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MOLECULAR MECHANISMS OF HYDROGEN PEROXIDE CYTOTOXICITY

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Summary. - *The molecular mechanisms of H_2O_2 toxicity have been investigated in both mammalian or bacterial cells. DNA breakage mediates cytotoxicity by low concentrations of H_2O_2 in mammalian cells, but DNA lesions do not appear as a direct consequence of the action of the hydroxyl radical; rather, these radicals may disturb intracellular Ca^{2+} homeostasis, which results in secondary reactions ultimately leading to DNA strand breakage and cytotoxicity. Studies that have used* Escherichia coli (E. coli) *as a cellular system have indicated that the two modes of killing detectable in cells exposed to increasing concentrations of H_2O_2 are mediated by different radical species. Mode-one killing seems to be produced by the superoxide anion whereas mode-two killing seems to be a consequence of the hydroxyl radical attack.*

Riassunto (Meccanismi molecolari di citotossicità del perossido di idrogeno). - *In questa rassegna vengono riportati recenti risultati da noi ottenuti in uno studio mirato alla comprensione delle basi molecolari dei meccanismi di citotossicità del perossido di idrogeno sia in sistemi di cellule di mammifero in coltura che nel batterio* Escherichia coli (E. coli). *Si suggerisce che il danno al DNA rappresenti il meccanismo di citotossicità in cellule di mammifero esposte a basse concentrazioni di H_2O_2. Le lesioni a livello del DNA non sembrano essere prodotte direttamente dal radicale ossidrilico, ma questa specie di ossigeno radicalico potrebbe essere responsabile dell'incremento dei livelli citoplasmatici di Ca^{2+} libero che stimolando l'attività nucleasica sarebbero poi responsabili della produzione di lesioni a livello del DNA e della risposta citotossica. Gli studi che hanno utilizzato il batterio* E. coli *come sistema cellulare, hanno dimostrato che i due modi di letalità che seguono l'esposizione di tali cellule a crescenti concentrazioni di H_2O_2 sono mediati da specie diverse. Più precisamente, sembrerebbe che il modo-uno sia riconducibile all'azione del radicale superossido, al contrario del modo-due che appare mediato dall'azione del radicale ossidrilico.*

Introduction

Cells are continuously exposed to free radicals produced by enzymatic processes, cell respiration and the action of specific drugs, radiation and hyperthermia [1]. Among these radical species, hydrogen peroxide is particularly important since it is produced in abundant quantities in many tissues, and high levels of the oxidant have been detected at inflammation sites [2]. The study of the molecular basis of H_2O_2 effects on cellular components will allow a better understanding of the physiological and toxicological relevance of the oxidant.

In recent years, research in our laboratory has been addressed to study the mechanisms of hydrogen peroxide cytotoxicity and our findings will be briefly summarized here.

Mammalian cells studies

It has been previously suggested that H_2O_2 toxicity is mediated by the hydroxyl radical (OH·) since iron chelators or OH· scavengers are capable of inhibiting the deleterious effects of the oxidant [3-5].

The proposed iron catalyzed reduction of H_2O_2 to OH· should occurr via the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow OH\cdot + Fe^{3+} + OH^-$$

It is unclear, however, whether OH· directly mediates the cytotoxic insult or whether initiates a series of events resulting in the induction of secondary lesions that ultimately lead to DNA damage and cytotoxicity.

We have attempted to answer this question by comparing DNA breakage and cytotoxicity in cultured mammalian cells exposed to the oxidant either under physiologic conditions (at 37 °C) or at 4 °C, a temperature at which enzymatic reactions are very slow and therefore where damage to critical targets should be directly induced by the OH·. Results obtained have indicated that H_2O_2 is far more toxic at 37 °C than at 4 °C and that DNA single strand breaks produced at ice temperature are

removed at least three times faster than those generated at 37 °C [6-7]. Another difference is that, although an inhibitor of the enzyme poly(ADP-ribose)transferase (ADPRT), 3-aminobenzamide (3AB), was capable of retarding the rejoining of DNA single strand breaks produced by the oxidant at both temperatures, toxicity was increased by 3AB only following exposure at 4 °C [6]. ADPRT is efficiently induced by H_2O_2 challenge at 37 °C [8] and although incubation of the cells for 1 h at 4 °C (followed by a 30 min post-incubation at 37 °C) seems itself to stimulate the transferase activity [9], no further induction is detectable following addition of concentrations of H_2O_2 up to 10 mM (manuscript in preparation). These results are in apparent contrast with those of Schraufstatter *et al.* [10] who have found that 3AB actually prevents cytotoxicity of the oxidant at 37 °C. It should be noted, however, that these authors have utilized very high concentrations of H_2O_2 (2.5 mM), which are at least 10 times higher, than the concentrations we have used. A chain reaction triggered by massive DNA breakage followed by induction of high levels of ADPRT activity resulting in dramatic and irreversible depletion in NAD^+ and ATP levels could, therefore, explain the molecular basis of the cytotoxicity of millimolar concentrations of H_2O_2. Not surprisingly, inhibition of ADPRT was found to slow down this process [10]. When concentrations of H_2O_2 in the micromolar range are used (as in our studies), however, a different mechanism of lethality may be involved, since the aforementioned processes are promptly and efficently reversed as the oxidant is consumed [8].

The results we have so far discussed clearly demonstrate that OH·, generated by H_2O_2 challenge at 37 °C or 4 °C, affects cellular homeostasis in a totally different manner. It would therefore appear that H_2O_2 toxicity, under physiological conditions, is mediated by a series of events that is initiated by the OH· and ultimately leads to DNA damage and cytotoxicity. The fact that metabolic poisons inhibit DNA breakage by H_2O_2 [11] further supports this notion.

It is known that H_2O_2, as well as other types of oxidant stress [12], is capable of producing a rise in cytosolic free calcium ions. It appears therefore plausible that the radical induced change in Ca^{2+} flux could result in the activation of Ca^{2+}-dependent nucleases or proteases. We have tested this hypothesis and found that quin 2, an intracellular calcium chelator, efficiently prevents DNA damage and cytotoxicity exerted by the oxidant (manuscript submitted).

Fig. 1 displays a scheme of the proposed mechanism of H_2O_2 cytotoxicity in cultured mammalian cells.

Finally, we have investigated whether DNA damage was responsible for the killing of cultured mammalian cells by H_2O_2. Although our results do not allow a definitive elucidation of the molecular mechanism, the following conclusions can be drawn from our study: 1) DNA repair inhibitors retard DNA single strand break

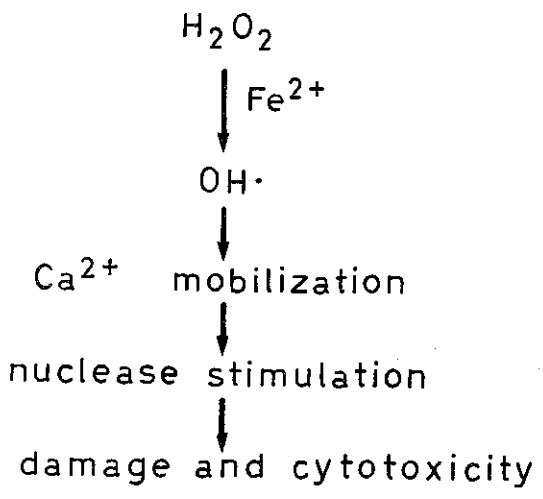

Fig. 1. - Hypothetical mechanism for the induction of DNA breakage and cytotoxicity in cultured mammalian cells by hydrogen peroxide.

rejoining and increase the cytotoxicity by low concentrations of H_2O_2 [6, 7], and 2) a DNA-repair-deficient mutant of Chinese hamster ovary cells displays, with respect to the parental cell line, a slower rate of removal of DNA single strand breaks produced by H_2O_2 and an increased sensitivity to the lethal effect exerted by low concentrations of the oxidant [7]. Taken together, these results suggest that DNA damage may be responsible for the toxicity generated by H_2O_2, at least in a low dose region of the survival curve.

Bacterial cell studies

The toxicity of hydrogen peroxide to bacteria is thought to be mediated by the hydroxyl radical which is formed *via* the reaction of the oxidant with divalent iron (Fenton reaction) [13].

Exposure of *Escherichia coli (E. coli)* to increasing concentrations of H_2O_2 results in a bimodal pattern of lethality (Fig. 2) characterized by a zone of partial resistance occurring at concentrations in the range 5 to 12.5 mM and two modes of killing, modes one and two, occurring at concentrations of H_2O_2 below 5 mM or higher than 12.5 mM, respectively [14-17]. In contrast to mode-two killing, mode-one lethality requires active cellular metabolism and is enhanced in DNA repair-deficient strains or by an anoxia-induced cell function [14].

In a previous study [18] we have utilized iron chelators and hydroxyl radical scavengers in an attempt to investigate the role of hydroxyl radicals in these two modes of lethality. We found that these species might be responsible only for the killing in the mode-two region. This hypothesis was further supported by the finding that *E. coli* mutants lacking Fe-superoxide dismutase *(sod B)* or Mn-superoxide dismutase *(sod A)*

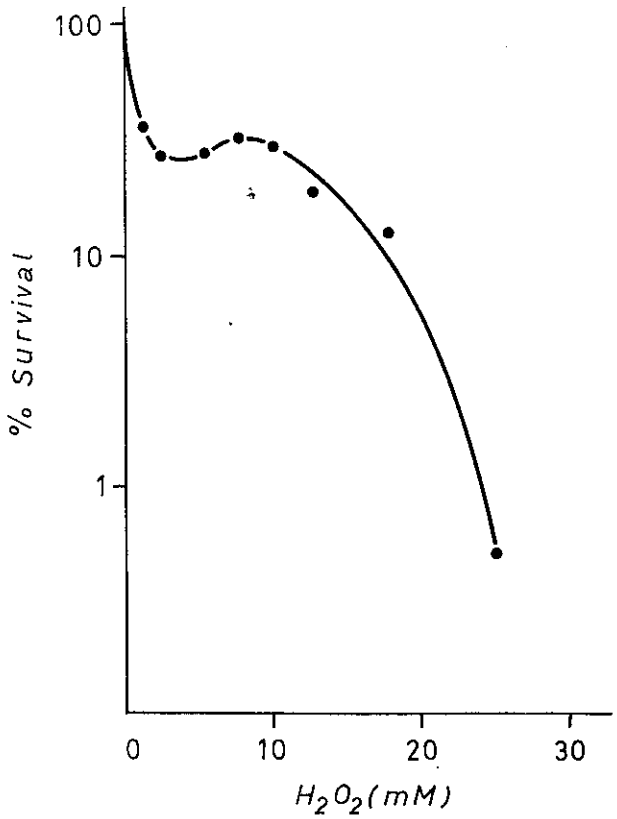

Fig. 2. - Survival of *Escherichia coli* cells exposed to increasing concentrations of H_2O_2 in K medium at 37 °C for 15 min.

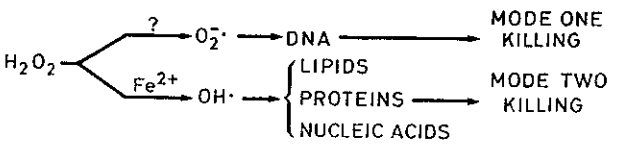

Fig. 3. - Hypothetical mechanism for the induction of modes one and two killing of *Escherichia coli* by hydrogen peroxide.

are hypersensitive to mode-two killing by H_2O_2 [19]. This observation may in fact be explained by the increased intracellular availability of superoxide ions (which should occurr in a condition of superoxide dismutase deficiency) which, in turn, may increase the rate of reduction of trivalent iron to the divalent form, thereby allowing further Fenton reactions (*i.e.*, more hydroxyl radicals should be produced). Under the experimental conditions utilized in this study (which involved treatment of cells in M9 salts), mode-one killing was not significantly different in wild type and superoxide dismutase mutants [19]. We have therefore performed a more detailed study on the toxicity of H_2O_2 in *E. coli* wild type and its mutants *sod A* or *sod B*.

The rationale of this study was that, in order to understand the role of superoxide ions in H_2O_2-induced inactivation of *E. coli*, it was necessary to investigate the sensitivity of superoxide dismutase mutants under experimental conditions known to be favorable for the occurrence of mode-one killing. Cells were therefore

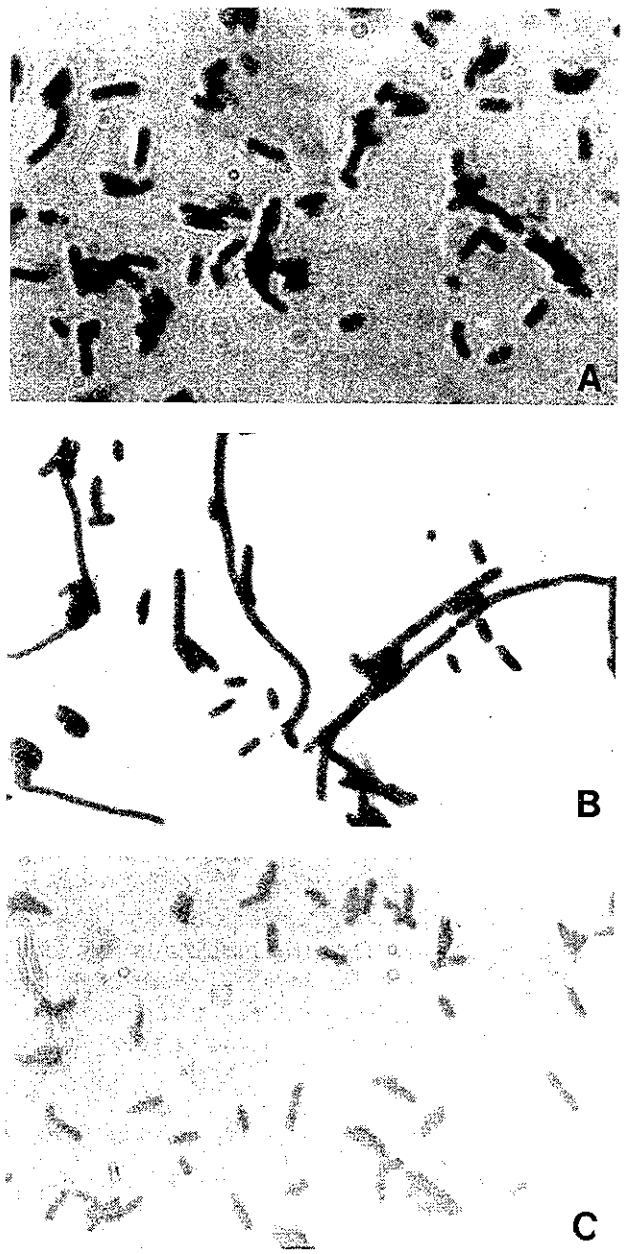

Fig. 4. - Effect of hydrogen peroxide on the cell shape of *Escherichia coli*. Cells were grown in K medium in the absence (A) or presence of 1.75 (B) or 17.5 (C) mM H_2O_2 for 300 min.

grown either euoxically, and then treated in a complete medium (in fact mode-one killing requires active cellular metabolism [14]) or anoxically, and then exposed to the oxidant in a complete medium (in fact anoxic growth induces the synthesis of one or more proteins that augment the sensitivity of cells to mode-one killing by H_2O_2 [14]). Results have shown that *sod B* cells, as well as the superoxide dismutase double mutant [15], are hypersensitive to mode-one killing by H_2O_2 under conditions of either euoxic or anoxic growth, thus suggesting that the superoxide ion may mediate this type of lethality [20]. Data obtained with the *sod A* strain are in apparent contradiction since this mutant was actually more resistant than wild type cells under any of the

experimental conditions utilized in this study. A more detailed investigation has indicated that low concentration of H_2O_2 (mode-one killing region) kill *sod A* cells by a different mechanism, as compared to wild type or *sod B* cells (manuscript in preparation). In fact, whereas mode-one lethality in the latter two strains is not affected by iron chelators or OH· scavengers, it would appear that these agents efficiently prevent the lethality exerted by low concentrations of H_2O_2. Thus, OH· mediates mode-one killing in *sod A* cells but not in wild type or *sod B* cells. Our previous conclusion that the superoxide ion may mediate mode-one killing by H_2O_2, therefore, should not be affected by the results obtained with *sod A* cells. Mode-two killing, as previously mentioned should be generated by the hydroxyl radical.

Fig. 3 displays a scheme for a hypothetical mechanism of cytotoxicity of H_2O_2 in *E. coli*.

We have also studied the morphology of *E. coli* cells challenged with concentrations of the oxidant resulting in the two modes of lethality (manuscript in preparation).

Cells were allowed to grow in the presence of 1.75 mM H_2O_2 (mode-one lethality) or 17.5 mM H_2O_2 (mode-two lethality) and, at various time intervals, the optical density, the colony forming units and the shape of the cells were examined and compared with those of control cells. Results have shown that cells treated with 1.75 mM H_2O_2 displayed an impaired ability to replicate; in fact, optical density values remained constant up to 90 min following exposure to the oxidant and then progressively increased reaching at 300 min an optical density that was half of that of control cells. In addition, a linear decrease with time of exposure was observed by assaying the colony forming units and, after 90 min, only 10^7 cells were viable as compared to the 10^8 cells before the challenge with oxidant. After this time cells recovered their ability to form colonies. Treatment with 17.5 mM H_2O_2 produced a progressive decrease in either optical density or colony forming ability; in fact, optical density values were reduced by 50% in 300 min of exposure to H_2O_2 and colony forming units were reduced of 4 order of magnitude in only 90 min.

Microscopic examination of cells treated with the same concentrations of the toxin revealed dramatic differences in the cells exposed to levels of H_2O_2 resulting in modes one or two killing as compared to control cells. As previously shown by Imlay and Linn [14], we have found that mode-one killed cells underwent extensive filamentation but did not septate (Fig. 4B). This change of morphology occurred after about 90 min exposure to H_2O_2 and was maximal after 180-210 min. Cells treated with 17.5 mM H_2O_2 remained unit-sized (Fig. 4C) but cell volume was reduced to about 50% of control cells, following a 300 min exposure to the oxidant (Fig. 4A).

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