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L-Glutamine prevents the L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity

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Abstract—Results presented in this study demonstrate that L-glutamine, a competitive inhibitor of L-histidine uptake, inhibits in a concentration-dependent fashion the L-histidine-mediated enhancement of H_2O_2-induced cytotoxicity. L-Glutamine also prevents the induction of DNA double strand breaks (DSB) but does not affect the enhancing effect of L-histidine on DNA single strand break induction by H_2O_2. Taken together, these data demonstrate that L-histidine, in order to allow the formation of DNA double strand breakage and increase the toxicity elicited by the oxidant, has to enter the cell. In addition, these results indicate that the enhancement of DNA single strand breakage is a consequence of the action of the amino acid at the extracellular level and/or outer surface of the plasma membrane and does not appear related to the mechanism whereby L-histidine increases the cytotoxic response to H_2O_2. The latter mechanism very likely involves the formation of DNA DSB.

A growing body of literature suggests that L-histidine is a powerful modifier of the cellular responses to oxidative stress [1–7]. It has been shown that, in the presence of this amino acid, hydrogen peroxide more effectively kills cells [1–3] and induces DNA single strand breaks (SSB*) [3], micronuclei [1, 2, 4], sister-chromatid exchanges [1, 2, 4] and chromosomal aberrations [5]. The mechanism whereby L-Histidine exerts its effects in oxidatively injured cells is, however, poorly understood. In a previous paper [3], we have reported that, in the presence of L-histidine, H_2O_2 produces DNA double strand breakage, a type of lesion which is not detectable even following cell exposure to exceedingly high concentrations of the oxidant alone. It was suggested that the production of this lesion may account for, or contribute to, the increased vulnerability of the cells treated with hydrogen peroxide in the presence of L-histidine.

In this study we report that the enhancing effect of L-histidine on the cellular killing induced by hydrogen peroxide, as well as the appearance of DNA double strand breaks (DSB), is dependent on the cellular uptake of the amino acid. In marked contrast, the increased level of DNA SSB does not appear related to intracellular L-histidine but, rather, to the amount of the amino acid which is present extracellularly and/or bound to the plasma membrane. The association that has been observed between the appearance of DNA DSB and the increased cytotoxic response suggests a cause–effect relationship. In contrast, the higher level of DNA SSB appears unrelated to the L-histidine-mediated increase in the cytotoxic response to hydrogen peroxide.

Materials and Methods

Cells. Chinese Hamster Ovary (CHO) cells were routinely grown in McCoy's 5a medium supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin in an atmosphere of 5% CO_2 in air, at 37°.

Cell growth inhibition studies. Cells were seeded at a density of about 5×10^5 cells/60 mm dish and, after 6 hr, were treated for 30 min with various concentrations of hydrogen peroxide in Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$ and 5 mM glucose), either in the absence or presence of 1 mM L-histidine and/or other amino acids. The monolayers were then rinsed twice with Saline A and incubated for 48 hr in a drug-free medium. Cell number was estimated after trypsinization with a Coulter Counter.

Alkaline elution assay. Cells were labelled for 24 hr with [^{14}C]thymidine (0.05 μCi/mL), seeded at a density of 5×10^5/60 mm dish and incubated for a further 6 hr in a medium containing unlabelled thymidine (1 μg/mL). Cells were then treated as detailed above, removed from the dishes by trypsinization (1% trypsin for 5 min at ice temperature) and analysed for DNA damage by the filter elution technique. This assay was carried out by a procedure virtually identical to that described by Kohn *et al.* [8] with minor modifications [9]. Briefly, 5×10^5 cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and rinsed twice with 10 mL of ice-cold Saline A containing 5 mM EDTA (disodium salt). Cells were then lysed with 5 mL of 2% SDS, 0.025 M EDTA (tetrasodium salt), pH 10.1. Lysates were rinsed with 7 mL of 0.02 M EDTA (tetrasodium salt) and the DNA was eluted in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% SDS (pH 12.1), 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° in 0.4 mL of 1 N HCl followed by the addition of 0.4 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.

Strand scission factors (SSFs) were calculated from the resulting elution profiles by determining the absolute log of the ratio of the percentage of DNA retained in the filters of the drug-treated sample to that retained for the untreated control sample (both after 8 hr of elution).

Neutral elution assay. The procedure is similar to that described above except that a pH 9.6 elution solution, which does not denature the DNA, was used [8].

L-Histidine transport. Cultures containing 5×10^5 cells/60 mm dish were washed twice with prewarmed Saline A and the transport was then initiated by addition of 1 mL of Saline A containing unlabelled (300 μM) and ^{3}H-labelled (2 μCi/mL) L-histidine (51.50 Ci/nmol) in the absence or presence of 20 mM L-glutamine. After incubation at 37° for various time intervals, the uptake was terminated by rinsing four times with ice-cold Saline A containing 50 mM unlabelled L-histidine. Monolayers were extracted with 2 mL of ice-cold 10% trichloroacetic acid (TCA) and radioactivity was determined by counting 1 mL of the TCA soluble fraction.

* Abbreviations: SSB, DNA single strand break; DSB, DNA double strand breaks; CHO, Chinese Hamster Ovary; TCA, trichloroacetic acid.

Results

L-Histidine is transported across the plasma membrane of rat hepatocytes by a Na-dependent system ("N") specific for L-histidine and L-glutamine [10]. The latter amino acid, therefore, should competitively inhibit the uptake of L-histidine. Indeed, as shown in Fig. 1, we find that 20 mM L-glutamine prevents the uptake of L-histidine (300 μM) in CHO cells. This effect was concentration-dependent (not shown). We have therefore tested the effect of L-glutamine (1 or 10 mM) on the cytotoxicity of hydrogen peroxide alone (25 μM) or associated to L-histidine (1 mM). Treatments were performed in a glucose-containing saline. Data illustrated in Fig. 2 indicate that L-glutamine reduces the L-histidine-mediated enhancement of H_2O_2-induced cytotoxicity in a concentration-dependent fashion. At the highest concentration, L-glutamine abolishes the effect of L-histidine. It should be noted also that L-glutamine does not itself affect the toxicity of hydrogen peroxide (Fig. 2). L-Asparagine was less efficient than L-glutamine in inhibiting the effects of L-histidine (not shown). Indeed, as previously demonstrated [10] system "N" transports L-glutamine faster than L-histidine and far faster than L-asparagine.

Taken together, these results strongly suggest that L-histidine has to enter the cell in order to increase the cytotoxicity of hydrogen peroxide. It is important to note that, in these experiments, cytotoxicity was measured as inhibition of cell growth and that similar results were obtained when cytotoxicity was measured by using cloning efficiency assays (not shown).

We next attempt to assess whether the internalization of the amino acid was also necessary to increase the level of DNA SSB and to result in the appearance of DNA DSB. Cells were treated for 30 min with increasing concentrations of H_2O_2 in the presence or absence of L-histidine (1 mM) and then analysed by alkaline or neutral elution assays in order to detect the formation of DNA SSB and DSB, respectively. The results reported in Fig. 3 confirm our previous data [3] indicating that the amino acid significantly increased the level of DNA SSB (A) and resulted in the appearance of DNA DSB (B) in oxidatively injured cells.

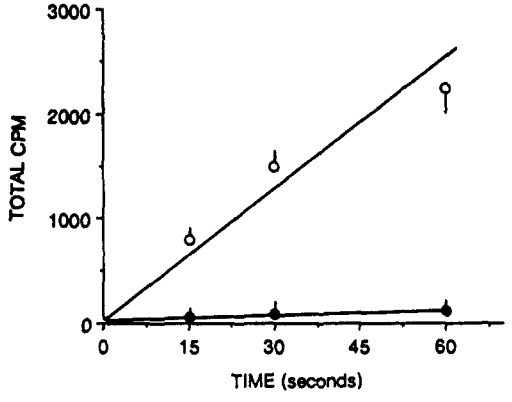

Fig. 1. Effect of L-glutamine on L-histidine transport. CHO cells were exposed to a prewarmed (37°) Saline A solution containing 2 μCi/mL [^{3}H]L-histidine/300 μM cold L-histidine in the absence (○) or presence (●) of 20 mM L-glutamine. At specific time intervals, cells were rinsed four times with ice-cold saline A containing 50 mM L-histidine and were then extracted with 1 mL of 10% TCA. The radioactivity was determined in the TCA-soluble fraction. Data represent the cpm/dish associated to the cells. Values are the means ± SEM calculated from three separate experiments, each performed in duplicate.

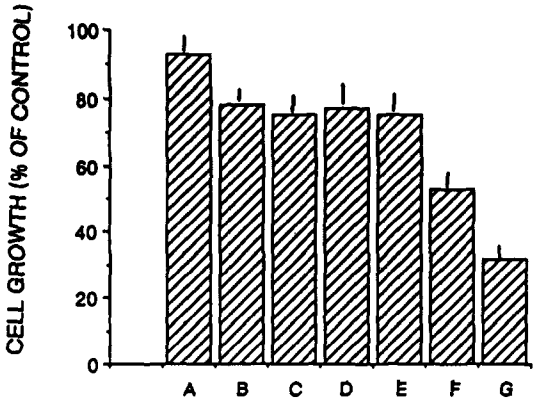

Fig. 2. Effect of L-glutamine on the L-histidine-mediated enhancement of H_2O_2-induced cytotoxicity. Cells were treated for 30 min as detailed below and were then allowed to grow for 48 hr. The cell number was then assessed with a Coulter Counter. Each point represents the mean ± SEM of three separate experiment, each performed in duplicate. Key: (A) 10 mM L-glutamine; (B) 10 mM L-glutamine + 25 μM H_2O_2; (C) 1 mM L-glutamine + 25 μM H_2O_2; (D) 25 μM H_2O_2; (E) 10 mM L-glutamine + 25 μM H_2O_2 + 1 mM L-histidine; (F) 1 mM L-glutamine + 25 μM H_2O_2 + 1 mM L-histidine; (G) 25 μM H_2O_2 + 1 mM L-histidine.

Interestingly, L-glutamine (10 mM) did not affect the L-histidine (1 mM)-mediated enhancement of H_2O_2-induced DNA SSB (Fig. 3A) but abolished the production of DNA DSB (Fig. 3B). Thus, L-histidine has to enter the cell in order to allow the production of DNA DSB in oxidatively injured cells, whereas it may be suggested that the intracellular L-histidine is not an efficient sensitizer for induction of DNA SSB, as it is the same amino acid as present in the extracellular compartment during oxidative stress.

Discussion

The results presented in this paper demonstrate that, in order to increase the cytotoxicity of hydrogen peroxide, L-histidine has to enter the cell. Indeed, this enhancing effect was abolished by competitive inhibitors of L-histidine uptake (L-glutamine and L-asparagine). Intracellular L-histidine was also responsible for the appearance of DNA DSB since addition of L-glutamine eliminated the production of this lesion. These observations are in keeping with our previous findings indicating that treatment of the cells at 4° with both L-histidine (under these experimental conditions the amino does not enter the cell) and hydrogen peroxide neither increases the cytotoxicity of the oxidant nor results in the appearance of DNA DSB, although increasing the level of DNA SSB [3]. The strict association that has been observed between the appearance of DNA DSB and the increased cytotoxic response strongly suggests a cause–effect relationship.

DNA SSB were differently regulated by L-histidine, as compared to DNA DSB. Results presented in this paper indicate that the production of this lesion in oxidatively injured cells is increased by the presence of L-histidine in the extracellular compartment. Indeed, the L-histidine-mediated enhancement of DNA SSB detected in cells exposed to the amino acid and the oxidant was not affected by the addition of L-glutamine. Thus, it appears that extracellular/membrane bound L-histidine accounts for the increased production of DNA SSB and that the latter lesion is totally unrelated to the L-histidine-mediated increase in

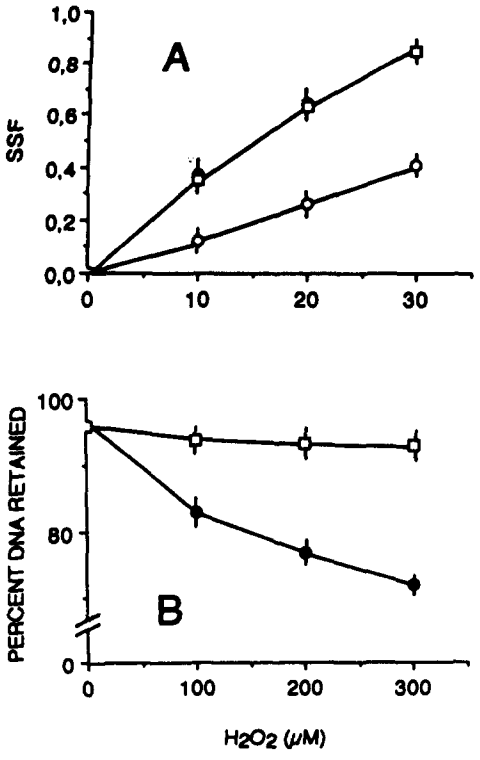

Fig. 3. Effect of L-glutamine on DNA single- and double-strand breakage produced by H_2O_2–L-histidine. Cells were treated for 30 min with increasing concentrations of H_2O_2 plus 1 mM L-histidine in the absence (●) or presence (□) of 10 mM L-glutamine and then analysed for either DNA single (A) or double (B) strand breakage. For comparison, results from experiments where cells were treated with increasing concentrations of the oxidant alone (○) are also shown. Results are expressed as (A) strand scission factor (SSF) values, and (B) per cent of DNA retained in the filter after 12 hr of elution (under neutral conditions). Each point represents the mean ± SEM calculated from at least three separate experiments, each performed in duplicate.

the cytotoxic response to hydrogen peroxide. Oya and Yamamoto [5] have also reported that extracellular L-histidine is responsible for the increase in chromosomal aberrations (of both the chromosome and chromatid type) observed in human fibroblasts exposed to the oxidant. It was suggested that this enhancing effect was mediated by the formation of a L-histidine–hydrogen peroxide adduct that was isolated and demonstrated to be more stable compared to the oxidant alone [5, 11]. These observations led the authors to speculate that the adduct could reach more easily the DNA than H_2O_2 alone and, by reacting with the chromatin-bound divalent iron, the oxidant could produce hydroxyl radicals species (site-specific Fenton reaction) [5, 11]. This hypothesis, however, contrasts with our experimental results since we have shown that inhibition of L-histidine uptake (and presumably of the L-histidine–H_2O_2 adduct) does not affect the L-histidine mediated enhancement of H_2O_2-induced DNA SSB. Thus, the mechanism whereby the extracellular fraction of the amino acid enhances the formation of hydrogen peroxide-induced DNA SSB remains a mystery and future research will address this intriguing mechanism.

We are also willing to test whether the L-histidine–H_2O_2 adduct is responsible for the production of DNA DSB and for the increased cytotoxic response. Indeed, the mechanism proposed by Oya and Yamamoto [5, 11] could explain these effects of L-histidine in oxidatively injured cells, since internalization of the amino acid is necessary.

In conclusion, data presented in this paper strongly suggest that the L-histidine-mediated enhancement of hydrogen peroxide cytotoxicity is related to the appearance of DNA DSB, both events being dependent on the intracellular fraction of the amino acid. In contrast, the increased production of DNA SSB is caused by the extracellular/membrane bound fraction of the amino acid and appears unrelated to the higher lethality elicited by the oxidant in the presence of L-histidine.

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