were defined as those producing colonies of 50 cells or more. The surviving fraction was calculated as the ratio of the plating efficiencies of hydrogen peroxide-treated and untreated control cells. L-histidine displayed no cytotoxicity towards these CHO cells.

Measurement of DSBs by pulsed field gel electrophoresis (PFGE)

Cells were labelled overnight with 0.05 μCi/mL [methyl-^{14}C]-thymidine (55.60 mCi/mmol) and incubated for a further 6 hr in a medium containing unlabelled thymidine (1 μg/mL).

The cells were treated with H_2O_2 and immediately subjected to PFGE as described by Blocher *et al.* [17]. To prepare agarose plugs, the cells were sedimented at 1000 rpm for 5 min at 4°C and the pellet resuspended in phosphate-buffered saline (PBS) containing 5 mM EDTA, pH 8.3. This procedure was repeated 3 times, and the final suspension in PBS adjusted to give a density of 3.64×10^6 cells/ml. This suspension (300 μL) was mixed with 300 μL of melted agarose (low-melt agarose, Biorad, Richmond, CA, U.S.A.; 1% solution in PBS) in a 15-mL conical tube maintained at 45°C. The cell-agarose mixture was vortexed, transferred to a gel plug former on ice, and refrigerated for 15 min. The plugs (4 per experimental point) were removed and incubated in 1 mL of ESP (0.5 M EDTA, 1% sarkosyl, 1 mg/mL proteinase K, pH 9) for 1–2 hr at 4°C and then at 45°C for 20 hr. The plugs were washed 5 times for 1 hr in sterile 0.5 × TE buffer (a 1:1 v/v dilution of TE buffer: 10 mM Tris HCl, 1 mM Na_2EDTA, pH 7.5) and stored at 4°C in 0.5 M EDTA (pH 8).

The DNA in the plugs was separated by PFGE in a 0.5% w/v agarose gel (chromosomal grade, Biorad, Richmond, CA, U.S.A.) in 0.5 × TBE buffer (a 1:1 v/v dilution of TBE buffer: 89 mM Tris base, 69 mM sodium borate, 2 mM Na_2EDTA, pH 7.2). The gel was run for 20 hr on a CHEF-DR II system (Bio-Rad, Richmond, CA, U.S.A.) operating at 1.21 V/cm with a switch time of 75 min. The buffer (0.5 × TBE) was maintained at 25°C. The gel was stained with ethidium bromide, viewed under a UV transilluminator, and photographed. The distribution of radioactivity in the gel was determined by cutting portions of the gel containing the DNA (well and lane). These portions were then melted in glass scintillation vials in the presence of 50 μL of 1 N HCl and processed for liquid scintillation counting.

The CHEF assay was also used to resolve DNA over a broad range of DNA sizes. For this purpose, we adopted an experimental protocol that we had recently developed [18] to permit the simultaneous resolution of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* chromosomes (ranging 5.7 to 0.2 Mb) in 68 hr with a sensitivity comparable to that of the DSB assay developed by Blocher *et al.* [17].

Briefly, these gels were cast using 0.8% w/v agarose in 0.5 × TBE buffer and run with a three-block assay. The switch time was 75 min for 15 hr at 25°C, with a field strength of 1.46 V/cm during the first block; in the second block, the temperature was decreased to 14°C and the switch time was 30 min for 48 hr using a field strength of 2.2 V/cm; in the third block, the switch time was linearly ramped from 5 to 110 sec for 6 hr and the field strength increased to 6 V/cm. The reorientation angle was kept at 120°.

This assay allows a resolution similar to that obtained by Elia and Nichols [19] using a 48-hr PACE technology assay.

Measurement of DSBs produced in lysed-cell DNA by the neutral elution assay

Cells (labelled with [methyl-^{14}C]thymidine as described above) were removed from the dishes, gently

loaded (5×10^5 cells/barrel) onto 25 mm, 2 μm pore polycarbonate filters and rinsed twice with 10 mL of ice-cold Saline A. The cells were then lysed with 5 mL of 2% sodium dodecyl sulfate, 0.025 M EDTA (tetrasodium salt), pH 10.1 and the lysates incubated for a further 30 min in the same solution containing 1 mg/mL proteinase K. The filters were finally rinsed with 10 mL of Saline A and then challenged with hydrogen peroxide/L-histidine. DNA DSBs were assayed as described [20]. The DNA was eluted in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulfate (pH 9.6), at a flow rate of ca. 0.04 mL/min.

Fractions of approximately 4.5 mL were collected and counted in 7 mL of Lumagel containing 0.7% glacial acetic acid. DNA remaining on the filters was recovered by heating for 1 hr at 60°C in 0.4 mL of 1N HCl, followed by the addition of 0.4 N NaOH (2.5 mL), and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 mL of 0.4 N NaOH. This solution was processed for scintillation counting as described above.

RESULTS

As outlined in the beginning, previous results obtained in our laboratory have provided circumstantial evidence suggesting that the formation of DNA DSBs was responsible for the L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity [5–8]. In this study, we address the question of whether temperature-dependent mechanisms mediate these events. Indeed, the effect of L-histidine on the cytotoxic response elicited by hydrogen peroxide at physiologic temperature could either reflect the exclusive action of chemical–free radical processes or involve biochemical/enzymatic processes. The latter mechanism, unlike the former, should be operative only at 37°C; therefore, a comparison of the effects of L-histidine in cells oxidatively injured at the 2 temperatures may prove fruitful. This experimental approach has been used successfully in previous investigations [12–16].

Effect of L-histidine on the cytotoxic response of CHO cells challenged with hydrogen peroxide at 37° or 4°C

To investigate and compare the effects of L-histidine in cells challenged with hydrogen peroxide at physiologic temperature or at 4°C, we adopted an experimental protocol involving a 20-min pre-exposure (37°) to the amino acid (1 mM) and, following accurate rinsing of the cultures, treatment with increasing concentrations of the oxidant for 30 min in either prewarmed or prechilled Saline A. This approach was selected on the basis of our previous findings indicating that, under these conditions, the amino acid is not toxic, is readily taken up by the cells, and only a very minor proportion is released during the subsequent 30 min of challenge with the oxidant [7]. In addition, the degree of enhancement of cell killing and DNA double-strand breakage caused by this pretreatment was similar to that observed following concomitant exposure to the oxidant and the amino acid, possibly reflecting the short time required for both the amino acid and the oxidant to produce maximal effects [7]. Results shown in Fig. 1A indicate that the toxicity of hydrogen peroxide alone is highly dependent on the temperature of exposure because the oxidant at 4°C resulted in essentially no cell kill at the same concentrations used for treatments at 37°C (inset). To produce a significant level of cell killing, very high levels of H_2O_2 had to be used and, from the resulting dose-survival curve (Fig. 1A), an IC_{50} value of approximately 1.9 mM could be calculated (Table 1). In contrast, treatment with hydrogen peroxide at physiological temperature produces a remarkable cell kill, reducing the surviving cell fraction to approximately 0.5 at a concentration of 75 μM (inset and Table I). L-Histidine increased the toxicity elicited by the oxidant at both temperatures, although its effect appeared remarkably more pronounced in cells challenged at 4°C (compare Fig. 1A and inset).

The relative cytotoxic activity obtained under the 4 treatment conditions is shown in Table I. At 4°C, H_2O_2 was approximately 25 times more effective in killing L-histidine-preloaded than non-preloaded cells. In agreement with our previous results [5–8], H_2O_2 was only twice as effective in killing preloaded cells challenged at 37°C.

We also investigated the L-histidine concentration-dependence for the enhanced cytotoxic response of oxidatively injured cells. As shown in Fig. 1B, pretreatment with increasing levels of the amino acid resulted in a progressive increase in the toxicity elicited by the oxidant (300 μM) at 4°C, this effect being apparent at concentrations as low as 10 μM and linear over a range of 10–300 μM. Similar experiments were also performed in cells oxidatively injured (50 μM) at 37°C with results (inset) demonstrating that the effect of the amino acid was once again concentration-dependent, although its onset was shifted at slightly higher concentrations (30 μM).

Effect of L-histidine on the level of DNA DSBs in CHO cells challenged with increasing concentrations of hydrogen peroxide at 4° or 37°C

To determine whether the enhanced cytotoxicity of hydrogen peroxide at 4°C in L-histidine preloaded cells was accompanied by a more effective induction of DNA double-strand breakage, as was previously observed in cells challenged with the oxidant at physiologic temperature [8], we assessed the level of this lesion in cells treated under conditions similar to those utilized in toxicity studies. For this purpose, we used the CHEF assay, a technique recently developed to measure DNA DSBs [17], although its use has so far been limited primarily to investigate the effect of ionizing radiation [21–23]. We recently adopted this assay for measuring DNA DSBs produced by concomitant exposure to hydrogen peroxide and L-histidine at physiologic temperature; its sensitivity allowed DNA DSBs to be measured over the same oxidant concentration range resulting in cytotoxicity [8]. Results illustrated in Fig. 2A indicate that no DNA DSBs are produced when cells are treated at 4°C with concentrations of hydrogen peroxide up to 750 μM. In L-histidine (1 mM) preloaded cells, however, the oxidant induces a significant level of this lesion at concentrations as low as 100 μM, and this response linearly increases at progressively higher oxidant concentrations. It is important to note that these levels of hydrogen peroxide are toxicologically relevant because the dose-response curve for DNA DSBs was obtained at survival-curve range concentrations of the oxidant (100–750 μM). Very high concentrations of hydrogen peroxide alone (in the millimolar range) can induce DNA DSBs at ice-bath temperature, although this lesion can be detected only in a

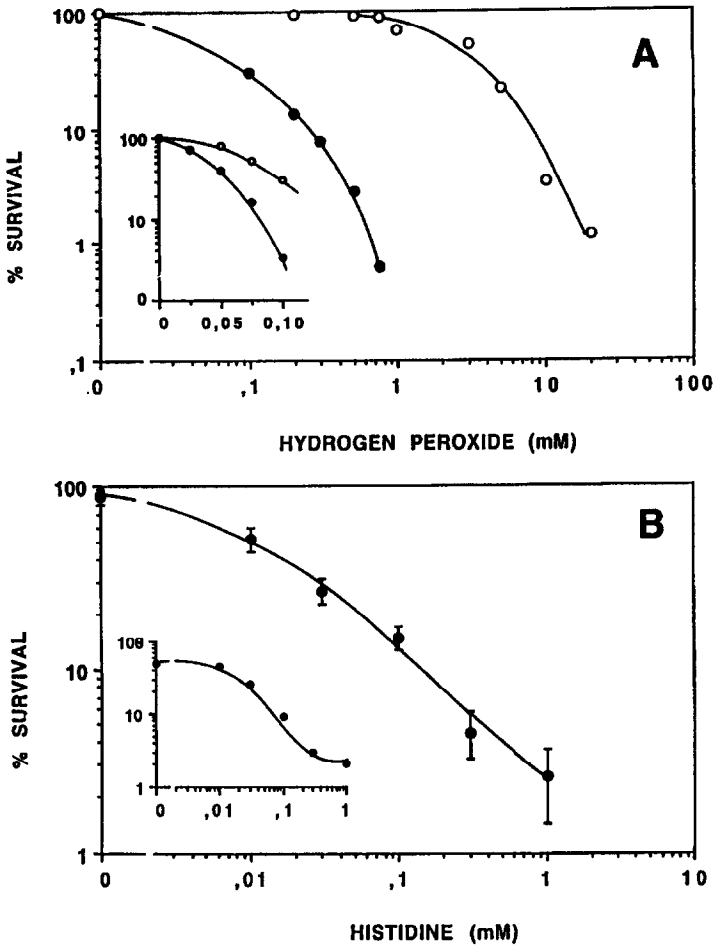


Fig. 1. Effect of pretreatment with L-histidine on the cytotoxic response of CHO cells to hydrogen peroxide. (A) Cells were incubated with 0 μM (○) or 1 mM (●) L-histidine for 20 min at 37°C and then challenged for 30 min with increasing concentrations of hydrogen peroxide. Treatments with the oxidant were performed either at physiologic temperature (inset) or at 4°C (main graph). Cytotoxicity was assessed by using a clonogenic assay, as detailed in the Methods section. Results are the mean ± SEM calculated from 3–4 separate experiments, each performed in duplicate. (B) Cells were incubated with increasing concentrations of L-histidine for 20 min at 37°C and then challenged with 300 μM or 50 μM H_2O_2 for a further 30 min at 4°C (main graph) or 37°C (inset), respectively. Cytotoxicity was assayed as described in (A). Results are the mean ± SEM calculated from 3–4 separate experiments, each performed in duplicate.

2–5 mM H_2O_2 concentration range, disappearing thereafter at higher levels (not shown). Fig. 2A (inset) shows the results obtained with the CHEF assay in cells preexposed to 1 mM L-histidine and then challenged with hydrogen peroxide at physiologic temperature. As we previously reported [8], the amino acid enhanced the formation of DNA DSBs that were produced at concentrations resulting in the first two logs of killing.

Table 1. Cytotoxic response of CHO cells exposed to H_2O_2 under various experimental conditions

Treatment[a]		
L-histidine	H_2O_2	IC_{50}
–	37°C	75 μM
+	37°C	39 μM
–	4°C	1.9 mM
+	4°C	78 μM

[a] Cells were incubated for 20 min at 37°C in a Saline A containing 0 (–) or 1 mM (+) L-histidine prior to challenge with increasing concentrations of the oxidant at either 37° or 4°C. Cytotoxicity was measured as detailed in the Methods section.

We also investigated the L-histidine concentration dependence for the enhanced formation of DNA DSBs in CHO cells oxidatively injured at 4°C. Results illustrated in Fig. 2B indicate that, in analogy to the results obtained in toxicity studies, pretreatment with increasing levels of the amino acid resulted in a progressive increase in the level of DNA DSBs generated by the oxidant (300 μM) at 4°C. The enhancing effect of L-histidine became apparent at levels as low as 10 μM and increased over a concentration range of 10–300 μM. Similar results were obtained in cells treated with the oxidant (100 μM) at 37°C. Indeed, the effect of the amino acid was, once again, concentration-dependent although its onset was shifted at slightly higher concentrations of the amino acid (30 μM) (inset).

Finally, we investigated whether or not the amino acid was capable of promoting the formation of DNA DSBs in partially purified DNA. This experimental approach

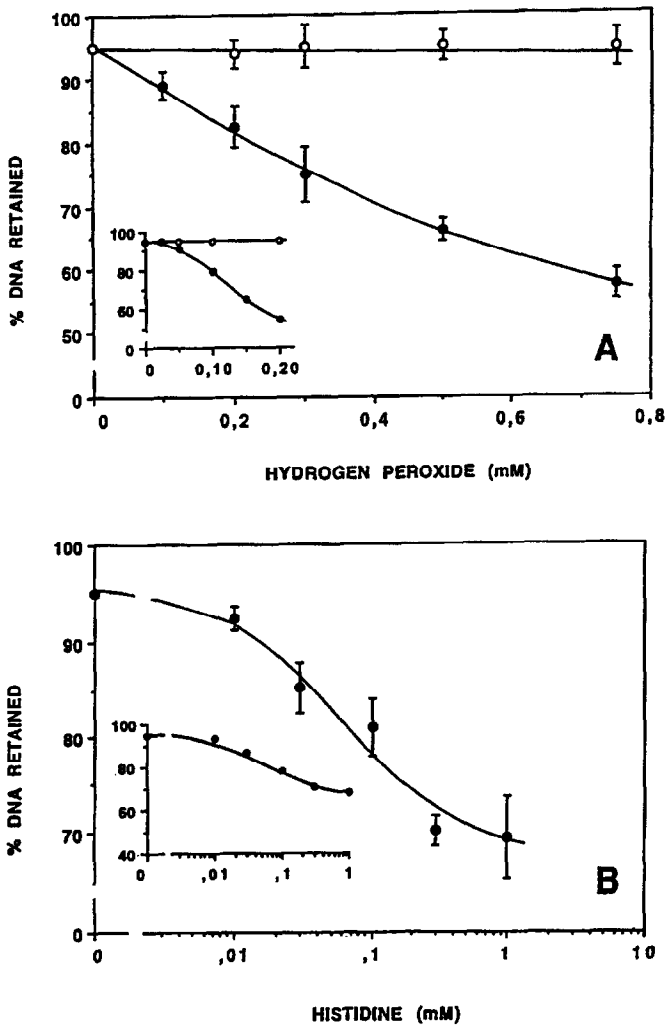


Fig. 2. Effect of pretreatment with L-histidine on the yield of DNA DSBs induced by hydrogen peroxide. Cells were treated as detailed in the legend to Fig. 1A and B and then analyzed for DNA double-strand breakage by the CHEF assay. Results are the mean ± SEM calculated from 3 separate experiments, each performed in duplicate.

was adopted to prove that cellular metabolism was not necessary for DNA DSB-induction by the cocktail hydrogen peroxide/L-histidine. For this purpose, CHO cells were lysed onto 2-μm pore size polycarbonate filters and, following protein digestion and accurate rinsing, hydrogen peroxide (200 μM) was added along with 300 μM L-histidine. Treatments (30 min at room temperature) were followed by neutral elution analysis (see Methods). Experimental results have demonstrated DNA DSBs are also generated under these conditions (Fig. 3).

Correlation between the formation of DNA DSBs and cytotoxicity

Figure 4 shows a correlation analysis curve obtained by plotting the level of DNA DSBs and cytotoxicity resulting from the various treatment protocols utilized in this study. It is of interest to observe that all the experimental points fit in one single curve ($r = 0.931$), irrespective of whether the above molecular and cellular events were varied by changing the L-histidine or hydrogen peroxide concentrations or even the temperature of exposure to the oxidant from physiologic to the metabolic nonpermissive 4°C temperature.

Removal of DNA DSBs and resolution of fragment size

We first compared the rate of rejoining of DNA DSBs produced in L-histidine-preloaded cells challenged with hydrogen peroxide at either 37° or 4°C. In these experiments, cells were pre-exposed to 1 mM L-histidine for 20 min at 37°C and, following washing, were challenged for 30 min with either 100 or 300 μM hydrogen peroxide, depending on whether the treatment with the oxidant was performed at 37° or 4°C. Under these experimental conditions, similar levels of initial DNA double-strand breakage, as measured by the CHEF assay, could be produced (see data shown in Fig. 2A and inset). After treatment, cells were allowed to repair in fresh prewarmed medium for various time intervals and assayed for residual DNA damage. Results illustrated in Fig. 5 indicate that the kinetics describing the rate of removal of DNA DSBs generated under the 2 treatment conditions were virtually superimposable, with $T_{1/2s}$ values of approximately 50 min. These results suggest that the same repair pathway(s) is/are involved in the removal of DNA lesion generated at 37° or 4°C.

Lastly, we investigated the size distribution of double-

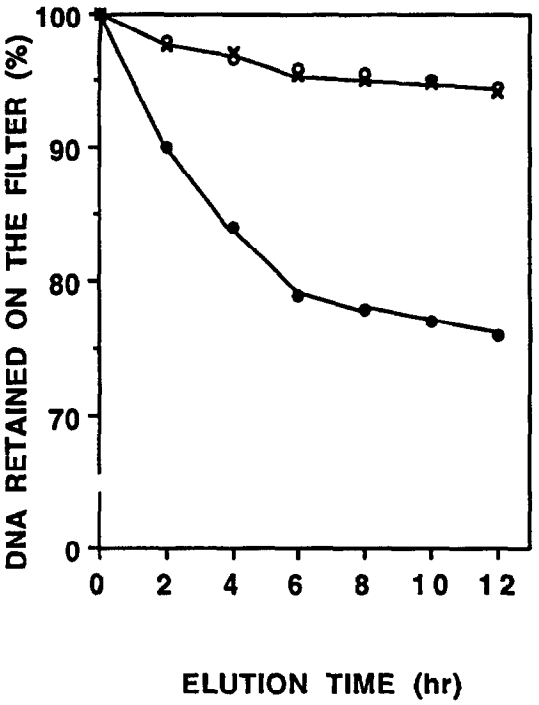


Fig. 3. Formation of DNA DSBs in cells treated postlysis with hydrogen peroxide/L-histidine. Cells were inoculated onto polycarbonate filters, lysed as detailed in the Methods section and exposed to proteinase K for 1 hr. Following treatment with 150 μM H_2O_2 alone (open circles) or associated with 1 mM L-histidine (closed circles), the DNA fragments were separated by neutral elution. The symbol (x) indicates the rate of elution of untreated double-stranded DNA. Results are from a representative experiment.

stranded DNA fragments produced under the 2 treatment conditions. For this purpose, we utilized a CHEF protocol differing from the one we adopted to measure DNA DSBs in some critical points. Although a detailed description is given in the Methods section, it is important to point out here that these techniques have a very similar sensitivity and a remarkably different resolution. As illustrated in Fig. 6 (photograph) the modified CHEF technique is capable of resolving a broad range of DNA sizes (from 0.2 Mb to approximately 6 Mb). Indeed, on a single run it was possible to separate very large DNA fragments, as demonstrated by the good resolution of *Schizosaccharomyces pombe* chromosomes (currently estimated to be 3.5, 4.6, and 5.7 Mb [19]) as well as smaller DNA fragments (indicated by the good separation of *Saccharomyces cerevisiae* chromosomes). In marked contrast, our conventional DNA DSB assay had a very poor ability to modulate the DNA size mobility across the gel because the DNA of both *S. pombe* and *S. cerevisiae* marker chromosomes appears as a single compressed band just below the well (not shown). To assay the size distribution of double-stranded DNA fragments produced by hydrogen peroxide (at either 37° or 4°C) in L-histidine-preloaded cells, we utilized concentrations of the oxidant resulting in similar cytotoxic responses (75 μM at 37°C and 250 μM at 4°C). Results illustrated in the gel (lanes 5–6 and 7–8) indicate that the pattern of DNA fragment distribution obtained under the 2 treatment conditions was basically identical; this is indicative of remarkable similarities in the topology of chromosomal domains where DSBs are generated. This inference is better illustrated in the bar graph displayed below the photograph. Indeed, the distribution of radiolabelled DNA released from the wells according to the fragment size indicated in the gel appears absolutely identical in L-histidine preloaded cells treated with the oxidant at either 37° or 4°C. The bar graph also shows that most of the DNA fragments generated by the cocktail hydrogen peroxide/L-histidine are greater than 5.7 Mb (approximately 40%) or have a size ranging from 3.5 to 5.7 Mb (approximately 40%).

DISCUSSION

Previous results from our laboratory have demonstrated that the L-histidine-mediated enhancement of the toxic response of cultured CHO cells to hydrogen peroxide at physiologic temperature is paralleled by the appearance of DNA DSBs [5–8]. The increased toxicity and formation of DNA DSBs was dependent on the intracellular fraction of the amino acid because both effects were apparent and similar under conditions of coexposure to L-histidine and hydrogen peroxide or preexposure to L-histidine followed by challenge with the oxidant [7], whereas no effect was produced by concomitant treatment with the 2 agents in the presence of an excess of L-glutamine, virtually abolishing the L-histidine uptake [6].

The present data extend these observations to show that pretreatment with L-histidine under metabolic permissive conditions—allowing the internalization of the amino acid—enhances the formation of DNA DSBs as well as the killing of cells subsequently treated with hydrogen peroxide, regardless of whether the latter treatment is administered at 37°C or 4°C. Thus, the ability of L-histidine to potentiate DSB-induction and cytotoxicity

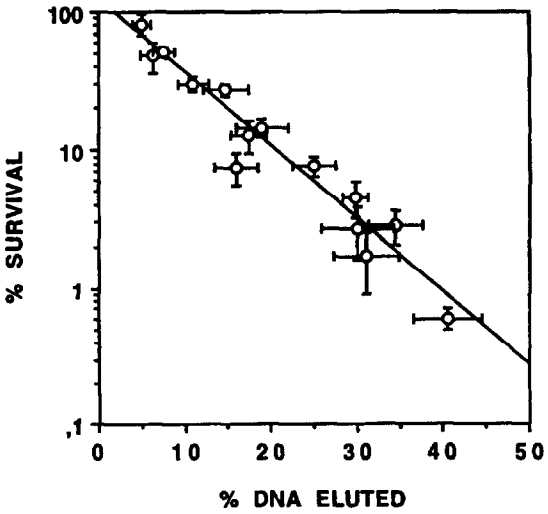


Fig. 4. Correlation analysis for DNA DSB and cytotoxicity in cells treated with hydrogen peroxide and L-histidine under different experimental conditions. The effect of hydrogen peroxide on cell-plating efficiency and DNA double-strand breakage was determined as detailed in the Methods section. Each point represents the results obtained using the treatment protocols detailed in the legend to Fig. 1.

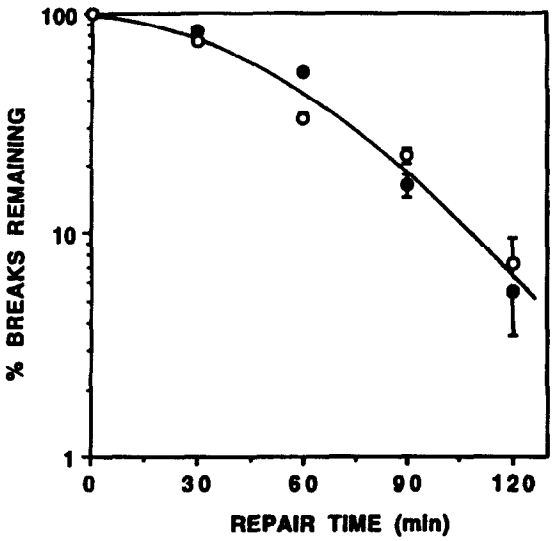


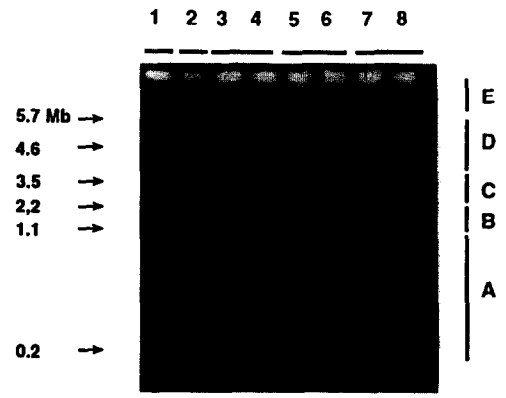
Fig. 5. Kinetics of removal of DNA double-strand breaks produced by hydrogen peroxide in the presence of L-histidine. Following treatment for 20 min with 1 mM L-histidine at 37°C, cells were then challenged for 30 min with hydrogen peroxide at either 37° or 4°C and then allowed to repair at 37°C for various time intervals. The CHEF assay was performed on these cells, as detailed in the Methods section. The initial damage was induced with 300 μM or 100 μM H_2O_2, depending on whether treatment was performed at 4° or 37°C. Open and closed circles represent residual damage at various time intervals following challenge with the oxidant at physiologic or ice-bath temperatures, respectively. Data points represent the percent ratio between the DNA eluted at the indicated repair times and the DNA eluted immediately after treatment (initial damage). Values represent the mean ± SEM of 3 to 5 separate experiments, each performed in duplicate.

was independent of the temperature of hydrogen peroxide exposure.

Evidence for the lack of involvement of metabolic processes in the L-histidine-mediated enhancement of DNA DSB induction in CHO cells treated with hydrogen peroxide is also derived from experimental work indicating that, in the presence of the amino acid, hydrogen peroxide effectively generated DNA DSBs in postlysed DNA samples.

The enhancing effect of L-histidine in cells challenged at 4°C appeared to trigger an all-or-nothing response because, in its presence, both the formation of DNA DSBs and the cytotoxic response were remarkably similar to those observed in cells treated at 37°C whereas, in its absence, the oxidant did not produce DNA DSBs and was virtually nontoxic, at least at concentrations in the micromolar range.

The striking correlation between the formation of DNA DSBs and enhanced cytotoxicity, observed under a variety of experimental conditions (*e.g.*, different concentrations of either hydrogen peroxide or L-histidine at different temperatures of exposure to the oxidant), strongly suggests that the mechanism whereby L-histidine mediates the increased cytotoxicity in oxidatively injured cells involves the formation of DNA DSBs, and that this effect does not require cellular metabolism or, more generally, energy-requiring reactions. This would also imply that DNA DSBs generated at the 2 temperatures are qualitatively identical. Three lines of evidence support this inference. Indeed, DNA DSBs produced by hydrogen peroxide at 4° or 37°C in L-histidine preloaded cells are: (1) equally cytotoxic; (2) removed with similar repair kinetics; and (3) result in superimposable patterns of double-stranded DNA fragments. These observations strongly suggest remarkable similarities in the type of DNA lesions generated by hydrogen peroxide in L-histidine preloaded cells at the 2 temperatures. The mechanism(s) involved in these processes, however, remains



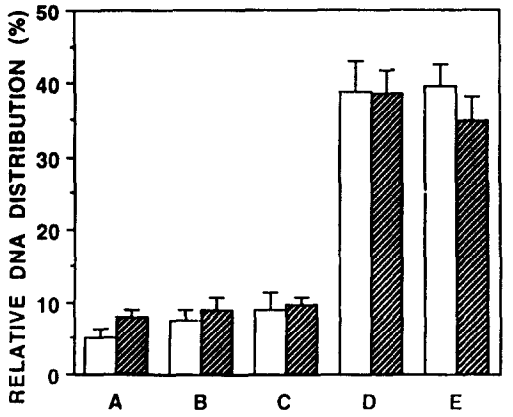


Fig. 6. Analysis of H_2O_2/L-histidine-induced DNA DSBs in CHO cells using the 3-block-high resolution CHEF assay. (A) Photograph of ethidium bromide-stained gel of DNA from untreated (lanes 3–4) or treated (lanes 5–8) CHO cells. Cells were treated for 20 min with 1 mM L-histidine at 37°C and then exposed for 30 min to either 75 μM H_2O_2 at 37°C (lanes 5–6) or 250 μM H_2O_2 at 4°C (lanes 7–8). The mobility of megabase DNA standards *S. pombe* and *S. cerevisiae* is shown in lanes 1 and 2, respectively. (B) Bar graph showing the distribution of radiolabelled DNA released from the wells according to the fragment size indicated in Fig. 1A. Open bars refer to lanes 5–6 and dotted bars to lanes 7–8. Each lane of the gel was cut into discrete areas identified by the letters A–E (right of the photograph) according to the migration pattern of the standards (A = DNA double stranded fragments ranging in size from 0.2 to 1.1 Mb; B = from 1.1 to 2.2 Mb; C from 2.2 to 3.5 Mb; D = 3.5 to 5.7 Mb; E = DNA fragments > 5.7 Mb). Results are expressed as the ratio between the radioactivity in each of the regions and the total radioactivity in the lane and represent the mean ± SEM of 3 separate experiments.

obscure and none thus far proposed is consistent with our results. For example, the fact that pre-exposure to L-histidine is as effective as co-exposure in enhancing the deleterious effects of H_2O_2 [7 and this paper] appears to rule out the possibility that L-histidine enhances the lethality of the oxidant via the formation of an adduct with the oxidant in the extracellular milieu [11]. It also seems unlikely that L-histidine exercises an effect on cellular NADPH, an event that would lead to a reduced function of the enzyme glutathione peroxidase [3]. Indeed, the effect of a treatment with the oxidant at 4°C should not be influenced by scavenging enzymes and, yet, we find that hydrogen peroxide is highly toxic in the presence of L-histidine at this temperature.

Our results also suggest that it is unlikely that the lack of H_2O_2 toxicity at 4°C is due to temperature-dependent inhibition of the reduction of trivalent iron to the divalent form because, in the presence of L-histidine, the oxidant recovered its cytotoxic potential. The possibility that the amino acid might switch the mechanism of cell killing normally operating at the physiologic temperature to some other mechanism may not be ruled out, although this would not explain why the enhancing effect elicited by L-histidine is lower when exposure to the oxidant is performed at 37° as compared to 4°C. The most likely explanation is, therefore, that there is a key step in oxidatively injured cells whereby temperature-dependent mechanisms play a pivotal role in the formation of the lethal lesion(s). Addition of L-histidine at physiologic temperature might promote this event, allowing the formation of the lethal lesion(s) irrespective of whether treatment with the oxidant is performed at 4° or 37°C. The result of the action of L-histidine would, then, be a slight potentiation of a mechanism already operative at metabolic permissive temperature and, therefore, a relatively small increase in toxicity in cells treated with hydrogen peroxide at 37° C and a tremendous enhancement in toxicity in cells challenged at 4°C.

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