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ACTION OF CYSTINE IN THE CYTOTOXIC RESPONSE OF ESCHERICHIA COLI CELLS EXPOSED TO HYDROGEN PEROXIDE

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Cystine markedly enhanced the cytotoxic response of *Escherichia coli* cells to concentrations of hydrogen peroxide resulting in mode one killing, but displayed little effect in mode two killed cells. The effect of cystine was concentration-dependent over a range of 5-50 μM and did not further increase at higher levels. Cystine had similar effects in other bacterial systems.

In order to sensitize the cells to the oxidative injury, the amino acid must be present during exposure to the oxidant since no enhancement of the cytotoxic response can be observed in cystine pre-loaded cells. In addition, no further enhancement of cytotoxicity could be detected when cystine was added before and left during challenge with the oxidant. The enhancing effect of cystine on oxidative injury of *E. coli* cells appears to be directly mediated by the amino acid and in fact cysteic acid, the most likely oxidation product, had no effect on the killing of bacterial cells elicited by hydrogen peroxide. Other disulfide compounds such as oxidized glutathione, cystamine and dithionitrobenzoic acid only slightly increased the susceptibility of bacteria to the oxidant. The effect of the disulfides was not concentration-dependent over a range of 200-800 μM and was statistically significant only for cystamine.

Taken together, these results indicate that cystine markedly increases the cytotoxic response of bacteria to hydrogen peroxide and suggest that the amino acid might impair the cellular defence machinery against hydrogen peroxide. This effect may involve a thiol-disulfide exchange reaction at the cell membrane level.

KEY WORDS: hydrogen peroxide, cytotoxicity, *Escherichia coli*, cystine.

INTRODUCTION

The cytotoxic effect of H_2O_2 has been investigated extensively in the microorganism *Escherichia coli*[1-9]. In these cells, the lethal response elicited by increasing concentrations of the oxidant is characterized by two regions of killing (mode one and mode two killing) which are produced by concentrations of H_2O_2 below 2.5 mM or higher than 12.5 mM, respectively[2]. Experimental evidence indicates that mutations in recA[2,10], polA[2,11] or xth[2,12] confer hypersensitivity to low concentrations of H_2O_2, suggesting an important role for the DNA repair system in the protection of the cells against H_2O_2-induced mode one killing. This type of lethality also requires active cellular metabolism, since starvation of the cells prior to treatment with the oxidant results

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in a marked protection[2]. Mode two killing, unlike the mode one type, does not require active cellular metabolism and is not enhanced in DNA repair deficient strains[2].

We have recently reported that the toxicity of hydrogen peroxide in *E. coli* cells is highly dependent on the composition of the extracellular milieu[13] and, in particular, we have shown that the amino acid cystine markedly sensitizes bacterial cells to the oxidative attack[13-14]. In this study, we have further characterized this effect of cystine in an attempt to elucidate the molecular basis for the cystine-mediated enhancement of H_2O_2-induced killing of *E. coli* cells.

MATERIALS AND METHODS

Materials

Chemicals/reagent-grade biochemicals were from Sigma Chemical Co., St. Louis, MO, USA. H_2O_2 was purchased as a 30% solution from J.T. Baker Chemicals B.V. (Deventer, The Netherlands).

Bacterial Strains and Growth

The *E. coli* K 12 wild-type was obtained from Dr R. Tyrrell of the Institut Suisse de Recherches Experimentales sur le Cancer (Epalinges s/Lausanne, Switzerland). Cells were initially grown overnight (16–18 hr) at 37°C in K medium (1% glucose, 1% acid hydrolysate of casein, tryptophane free, 1 μg/ml thiamine hydrochloride, 25 μg/ml $MgSO_4 \times 7H_2O$ and 2 μg/ml $CaCl_2$ in M9 salts – the composition of the M9 salts was 6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l $(NH_4)_2SO_4$). An aliquot of the overnight cell suspension was diluted 50 fold with fresh K medium and aerobic growth was achieved in an Erlenmeyer flask with 200 rpm of shaking. *E. coli* cells grown to an optical density (O.D.) of 0.2 (600 nm), corresponding to about $7–10 \times 10^7$ cells/ml, were harvested by centrifugation at room temperature, washed in M9 salts and resuspended at a density of $7–10 \times 10^7$ cells/ml in M9 salts containing 1% glucose. A similar experimental protocol was used for growing other microorganisms utilized in this study, i.e. *Salmonella anatum* (isolated in our laboratory), *Staphylococcus aureus* (obtained from the American Cell Type Collection).

Bacterial Cell Survival Experiments

Treatments with H_2O_2 (15 min) were performed in the absence or presence of cystine (or other disulfides) in 3 ml of cell suspension placed in a 20 ml scintillation counting vial with 200 rpm of shaking, at 37°C. At the end of the treatment, the cell suspension was diluted in M9 salts, cells were plated in quadruplicate in LB agar plates, and incubated for 24 hr at 37°C, to allow colony formation.

Cystine and other disulfides were dissolved in M9 salts/1% glucose prior to addition to the bacterial cell suspension.

Bacterial Cell Growth Inhibition Studies and Microscopic Analysis

Cells were processed as described for survival experiments, except that a volume of 30 ml per experimental condition was used. Following exposure to the oxidant in the

absence or presence of cystine, the cell suspension was centrifuged, rinsed with M9 salts and cells were resuspended in K medium at an O.D. of 0.2. Cells were then allowed to grow for up to 270 min and 2 ml samples were taken at various time intervals for microscopic analysis and to measure O.D. values.

RESULTS

Figure 1A shows the survival curves of *E. coli* cells treated with increasing concentrations of H_2O_2 in glucose-containing M9 salts, either in the absence or presence of 137 μM cystine (the concentration of the amino acid in the K medium). In agreement with data in the literature[2], we found that the survival curve of cells exposed to various concentrations of hydrogen peroxide displays a biphasic character with two regions of lethality and an intermediate zone of partial resistance (Figure 1A). In the presence of cystine, the survival curve remained bimodal but the mode one killing response (which occurs at concentrations of H_2O_2 below 5 mM) resulted markedly increased. The zone of partial resistance was also apparent in the presence of the amino acid, and mode two killing was only slightly increased. For comparison, the survival curve of *E. coli* cells treated with the oxidant in K medium is also shown in Figure 1A. Note that mode one killing, unlike mode two, is still higher in the presence of cystine as compared to the situation where all the amino acids were present along with other medium components such as inorganic salts and vitamins. For example, the survival of *E. coli* cells treated with 2.5 mM H_2O_2 in this extracellular milieu (K

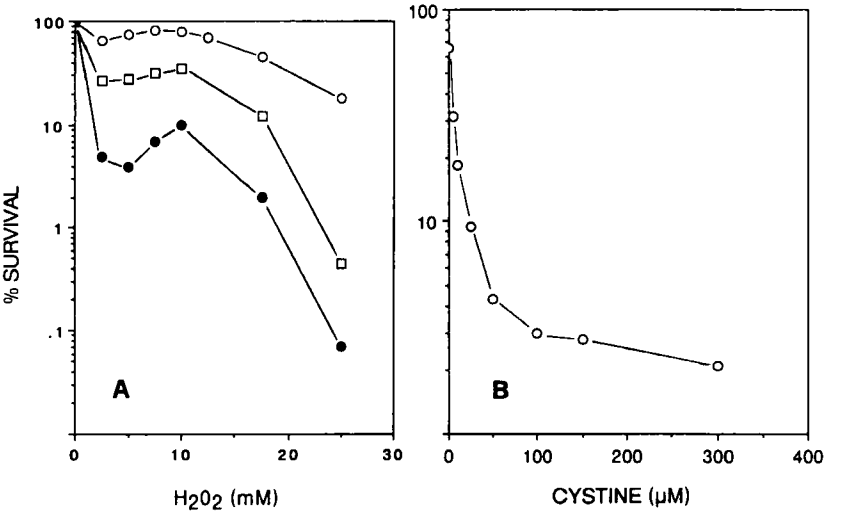

FIGURE 1 Cystine dependence of hydrogen peroxide cytotoxicity in *E. coli* cells.
(A) *E. coli* cells were exposed to increasing concentrations of H_2O_2 (15 min) in glucose containing -M9 salts, either in the absence (○—○) or presence (●—●) of 137 μM cystine, and assayed for survival as described in the Methods section. Also shown in the Figure is the survival curve for cells exposed to increasing concentrations of the oxidant in K medium (□—□). Each value is the mean of 5-7 separate experiments with a coefficient of variation of less than 10%.
(B) *E. coli* cells were exposed to 2.5 mM H_2O_2 in glucose containing-M9 salts either in the absence or presence of increasing concentrations of cystine and assayed for survival. Each value is the mean of 5-7 separate experiments with a coefficient of variation of less than 10%.

medium) is about 30%, and thus higher than that observed under conditions of treatment in a saline containing glucose and the amino acid (4%). Therefore, one or more components of the K medium, besides cystine, can modify the toxicity of the oxidant or counteract the potentiating effect exerted by the amino acid. Experiments were carried out to identify these components of the K medium. Table I shows the effects of inorganic salts, vitamins and the various amino acids present in the K medium on the toxicity of 2.5 mM H_2O_2 (a concentration of the oxidant representative of mode one killing) alone or associated to cystine. Note that, as we previously reported[13], histidine and cystine were the only two amino acids producing a statistically significant increase in the toxicity of H_2O_2 in bacteria. The amino acid mixture (AA) resulted in a degree of potentiation of the oxidative response similar to that obtained with histidine. In addition, none of the other constituents of the K medium, when added alone, were capable of affording protection in oxidatively injured cells. Similar results were obtained with cells treated with H_2O_2 in the presence of cystine (Table I). Histidine further increased this cytotoxic response ($p < 0.05$) and valine resulted in a low, although statistically significant, decrease ($p < 0.05$). All the other amino acids, as well as the remaining medium components, did not modify significantly the toxicity of the cocktail H_2O_2-cystine or, at the most, afforded a very low protection (reproducible but not statistically significant).

TABLE I

Effect of various amino acids and other constituents of the K medium on the cytotoxic response of *E. coli* cells challenged with hydrogen peroxide alone or associated to cystine[a]

Amino acid	Survival (%)	
	H_2O_2[b]	H_2O_2 plus cystine
-	72.0 ± 5	7.00 ± 1.0
L-Valine	68.3 ± 6	17.11 ± 3.1*
L-Tyrosine	67.8 ± 4	7.53 ± 1.2
L-Threonine	70.5 ± 3	8.29 ± 2.1
L-Serine	77.2 ± 7	7.01 ± 1.6
L-Proline	66.5 ± 4	6.04 ± 2.3
L-Phenylalanine	68.9 ± 4	7.04 ± 2.8
L-Methionine	69.5 ± 3	12.96 ± 3.9
L-Lysine	71.5 ± 7	6.04 ± 1.9
L-Leucine	76.0 ± 5	9.86 ± 1.2
L-Isoleucine	68.2 ± 8	9.15 ± 0.8
L-Histidine	29.3 ± 3**	2.21 ± 0.3*
Glycine	72.1 ± 4	11.47 ± 2.5
L-Glutamic acid	73.8 ± 4	9.17 ± 3.1
L-Cystine	7.0 ± 1**	-
L-Aspartic acid	71.5 ± 4	5.20 ± 1.9
L-Arginine	75.1 ± 3	8.07 ± 2.1
L-Alanine	68.0 ± 5	10.94 ± 3.2
$CaCl_2$	72.2 ± 6	10.42 ± 2.9
$MgSO_4$	70.5 ± 7	9.20 ± 2.8
Thiamine	72.1 ± 7	8.31 ± 1.1
AA[c]	31.5 ± 2**	-

[a] *E. coli* cells were treated for 15 min with 2.5 mM H_2O_2 alone, or associated to 137 μM cystine, in the absence or presence of the specific amino acids (at the concentration of the K medium) and then assayed for cell survival. Each value is the mean ± S.E.M. of at least 3 separate experiments.

* $p < 0.05$ when compared with the corresponding basal value.

** $p < 0.001$ when compared with the corresponding basal value.

[b] Data from Brandi et al. [1992a].

[c] Amino Acid mixture.

The effect of increasing concentrations of cystine on the toxicity of a concentration of H_2O_2 representative of mode one killing (2.5 mM) was also investigated. As shown in Figure 1B, the amino acid was active at concentrations as low as 5 μM ($p < 0.01$) and, at 25 μM, less than 10% of the cells were still able to form colonies, as compared to the 65% of cells that remained viable following exposure to the oxidant alone. Note that the survival curve shown in Figure 1B is linear at low cystine concentrations and then saturates; indeed, the amino acid was highly effective in increasing the cytotoxic response at concentrations up to 50 μM, whereas at higher levels the relative increase was barely detectable.

Since cystine is a disulfide, it is possible that its potentiating action in oxidatively injured cells could be mediated by thiol disulfide exchange reactions. We therefore tested the effect of some disulfide compounds on the viability of *E. coli* cells challenged with H_2O_2 (Table II). In contrast to cystine, compounds such as oxidized glutathione, cystamine and dithionitrobenzoic acid only slightly increased the vulnerability of bacterial cells to hydrogen peroxide. This effect was not concentration dependent over a range of 200–800 μM and, although observed in all the experiments performed with the disulfides, it was statistically significant only for cystamine ($p < 0.05$).

Cystine, in order to augment the susceptibility of bacteria to the lethal action of the oxidant, had to be present during challenge with H_2O_2 and was not effective when exposure to the oxidant was performed before or following treatment with the amino acid (Figure 2). In addition, no further enhancement in the cytotoxic response was observed when cystine was present both before and during challenge with the oxidant (Figure 2).

The kinetics of killing elicited by hydrogen peroxide (2.5 mM) alone or associated

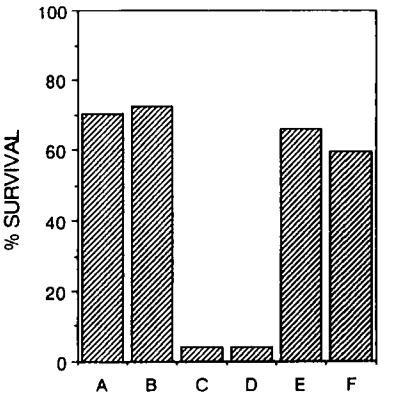

FIGURE 2 Expression of the cystine-mediated enhancement of H_2O_2 - induced killing of *E. coli* cells as a function of the protocol of cystine exposure.
Cells were treated with 2.5 mM H_2O_2 (15 min) in glucose containing - M9 salts before, during or following exposure (15 min) to 135 μM cystine, and assayed for survival as described in the Methods section. Each value is the mean of at least 5 separate experiments. Standard errors were less than 10%. Key:
- Exposure to H_2O_2 preceded by incubation in the absence (A) or presence (B) of cystine.
- Exposure to H_2O_2 preceded by incubation in the absence (C) or presence (D) of cystine and performed in the presence of the amino acid (C and D).
- Exposure to H_2O_2 followed by incubation in the absence (E) or presence (F) of cystine.

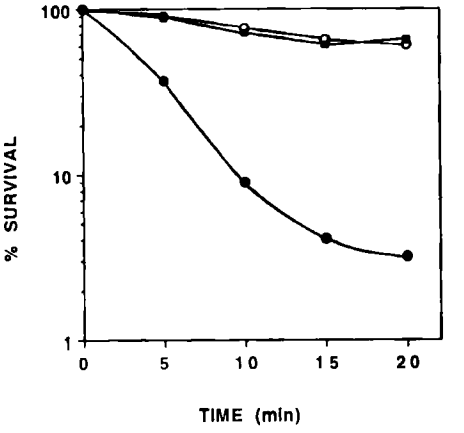

FIGURE 3 Kinetics of *E. coli* inactivation following exposure to hydrogen peroxide in the presence or absence of cystine.
Bacteria were treated with hydrogen peroxide as described in the legend to Figure 1A in the absence (○—○) or presence (●—●) of 137 μM cystine. Samples were taken at specific time intervals and assayed for survival. The results obtained with cells pre-incubated (15 min) with the amino acid and then challenged with the oxidant (■—■) are also shown. Each value is the mean of 5 separate experiments with a coefficient of variation of less than 10%.

to cystine (137 μM) are illustrated in Figure 3. It is apparent that the toxicity of the oxidant increased linearly over the first 15 min of exposure and leveled off at the 20 min time point; addition of cystine markedly increased the slope of the inactivation curve and once again a plateau was reached at about 20 min. Preincubation with cystine, followed by exposure to the oxidant, did not produce any significant effect and indeed the experimental curve obtained under these experimental conditions virtually overlapped the toxicity curve obtained with cells challenged with the oxidant alone.

The effect of cystine did not appear to be mediated by an oxidation product of the amino acid itself since cysteic acid, the most likely product of this reaction, was not cytotoxic at concentrations up to 30 μM and did not enhance the lethal action elicited by hydrogen peroxide (data not shown).

Microscopic analysis of cells challenged with H_2O_2 alone or associated to cystine (2.5 mM H_2O_2 ± 137 μM cystine) and then allowed to grow in a complete medium has indicated that the morphological response of *E. coli* cells to the oxidant is also affected by the amino acid. In fact, treatment with the oxidant in the presence of cystine increased both the number and length of the filaments (data not shown). It should be noted that, as previously shown by Imlay and Linn[2] and by us[15], *E. coli* cells treated with concentrations of hydrogen peroxide in the range of 0.5–5 mM filament but do not septate.

The action of cystine on the oxidative damage to bacterial cells did not appear to be confined to *E. coli* since the toxicity of hydrogen peroxide was also increased in *Salmonella anatum* and *Staphylococcus aureus* (not shown).

DISCUSSION

Previous studies from our laboratory have demonstrated that specific medium components significantly affect the cytotoxic response of *E. coli* cells to hydrogen peroxide[13-14]. In particular, the amino acids histidine and cystine were found to markedly increase the toxicity of the oxidant. In the present study we have attempted to characterize the enhancing effect of cystine with the specific aim of gathering information on the molecular basis of the cystine-mediated enhancement of the H_2O_2-induced killing of *E. coli* cells. Our results show that cystine markedly increases the killing of bacterial cells following treatment with low concentrations of hydrogen peroxide (Figure 1A). Under these experimental conditions, cells are killed via a mechanism which is referred to as mode one killing and requires active cellular metabolism[2]. Since the lethality elicited by high concentrations of the oxidant, resulting in mode two killing, is affected very little by cystine, it may be concluded that the amino acid selectively increases the sensitivity of the cells to killing by physiologically relevant concentrations of the oxidant. Thus, all the experiments that were aimed at characterizing the potentiating action of cystine were performed utilizing a concentration of the oxidant (2.5 mM) representative of mode one killing. Under these conditions, very low concentrations of the amino acid were highly effective in increasing the lethality of the oxidant (Figure 1B) and a linear dose-response curve was observed for concentrations of the amino acid below 25–50 μM. A second, practically flat, component of the inactivation curve was detected at higher concentrations of cystine, indicating that its action on the lethality elicited by hydrogen peroxide is augmented very little, if at all, by increasing the extracellular level of the amino acid from 50 to 300 μM. These data collectively indicate that the potentiating effect of cystine is mediated by a saturable process. In addition, cystine itself seems to cause this response since cysteic acid, the most likely oxidation product of cystine, was neither cytotoxic nor did it increase the toxicity of hydrogen peroxide.

Important information can be gathered from the analysis of Figure 2. The results depicted in this Figure indicate that an increased sensitivity of the cells was observed only when the amino acid was coadministered with hydrogen peroxide, whereas pre- or post-exposure to cystine did not modify the lethal response to the oxidant. In addition, no further enhancement of cytotoxicity was detectable when cystine was present both before and during challenge with the oxidant. These data are consistent with those obtained investigating the kinetics for the induction of the lethal response (Figure 3) and may suggest that the action of cystine is at the extracellular level. Assuming that this hypothesis is correct, it may also then be speculated that cystine acts via thiol disulfide exchange with a "thiol receptor". Other disulfides which have been tested, however, resulted in very low increases of the cytotoxic response to the oxidant and only in the case of cystamine was the effect statistically significant (Table II). We would have expected the disulfides to produce effects similar to that of cystine, since they should also be able to interact with the -SH groups of proteins which are located on the exofacial membrane. On the other hand, oxidized glutathione, dithionitrobenzoic acid and cystamine may not reach the "thiol receptor" because the access to this site might be limited by a number of factors such as steric hindrance and other stereochemical parameters. In addition, it is also possible that the thiol disulfide exchange reaction is enzyme-catalyzed and, therefore, it is not surprising that compounds like dithionitrobenzoic acid are not active in this system.

It is important, however, to stress that the fact that pre-exposure to cystine does

TABLE II
Effect of various disulfide compounds on hydrogen peroxide toxicity in *E. coli* cells[a]

Disulfide compounds (μM)	Survival (%)
-	70.1 ± 4.3
Oxidized glutathione	
200	65.5 ± 6.6
400	64.7 ± 3.9
800	59.9 ± 5.5
Cystamine	
200	52.4 ± 5.1*
400	57.3 ± 4.9*
800	51.3 ± 4.1*
Dithionitrobenzoic acid	
200	62.3 ± 5.2
400	61.2 ± 4.2
800	60.8 ± 5.3

[a] *E. coli* cells were treated with 2.5 mM H_2O_2 (15 min) in the absence or presence of increasing concentrations of the disulfide compounds and then assayed for cell survival. Each value is the mean ± S.E.M. of 3 separate experiments.
* $p < 0.05$ when compared with the corresponding basal value.

not enhance the toxicity of hydrogen peroxide cannot be taken as clearcut evidence for an extracellular location of the site of action of the amino acid. In fact, the possibility of an enhanced cystine-uptake under conditions of oxidative stress also exists. This would result in an increased cellular accumulation of the amino acid which, by acting intracellularly via some unknown mechanism, could then enhance the toxicity of the oxidant.

The enhancing effect of cystine on the killing of *E. coli* cells by hydrogen peroxide was somehow modulated by the other components of the extracellular milieu. Indeed, the lethality exerted by H_2O_2 in a minimal glucose medium was higher compared to that observed in a complete medium. Valine significantly reduced the effect of cystine (Table I) whereas other amino acids were either ineffective, or capable of reproducibly reducing the enhancing effect of cystine, although in a non statistically significant fashion. Histidine was the only amino acid which significantly increased the effect of cystine.

Finally, the effect of cystine was not limited to oxidatively injured *E. coli* cells but also occurred in *Salmonella anatum* and *Staphylococcus aureus*.

In conclusion, our results indicate that cystine is a powerful enhancer of hydrogen peroxide-induced mode one killing of bacteria. The facts that 1) the amino acid is directly responsible for producing this effect, 2) the enhancement of the cytotoxic response is mediated by a saturable process, and 3) the site of action of cystine seems to be located extracellularly, are all consistent with the theory that a "thiol receptor" might exist on the cell membrane of bacterial cells. Although we are aware that the above results could have alternative explanations, we are currently considering the hypothesis that the "putative" protein carrying this -SH group might represent a firstline defense mechanism against hydrogen peroxide present in the extracellular

milieu. Experiments are in progress in our laboratory to find support for or disprove this hypothesis.

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