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ROLE OF HYDROXYL RADICALS IN *ESCHERICHIA COLI* KILLING INDUCED BY HYDROGEN PEROXIDE

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Escherichia coli lethality by hydrogen peroxide is characterized by two modes of killing. In this paper we have found that hydroxyl radicals (OH·) generated by H_2O_2 and intracellular divalent iron are not involved in the induction of mode one lethality (i.e. cell killing produced by concentrations of H_2O_2 lower than 2.5 mM). In fact, the OH radical scavengers, thiourea, ethanol and dimethyl sulfoxide, and the iron chelator, desferrioxamine, did not affect the survival of cells exposed to 2.5 mM H_2O_2. In addition cell vulnerability to the same H_2O_2 concentration was independent on the intracellular iron content. In contrast, mode two lethality (i.e. cell killing generated by concentrations of H_2O_2 higher than 10 mM) was markedly reduced by OH radical scavengers and desferrioxamine and was augmented by increasing the intracellular iron content.

It is concluded that OH· are required for mode two killing of *E. coli* by hydrogen peroxide.

KEY WORDS: hydrogen peroxide, hydroxyl radicals, toxicity, *Escherichia coli*.

INTRODUCTION

There is a considerable amount of evidence indicating that hydroxyl radicals produced via the Fenton reaction are a major cause of hydrogen peroxide-induced cell killing in either mammalian [1-3] or bacterial cells.[4] In these investigations OH· scavengers and iron chelators were used to show that toxicity could be reduced by either protecting cells against OH radical attack or inhibiting their formation. Recently, Imlay and Linn [5] have pointed out that two modes of killing of *E. coli* by H_2O_2 can be distinguished, the first occuring at concentrations below 2 mM and the second at concentrations higher than 10 mM. Furthermore, they have shown that it is possible to differentiate mode one from mode two killing on the basis that the latter does not require active metabolism and is not enhanced in strains with DNA repair defects.[5] Therefore, at least in bacteria, an iron catalized reduction of H_2O_2 to OH· does not seem to be the sole mechanism involved in cell killing; rather, it would seem that the two modes of killing are mediated by different cytotoxic species, possibly acting on distinct target sites.

In the current work we have investigated this premise and found that hydroxyl

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radicals are involved in the production of mode two but not mode one killing by H_2O_2.

MATERIALS AND METHODS

Materials

H_2O_2 was purchased as a 30% solution from J.T. Baker Chemicals B.W. (Deventer, Holland); other chemicals and most reagent grade biochemicals were from Sigma Chemicals Co. (St. Louis, MO, U.S.A.) and Flow Labs (Mc Lean, VA, U.S.A.).

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Bacterial strain and growth

The *E. coli* strain used, AB1157, was routinely grown in our laboratory at 37°C.

Cells were initially grown overnight (16–18 hr) at 37°C in K medium (1% glucose, 1% casamino acids, 25 μg/ml $MgSO_4 \cdot 7 H_2O$ and 2 μg/ml $CaCl_2$ in M9 salts). Samples were diluted 50 fold in fresh K medium and grown to about 10^8 cells/ml under aerobic conditions. Aerated growth was achieved by incubation of 50 ml of K medium containing 1–5 × 10^7 cells/ml in a 500 ml Erlenmeyer flask with 200 rpm of shaking. In some experiments $FeSO_4$ was added to the K medium.

Iron content

Cells were grown in the absence or presence of increasing concentrations of $FeSO_4$ (0.5, 5, 12.5 or 25 μg/ml) and the iron content was determined using the bathophenatroline method without deproteinization.[6,7]

Survival curves

Cells grown to about 10^8 cells/ml were harvested by centrifugation at room temperture, washed once with M9 salts and resuspended at 5 × 10^7 cells/ml in prewarmed M9 salts (pH 7.4) or K medium (pH 7.4). Treatments with H_2O_2 (for 15 min, or for longer time intervals, as stated in the text) were performed in 3 ml of cell suspension in a 20 ml scintillation counting vial with 200 rpm of shaking, at 37°C. The challenge was terminated by dilution in M9 salts. Cells were immediately plated in quadruplicate in LB agar plates and incubated at 37°C for 24 hr to allow colony formation.

RESULTS

The toxicity of hydrogen peroxide (treatment for 15 min at 37°C) on *E. coli* is presented in Figure 1.

As previously demonstrated by Imlay and Linn [5] two modes of killing are apparent; mode one killing refers to the lethality occurring at concentrations of H_2O_2 below 2.5 mM and mode two killing to the lethality which occurred at high, postshoulder concentrations (> 10 mM). An intervening zone of partial resistance is detectable between the two regions of killing. The objective of this study was to investigate the role of hydroxyl radicals generated by H_2O_2 and divalent iron via the Fenton reaction

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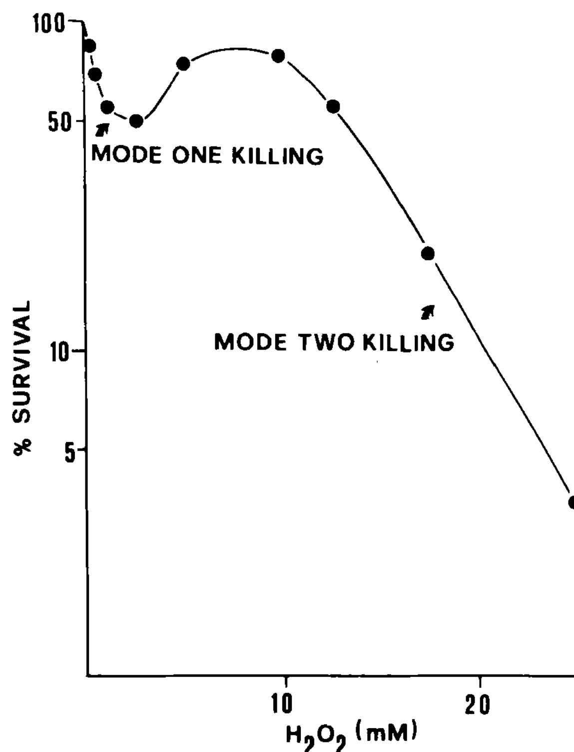

FIGURE 1 Survival of *E. coli* cells after challenge with H_2O_2. Cells were treated with increasing H_2O_2 concentrations for 15 min in M9 salts at 37°C. Cell viability was assessed as detailed in the Methods section. Each point represents the mean of 6–12 separate experiments. Standard errors were less than 10%.

in these two modes of lethality. In the first set of experiments, cells were exposed to 2.5 or 25 mM H_2O_2 (the first concentration being representative of mode one and the second of mode two killing) in the presence or absence of the OH· scavenger, thiourea (35 mM).[8] As illustrated in Figure 2, whereas mode one killing (A) remained unaffected, mode two killing (B) was markedly reduced ($p < 0.001$) by the scavengers of OH radicals, suggesting that this species might be of importance only in the lethality caused by high concentrations of H_2O_2 (i.e. mode two killing). The effect of thiourea was dose-dependent and 35 mM was selected since it was the maximal non toxic concentration (not shown). Dimethylsulfoxide (250 mM) and ethanol (100 mM) were also utilized as OH· scavengers,[8] giving comparable results to the ones obtained with thiourea (Figure 2). It should be noted, however, that the effect of thiourea was more pronounced than that of DMSO or ethanol. Since mode one killing by H_2O_2 occurs in actively metabolizing cells we have also investigated the effect of the scavengers on this type of lethality following exposure to the oxidant for 15 min in K medium (it should be noted that, unless specified in the text, experimental protocols utilized in this study, involved exposure to the oxidant in M9 salts). Not surprisingly, we have found that killing by 2.5 mM H_2O_2 in K medium is higher by about 30% with respect to the killing observed following treatment in M9 salts; the effect of the OH· scavengers, however, remained approximately the same (not shown), thus suggesting

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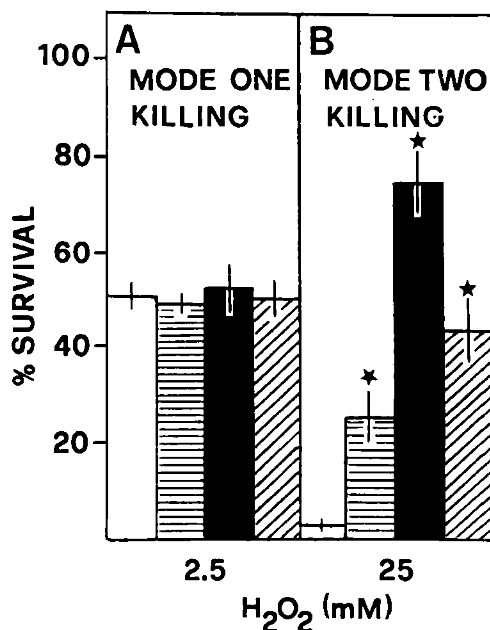

FIGURE 2 Prevention of H_2O_2 -induced cell killing by thiourea, ethanol or dimethyl sulfoxide. Cells were exposed to 2.5 mM (A) or 25 mM (B) H_2O_2 for 15 min at 37°C in the absence ( ) or presence of 100 mM ethanol ( ), 35 mM thiourea ( ) or 250 mM dimethyl sulfoxide ( ) and then tested for viability. Results are the mean ± S.E.M., (n = 4). * $p < 0.001$ (Student's t test's).

that the molecular mechanisms mediating mode one killing in bacteria treated in M9 salts or K medium do not differ significantly. The experiments that will be described later on in this section of the paper were all performed in M9 salts, some of them, however, were repeated by exposing the cells to the oxidant in K medium, and comparable results were obtained. The effect of the scavengers on the whole curve relating the concentrations of H_2O_2 to the ability of the cells to form colonies was also tested and, consistent with the results shown in Figure 2, the mode one killing region remained unaffected, on the contrary to the mode two killing region which was dramatically reduced by either thiourea or the other non-SH OH· scavengers, dimethyl sulfoxide and ethanol (not shown). Furthermore, experiments where cells were treated with 2.5 mM H_2O_2, alone or associated with hydroxyl radical scavengers, for various time intervals have indicated that the cytotoxic response was a linear function of the time of exposure (at least up to 90 min), independently of the presence of the OH· scavenger, thus suggesting that the lack of protection in mode one lethality may not be related to the inability of thiourea, dimethyl sulfoxide or ethanol to reach critical targets where OH· are produced and/or generate their insult (not shown).

In experiments similar to those depycted in Figure 2, the effect of desferrioxamine (500 μM) on H_2O_2 killing of *E. coli* was also evaluated. The iron chelator was added 5 min before H_2O_2 treatment and, as shown in Figure 3, was able to partially prevent mode two lethality ($p < 0.001$) without affecting mode one killing induced by the oxidant. Again, when various concentrations of desferrioxamine were tested for their

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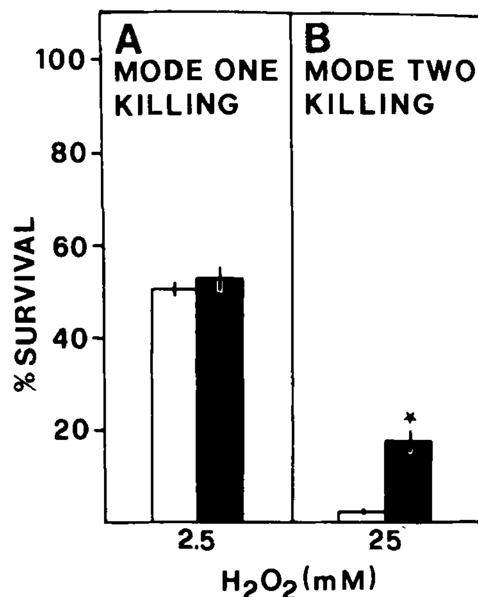

FIGURE 3 Effect of desferrioxamine on H_2O_2 induced *E. coli* killing. Cells were treated for 15 min with 2.5 mM (A) or 25 mM (B) concentrations of H_2O_2 in the absence (open bars) or presence (closed bars) of 500 μM desferrioxamine and then tested for viability. Results are the mean ± S.E.M. of at least 4 separate experiments. * $p < 0.001$ (Student's t test).

ability to improve the survival of *E. coli* challenged with 2.5 or 25 mM levels of H_2O_2, it was found that although no effect could be detected in mode one killing with concentrations of the iron chelator up to 10 mM, mode two lethality was inverse function of increasing concentrations of desferrioxamine over a range 0.25–5 mM (not shown). It should be also noted that, in analogy to our observations with OH· scavengers, desferrioxamine did not affect mode one lethality even when exposure was of 30 or 45 min (not shown). In the second set of experiments, cells were grown in the presence of various concentrations of $FeSO_4$ and tested for H_2O_2 sensitivity. Intracellular iron levels progressively increased by growing cells in media with increasing $FeSO_4$ concentrations and a 10 fold increase in intracellular iron content was achieved when $FeSO_4$ was added at a concentration of 25 μg/ml (not shown). Cells grown with 25 μg/ml $FeSO_4$ or with no extracellular iron added were compared for their sensitivity to the H_2O_2 insult. Again, two concentrations of H_2O_2, representative of modes one (2.5 mM) and two (17.5 mM) killing, were used. Figure 4 B shows that cells with an increased iron content were more susceptible to mode two killing by H_2O_2 as compared with control cells (cells grown with no extracellular iron added). In contrast, mode one killing was independent on intracellular iron concentrations (Figure 4 A). In other experiments the vulnerability of *E. coli* cells to 17.5 mM H_2O_2 was found to increase as a function of increasing intrinsic iron concentrations which were achieved by growing cells in the presence of 0.5, 5, 12.5 or 25 μg/ml $FeSO_4$ (not shown).

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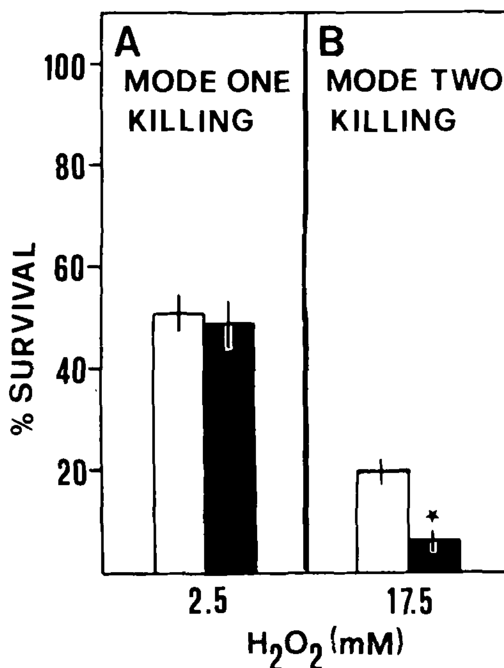

FIGURE 4 Dependence of H_2O_2 toxicity on cellular iron content. Cells were grown in K medium in the absence (open bars) or presence (closed bars) of 25 μg/ml $FeSO_4$. Cells were then exposed to 2.5 mM (A) or 17.5 mM (B) H_2O_2 for 15 min. Following drug challenge, cells were processed for survival studies. Results are the mean $\pm$ S.E.M., (n=4). * $p < 0.001$ (Student's t test).

DISCUSSION

Our results indicate that mode one killing is not affected by hydroxly radical scavengers and is not function of intracellular iron levels. The hydroxyl radical, therefore, does not seem a likely candidate for the production of this type of lethality and the possibility that thiourea, dimethyl sulfoxide, ethanol or desferrioxamine fail to reduce killing by low concentrations of H_2O_2 since these compounds may not reach critical sites where OH• are produced seems to be ruled out by the results we have obtained in a number of experimental approaches. In fact, mode one killing was independent on intracellular iron concentrations (cells grown in 25 μg/ml $FeSO_4$ displayed iron levels 10 times higher than control cells), on the presence of increasing concentrations of desferrioxamine or OH• scavengers and on the length of exposure to these same agents (Figures 2–4 and text of the Results section). These results, however, do not necessarily prove that iron is not required for mode one killing by H_2O_2. In fact, very low amounts of iron in specific sites that cannot be approached by OH• scavengers or iron chelators, may suffice to react with the oxidant and generate the lethal event; in addition, it should be noted that, in our study, bacteria were grown in a high-iron high-phosphate medium and, therefore, the growth in the presence of exogenous $FeSO_4$ may have increased only the amount of iron associated at the membrane level, a site which does not seem important as far as mode one killing is concerned. Imlay and Linn [5] have previously reported that this mode of lethality requires active

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metabolism and is enhanced in DNA repair deficent strains or by an anoxia induced, chloramphenicol sensitive, cell function. Furthermore, we have previously shown that mode one killing is independent of O_2 tension during drug challenge and is abolished when drug exposure is performed at 4°C.[9] Taken together, these results suggest that DNA seems to be the target of mode one lethality and that the ultimate oxidant should not be the hydroxyl radical but H_2O_2 itself or a so far unknown radical species produced via H_2O_2 metabolism. It should be noted, however, that H_2O_2 does not directly damage the DNA [10] and therefore is not a likely candidate for producing mode one lethality since the latter is mediated by DNA damage.[5] All we know of the hypothetical, unknown species is that a) the machinery responsible for the putative reduction of H_2O_2 requires active cellular matabolism; [5,9] b) the lesions that this species produces in the DNA are not efficiently repaired in strains carrying mutations in recA, [5,11] polA [5,12] or xth [5,13] genes; c) the lesions that this species produces in the DNA are either formed or repaired with the same efficiency, independently of O_2 tension [9] and d) anoxic growth induces the synthesis of proteins which dramatically enhances its lethality. [5] It could be speculated that anoxia induces the synthesis of a repressor of the scavenging enzyme of the unknown radical species, thus increasing the vulnerability of the cells to this type of lethality. This hypothesis is supported by the fact that since anoxically growing bacteria produce during normal metabolism less reactive oxygen species than aerobically growing bacteria, cells grown in anaerobiosis may not need an efficient radical scavenging system. It should be noted that when *E. coli* cells are grown anaerobically, the enzyme manganese superoxide dismutase is not expressed. [14] For this reason and since a mutant of *E. coli* lacking both manganese and iron superoxide dismutases is sensitive to mode one lethality, [15] we are now in the process of investigating the possibility of superoxides being the unknown species involved in the production of mode one killing.

In agreement to Imlay and Linn [5] we have found an intervening zone of partial resistance between modes one and two killing. These Authors [5] have suggested that "this protective effect occurred by adjustment of production, rather than resolution, of lesions". We do agree with their hypothesis and we propose that the levels of the unknown species are reduced at concentrations of H_2O_2 above 2.5 mM because they are directly quenched by H_2O_2 or $OH\cdot$. Alternatively, hydroxyl radicals or hydrogen peroxide may inhibit their formation.

OH radicals produced by H_2O_2 concentrations below 10 mM are not lethal and, therefore, cells are either protected or the lesions that are generated are efficiently repaired by the cells. Above this concentration, however, toxicity occurs (mode two killing) possibly because scavenging or repair systems become progressively saturated.

Since hydroxyl radical scavengers such as thiourea, dimethyl sulfoxide or ethanol and the iron chelator desferrioxamine prevent mode two killing by H_2O_2 (Figures 2 and 3), we suggest that $OH\cdot$ mediate this type of lethality. Furthermore, additional evidence that mode two killing is produced by $OH\cdot$ is given by our findings that this lethality was function of intracellular iron content (Figure 4). It should be noted that among the $OH\cdot$ scavengers utilyzed in this study, one is a SH-compound (thiourea), on the contrary to the other ones (DMSO and ethanol). Since the first displayed an higher protection, as compared to the latter compound, it could be speculated that the action of thiourea is also mediated by chemical restitution (in fact, the ability of H_2O_2 to deplete intracellular pools of protein- and non-protein-thiols is well documented). Of obvious interest would be to know whether $OH\cdot$ formation is direct function of H_2O_2 concentration or is dependent on intracellular ferrous ions levels. Starke and

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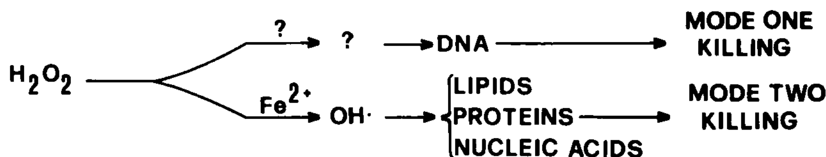

FIGURE 5 Proposed mechanism of H_2O_2-induced cytotoxicity in *E. coli.*

Farber [16] have recently shown that in cultured hepatocytes treated with H_2O_2, Fe^{3+} is reduced to Fe^{2+} by the superoxide anion thereby allowing further Fenton reactions. This sequence of reactions is known as Haber–Weiss reaction. Assuming that the same sequence of events occurs in bacteria, a continous production of OH·, at increasing H_2O_2 concentrations, may be expected. Consistent with this hypothesis is the fact that mode two lethality is proportional to H_2O_2 concentrations to at least 50 mM (not shown, or ref.)[5]. Also consistent is the fact that cells lacking either manganese or iron superoxide dismutases are hypersensitive to mode two killing by H_2O_2; [17] in fact, assuming that the superoxide anion is responsible for the reduction of Fe^{3+} to Fe^{2+}, a deficency in superoxide dismutase activity would be expected to exacerbate mode two killing since more superoxide anions are available for the reduction of trivalent iron. The fact that desferrioxamine preferentially chelates Fe^{3+} would indicate that the latter, and not Fe^{2+}, plays a critical role in H_2O_2 induced inactivation of *E. coli.* It should be noted, however, that as shown by Halliwell and Gutteridge, [18] Fe^{3+} bound to desferrioxamine cannot be reduced by the superoxide anion, and hence it inhibits OH· formation. An obvious question is whether the target of OH· — induced cell death is the DNA, as we have hypothesized for mode one killing. The fact that strains with DNA repair defects were not especially susceptible to mode two killing would indicate that the critical lesions are generated on targets other than the DNA. Alternatively, it can be suggested that DNA is not the sole target for this type of toxicity. Therefore, mode two killing is more likely to be produced by inactivation of multiple targets such as lipids, proteins, and eventually, nucleic acids. Recently, Imlay and Linn [15, 19] have reported that DNA is definetly a target of the OH· generated by high concentrations of H_2O_2 since, at those levels, mutagenesis has been shown to occur.

We summarize the above discussion by concluding that the bimodal pattern of *E. coli* killing by H_2O_2 is best explained by the involvement of at least two distinct radicals species which actually mediate the two modes of lethality. Figure 5 outlines a schematic diagram of the proposed mechanism.

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