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Mitochondrial Biosynthesis Controls the Sensitivity of Chinese Hamster Cells to Hydrogen Peroxide

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The mechanism of H_2O_2-resistance of Hpr-4, a variant of Chinese hamster V79 cells, was investigated. The effect of H_2O_2 on the mitochondria of the parental and Hpr-4 cells was compared. First, both biochemical and ultrastructural results showed that mitochondria in the parental cells were damaged by exposure to H_2O_2, while those in Hpr-4 cells recovered from the damage. Second, the H_2O_2-resistance of Hpr-4 cells was reversibly reduced or recovered by the addition or removal of inhibitors of mitochondrial biosynthesis, respectively. Third, the parental cells were auxotrophic to pyruvate after exposure to H_2O_2. Fourth, H_2O_2-sensitivity of the parental cells was also enhanced by the inhibition of mitochondrial biosynthesis. From these results, it was concluded that the mitochondria of Hpr-4 cells apparently had a greater resistance to H_2O_2 than those of the parental cells and that functional mitochondria were involved in the recovery of Chinese hamster V79 cells from H_2O_2-induced damage.

INTRODUCTION

Many steps are involved in the cytotoxicity of H_2O_2.[1] H_2O_2 is first converted to reactive intermediates such as ·OH radicals via a metal-catalyzed Fenton reaction, which generates many cellular lesions such as oxidation of proteins and DNA, membrane peroxidation, changes in nucleotide levels, increase in cytosolic Ca^{+2}, and mitochondrial damage. None of these lesions, however, has been well established as a cause of cell death.[2]

In order to determine the lethal targets in cells exposed to cytotoxic drugs, an investigation using a drug resistant mutant is a profitable approach. Actually several investigators have used H_2O_2-resistant cells to clarify the mechanism of resistance.[3,4,5] Except for cells with higher levels of detoxicating enzyme,[3,4] however, the mechanism of H_2O_2-resistance remains obscure.[5] Previously, we isolated H_2O_2-resistant variant cells (Hpr-4) from a spontaneously expanded pool of the parental V79 cells.[6] No difference was found between the parental and Hpr-4 cells in the level of detoxicating enzymes and in the level of OH radicals produced.[7] The above characteristics of H_2O_2-resistant cells are essentially similar to those of cells reported by Cantoni *et al.*[5,8] According to their recent report,[8] there was a weak correlation between H_2O_2-cytotoxicity and DNA strand

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break-level, which we confirmed for Hpr-4 cells (unpublished results).

Since mitochondrial damage and ATP depletion seem to be closely related to cell death by H_2O_2,[2,9,10] an investigation was attempted to clarify whether mitochondria are responsible to H_2O_2-resistance of Hpr-4 cells. We concluded that functional mitochondria were essential for cells to recover from lethal damage from H_2O_2 based on the results that exposure to H_2O_2 led the parental cells to auxotrophy to pyruvate and that inhibitors of mitochondrial biosynthesis reversibly controlled the H_2O_2-resistance of Hpr-4 cells.

MATERIALS AND METHODS

Chemicals

Catalase, ATP, pyruvate and chloramphenicol were purchased from Sigma Chemical Co. (St Louis, MO). Ethidium bromide was purchased from Boots Pure Drug Co. LTD (Nottingham, England). Luciferase-luciferin was purchased from Wako Pure Chem. Ind. LTD (Osaka, Japan). ^{35}S-labelled methionine was purchased from ICN (Irvine, CA).

Cell Culture

Chinese hamster V79 and H_2O_2-resistant cells(Hpr-4) were cultured in MEM (Nissui Pharmaceutical Co., Tokyo, Japan) with 10% fetal bovine serum (FBS, Cell Culture Laboratories, Cleveland, OH) in a humidified (5% CO_2) incubator. When cells were cultured in the presence of ethidium bromide (250 ng/ml) or chloramphenicol (100 μg/ml), they were fortified with pyruvate (0.1 mg/ml).

Oxygen consumption

Cells ($5–10 \times 10^6$) were trypsinized and suspended in 4 ml of complete culture medium to monitor the cell number. After centrifugation ($800 \times g$, 5 min), the cells were resuspended in phosphate-buffered saline (PBS). The oxygen consumption of cells was recorded in a polarographic cell (1.0 ml) at 37°C with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH).

ATP determination

Cellular ATP levels were assayed by bioluminometry, as described by Spragg *et al.*[10] Briefly, cell monolayers ($1–2 \times 10^6$ cells/60 mm Petri dish) were harvested by trypsinization and centrifuged to form a cell pellet. The cell pellet was suspended in PBS (4×10^6 cells/ml). 0.1 ml of the suspension was mixed with 2.0 ml of 10 mM KH_2PO_4, 4 mM $MgSO_4$ (pH 7.4), heated at 95–99°C and cooled in an ice bath. After centrifugation, 1 ml of the supernatant was supplemented with 5 ml of 50 mM $NaAsO_2$, 20 mM $MgSO_4$ (pH 7.4), and then 50 μl of luciferase/luciferin. Precisely 15 s later, the light emission was quantified in a liquid scintillation counter (Beckman, LS3801). Standard solutions of ATP were prepared in 10 mM KH_2PO_4, 4 mM $MgSO_4$ (pH. 7.4), using an extinction coefficient of 15 400 at 259 nm. Standard curves of log photon counts vs. log[ATP] were linear from 10^{-9} to 10^{-11} mol ATP.

Survival experiments

A) Dependence on H_2O_2 concentration

The relative plating efficiencies in the presence of different concentrations of H_2O_2 were determined as the ratio of the number of colonies at a given H_2O_2 concentration to that obtained in the control culture in the absence of any drug, as described previously.[7] Cells were seeded at 5×10^5/60 mm Petri dish in 4 ml of MEM supplemented with 10% FBS. On the next day, cells were exposed to H_2O_2 in BME (Basal Medium Eagle, Sigma Chemical Co.) without serum for 1 hr at 37°C, washed in PBS, trypsinized. 200 to 1000 cells were seeded in 60 mm Petri dishes with 4 ml of MEM containing 10% FBS with or without pyruvate as described in the figure legends for each experiment and were

cultured for 6 days. The colonies (>50 cells/colony) were counted under a dissecting microscope after fixation and staining. A group of three replicate dishes was used to assess the effect of different doses of H_2O_2 on plating efficiency.

B) Dependence on the exposure time

The relative plating efficiency after the treatment with H_2O_2 for different incubation times was determined as the ratio of the number of colonies at a given exposure time to that obtained in the control culture treated with 1 mM H_2O_2 in the presence of catalase. Exponentially growing cells were trypsinized and divided into 1.5 ml centrifugation tubes containing 2×10^5/ml BME without serum, and were incubated for different periods at 37°C in the presence of 1 mM H_2O_2, at which time catalase was added to decompose residual H_2O_2. 200 ~ 400 cells were seeded in 60 mm Petri dishes containing 4 ml of MEM plus 10% FBS with or without supplementation of pyruvate, and were cultured for 6 days.

Electron microscopy

Cells were incubated in BME without serum in the presence or absence of 1 mM H_2O_2 for 1 hr at 37°C. Then, they were washed with PBS, resuspended in fresh medium plus 10% FBS and incubated for 20 hr. After trypsinization, cells were centrifuged to form a pellet, which was fixed for 60 min at 4 °C with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), followed by 1% OsO_4 in the same buffer for 30 min. The cell pellet was then dehydrated with graded ethanol and embedded in Epok 812. Ultrathin sections were stained with uranyl acetate followed by lead citrate and were examined under a Hitachi H-600 electron microscope at an average accelerating voltage of 75 kV.

Incorporation of ^{35}S-methionine

^{35}S-methionine incorporation was performed by the method described by Attardi and Ching.[11] Briefly, exponentially growing cells were collected by centrifugation, resuspended at a concentration of 3×10^6 cells/ml in warmed MEM deprived of methionine with 10% FBS, and incubated for 30 min at 37°C. After supplement of emetine (100 mg/ml) 5 min prior to the labelling, 100 μCi of ^{35}S-methionine was added to the culture, then incubated for 2 hr and kept in ice cold water. The labelled cells were washed three times in PBS and homogenized in 0.5 ml of 0.25 M sucrose in PBS by a Potter homogenizer. The 5000 g pellet by differential centrifugation was suspended in water. A quantity equivalent to three quarters of the suspension was used for a liquid scintillation counting and the remaining quarter was used for the determination of protein content.

RESULTS

Comparison of mitochondrial respiration between the parental V79 cells and H_2O_2-resistant Hpr-4 cells

We measured the oxygen consumption rates of intact cells, in order to compare mitochondrial functional integrity of the parental V79 cells and H_2O_2-resistant (Hpr-4) cells which had been treated with H_2O_2. Figure 1 shows the time course of oxygen consumption rates of both the parental and Hpr-4 cells after treatment with 1 mM H_2O_2 for 1 hr at 37°C together with those of sham-treated cells. In the parental cells, the oxygen consumption rates dropped immediately after the treatment. After 2 ~ 18 hr post-treatment incubation, the rates decreased gradually to about 20% of the initial control levels. On the other hand, for Hpr-4 cells exposed to 1 mM H_2O_2, the initial drop of the rates was less than that of the parental cells and gradually recovered to the initial levels. During the post-incubation period, the viability of both parental and Hpr-4 cells treated with H_2O_2 was between 93 ~ 99% as measured by a dye exclusion test (data not shown). The oxygen consumption rates of the sham-treated parental and Hpr-4 cells increased slightly in an initial 2 to 8 hr and decreased afterwards to the initial level.

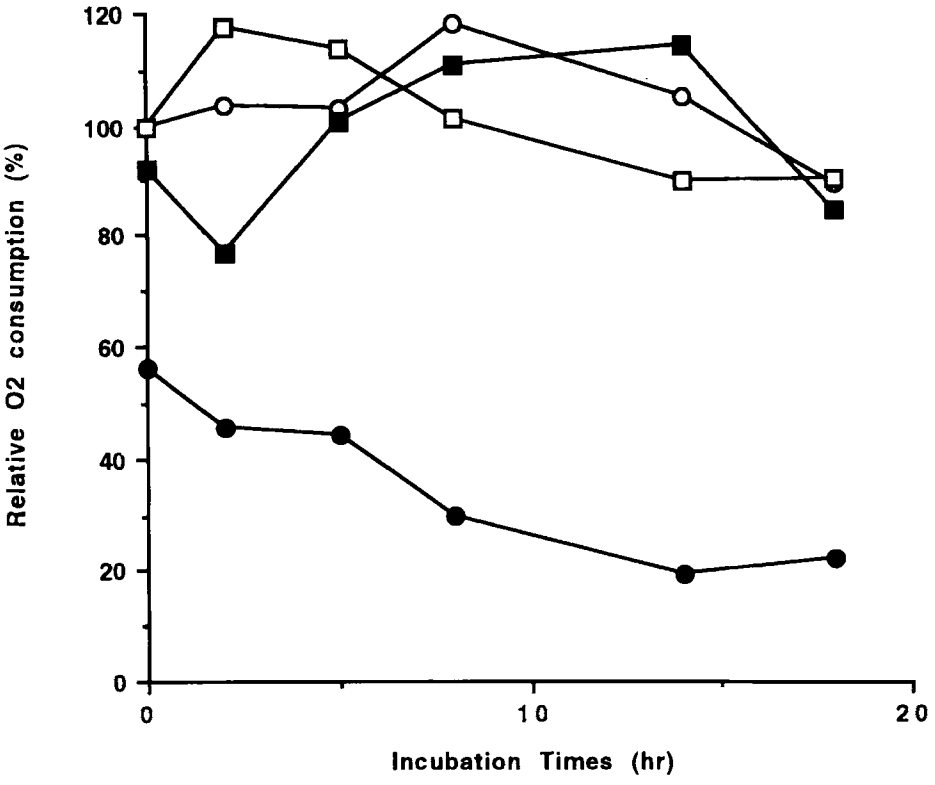

FIGURE 1 Time course of O_2 consumption after exposure to 1 mM H_2O_2 for 1 hr at 37°C. O_2 consumption was expressed as % of O_2 consumption of the sham-treated cells at 0 h. —○—, the sham-treated parental cells, 100% of O_2 consumption at 0 h was 28.5 ± 3.4 nmol $O_2/10^7$ cells/min (the average of 3 experiments); —●—, H_2O_2-treated parental cells; —□—, the sham-treated-Hpr-4 cells, 100% of O_2 consumption of sham-treated Hpr-4 cells at 0 h was 34.1 ± 2.4 nmol $O_2/10^7$ cells/min (the average of 4 experiments); —■—, H_2O_2-treated Hpr-4 cells.

In order to compare the mitochondrial functional integrity with cellular ATP levels after treatment with H_2O_2, we measured ATP levels in the parental and Hpr-4 cells treated with H_2O_2. Figure 2 shows the time course of ATP levels of the parental and Hpr-4 cells after treatment with 1 mM H_2O_2 for 1 hr at 37°C. In both sham-treated parental and Hpr-4 cells, the ATP levels increased 40 ± 20 and 80 ± 20%, respectively, during 5 hr post-incubation period, then decreased to the initial levels. On the other hand, the ATP levels of H_2O_2-treated parental and Hpr-4 cells changed differently, i.e. those of the parental cells dropped immediately after the treatment and maintained reduced levels during the post-incubation period, on the other hand, for those of Hpr-4 cells, the initial reduction was similar to the levels of the parental cells, gradually increasing to the levels of the sham-treated cells. The initial concentrations of ATP in both sham-treated parental and Hpr-4 cells were similar. There is apparent consistency between the change of oxygen consumption and that in ATP levels: both the oxygen consumption rates and the ATP levels of the parental cells which had been exposed to H_2O_2 remained at reduced levels during the post-incubation period, while those of Hpr-4 cells recovered from reduced levels during the same period. However, it was not possible to directly correlate the decrease of ATP levels with that of the oxygen consumption rates, since the former results from the imbalance of consumption and formation, which is by way of both mitochondrial respiration and glycolysis.

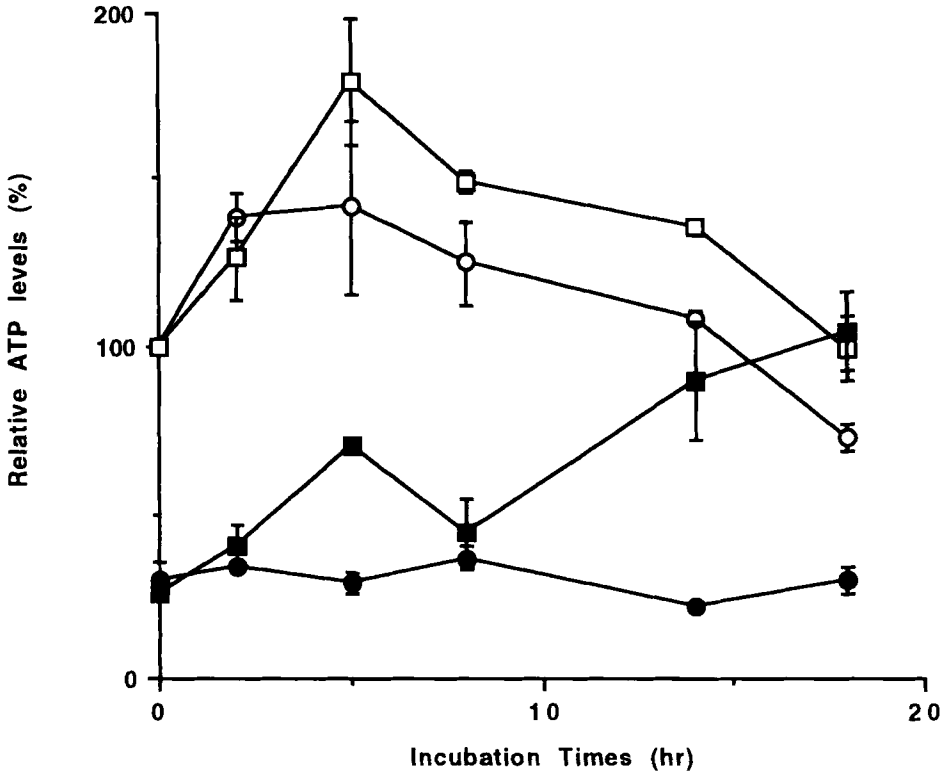

FIGURE 2 Time course of ATP levels after exposure to 1 mM H_2O_2. ATP levels were expressed by % of the sham-treated cells at 0 h. —○—, the sham-treated parental cells, 100% of ATP level at 0 h was 3.4 ± 0.1 nmol/10^6 cells (the average of 4 experiments); —●—, H_2O_2-treated parental cells; —❑—, the sham-treated Hpr-4 cells, 100% of ATP level at 0 h was 2.8 ± 0.4 nmol/10^6 cells (the average of 5 experiments); —■—, H_2O_2-treated Hpr-4 cells. Each time point is the mean of duplicate experiments, which are presented by an error bar.

Since there were differences between the parental and Hpr-4 cells exposed to H_2O_2 as to recovery from an impaired mitochondrial function and from the depletion of ATP levels, we compared mitochondrial ultrastructure of both types of cells. Figure 3 shows the ultrastructure of the parental and Hpr-4 cells 20 hours after H_2O_2- and sham-treatment. The mitochondria of the control samples generally appeared to be oblong with cristae traversing the entire width of the organelle. The density of the mitochondrial matrix was greater than that of the surrounding cytoplasm. H_2O_2-treatment accompanied by post-treatment incubation brought about important structural changes in the mitochondria of the parental cells. Most of the inner membranes appeared shrunken where cristae disappeared or segregated as electron dense particles. In contrast to the parent cells, the mitochondria of Hpr-4 cells remained intact, preserving the initial structure. Accordingly, both biochemical and ultrastructural observation indicated that mitochondria of Hpr-4 cells had a greater resistance to H_2O_2-exposure than those of the parental cells.

Alteration of the sensitivity of Hpr-4 cells to H_2O_2 by the inhibition of mitochondrial biosynthesis

If mitochondria were really responsible for the H_2O_2-resistance of Hpr-4 cells, inhibition of mitochondrial function in Hpr-4 cells should lead to a

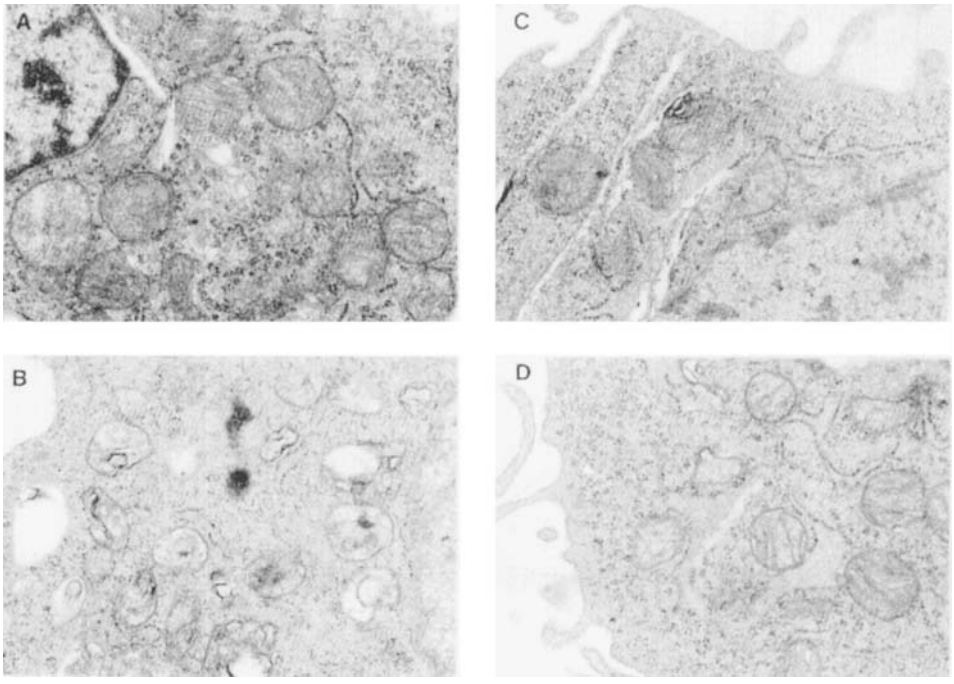

FIGURE 3 Electron micrographs of cells exposed to 1 mM H_2O_2 and post-incubated for 20 hr. A and B, the parental cells; C and D, Hpr-4 cells. A and C were sham-treated for 1 hr at 37 °C and incubated for 20 hr. B and D were H_2O_2 (1 mM)-treated for 1 hr at 37 °C and incubated for 20 hr. Bars (A, B, C, and D) represent 0.5 μm.

loss of their resistance to H_2O_2. Concerning the inhibition of mitochondrial function, it has been shown that ethidium bromide and chloramphenicol block mitochondrial transcription and protein synthesis, respectively.[12,13] As a consequence, it was expected that the mitochondrial function of Hpr-4 cells which had been cultured in the presence of ethidium bromide or chloramphenicol would be impaired. The inhibitory effect of these inhibitors on the mitochondrial protein synthesis and mitochondrial respiration is shown in Table 1 together with the results of the parental cells. Certainly, these inhibitors partially reduced the level of mitochondrial protein synthesis and oxygen consumption in both Hpr-4 cells and the parental cells in accord with previous observations.[12,13]

In the next step, we checked the possible modifying effect of these inhibitors on H_2O_2-sensitivity of Hpr-4 cells under the following two groups of experimental conditions. In the first group, Hpr-4 cells were cultured for 5 days in the presence of ethidium bromide or chloramphenicol. In the second group, cultured cells which had been exposed to these inhibitors for 5 days were cultured for a further 5 days in the absence of these inhibitors. The survival of these groups of cells in response to H_2O_2-dose is shown in Figure 4a and 4b for ethidium bromide and chloramphenicol, respectively, together with that of the control Hpr-4 cells and the parental V79 cells. At concentrations of H_2O_2 lower than 2 mM, Hpr-4 cells in the first group were much more sensitive to H_2O_2 than the control Hpr-4 cells. Their sensitivity resembled that of the parental cells. On the contrary, Hpr-4 cells in the second group recovered their H_2O_2-resistance and showed a survival similar to that of the control Hpr-4 cells. From these results, we concluded that mitochondrial function was essential to H_2O_2-resistance of Hpr-4 cells. In other words, the H_2O_2-resistance of Hpr-4 cells was

TABLE 1 Incorporation of ^{35}S-methionine and oxygen consumption of cells cultured in the presence of ethidium bromide or chloramphenicol.

Cells	Culture conditions	^{35}S-methionine incorporation (dpm/μg protein)	O_2 consumption ($nmolO_2/10^7$/min)
Hpr-4	control	338.7 ± 38.3	14.0 ± 0.6
	ethidium bromide (250 ng/ml, 5 days)	105.5 ± 58.6	6.0 ± 1.2
	chloramphenicol (100 μg/ml, 5 days)	62	4.8 ± 1.2
V79	control	319.3 ± 5.7	15.9 ± 0.6
	ethidium bromide (250 ng/ml, 5 days)	134.3 ± 19.5	7.1 ± 2.6
	chloramphenicol (100 μg/ml, 5 days)	181.9	5.0

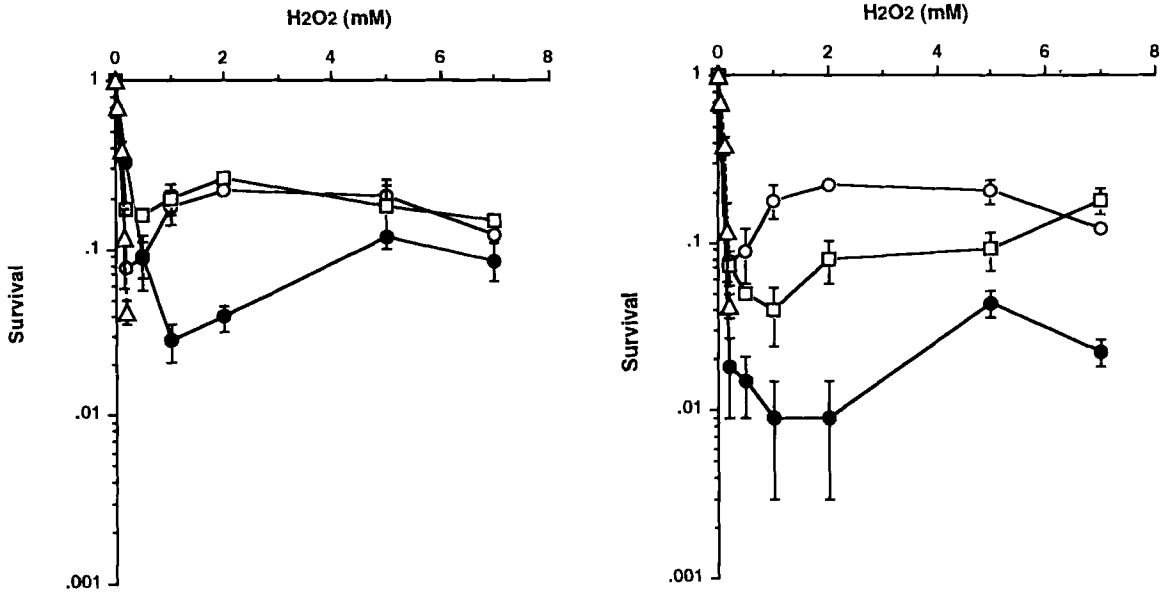

FIGURE 4 The relative plating efficiency of Chinese hamster Hpr-4 cells treated with H_2O_2 at 37°C for 1 hr. All cultures were supplemented with pyruvate (0.1 mg/ml) after seeding to 60 mm Petri dishes. a) Effect of ethidium bromide (250 ng/ml) on the plating efficiency. —○—, control Hpr-4 cells; —●—, ethidium bromide was added to the culture for 5 days;—❑—, ethidium bromide was added to the culture for 5 days and omitted from the successive 5 days' culture. b) Effect of chloramphenicol (100 μg/ml) on the plating efficiency. —○—, control Hpr-4 cells; —●—, chloramphenicol was added to the culture for 5 days;—❑—, chloramphenicol was added to the culture for 5 days and removed for 5 days. For both a) and b), - - -Δ- - - represents the control parental V79 cells. The error bars represent the standard deviation of triplicate experiments.

reversibly controlled by the presence or removal of the inhibitors of mitochondrial biosynthesis.

Involvement of functional mitochondria in H_2O_2-induced toxicity of the parental cells

It has been well established that mitochondria-deficient cells and cells treated with mitochondrial inhibitors such as antimycin or chloramphenicol require pyruvate.[15] Pyruvate is an essential precursor for various biosynthesis in the cells with an impaired function of mitochondria, but with sufficient ATP supply via glycolysis.[15] The auxotrophicity of pyruvate was used to check the possible involvement of mitochondrial function in H_2O_2-induced toxicity of the parental cells. After exposure to 1 mM H_2O_2 for increasing time intervals, cells were treated with catalase to decompose residual H_2O_2 and were diluted for the colony forming assay in dishes with or without the supplement of pyruvate. As shown in Figure 5, H_2O_2-treated parental cells cultured in the absence of pyruvate were more sensitive to H_2O_2 than those in the presence of pyruvate. The

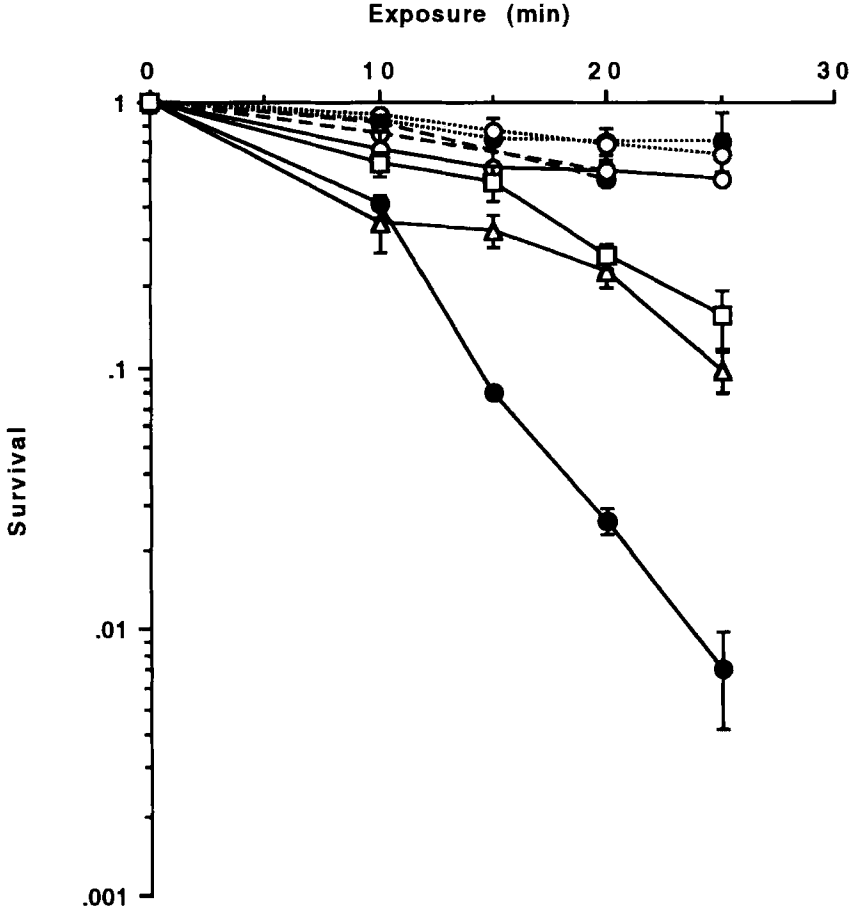

FIGURE 5 Time course of the plating efficiency of cells exposed to H_2O_2. —O—, Control parental cells exposed to 1.5 mM H_2O_2; —Δ—, parental cells cultured in the presence of ethidium bromide (250 ng/ml) for 5 days and exposed to 1.5 mM H_2O_2 —❑—, parental cells cultured in the presence of chloramphenicol (100 μg/ml) for 5 days and exposed to 1.5 mM H_2O_2. All cultures were assayed in the presence of pyruvate (0.1 mg/ml). —●—, control parental cells exposed to 1.5 mM H_2O_2 and assayed in the absence of pyruvate. ....O...., control Hpr-4 cells exposed to 1.5 mM H_2O_2 and assayed in the presence of pyruvate; ....●...., control Hpr-4 cells exposed to 1.5 mM H_2O_2 and assayed in the absence of pyruvate; - - -O- - -, control Hpr-4 cells exposed to 5 mM H_2O_2 and assayed in the presence of pyruvate; - - -●- - -, control Hpr-4 cells exposed to 5 mM H_2O_2 and assayed in the absence of pyruvate.

requirement of pyruvate for H_2O_2-treated cells was consistent with the interpretation that mitochondria received damage from the H_2O_2-treatment. In contrast with the result which was achieved with the parental cells, the sensitivity of Hpr-4 cells was not altered by the supplementation with pyruvate at 1.5 and 5 mM H_2O_2. This lends further support to the assumption that H_2O_2-resistance of Hpr-4 cells compared to the parental cells might be due to H_2O_2 resistance of mitochondria in Hpr-4 cells.

The above interpretation was rechecked by inhibiting the mitochondrial function of the parental cells in the presence of ethidium bromide or chloramphenicol in the medium supplemented with pyruvate. The inhibiting effect of these substances on protein synthesis and oxygen consumption in the parental cells was confirmed by the result shown previously in Table 1. These cells were exposed to 1 mM H_2O_2 for different length of times and cultured to measure plating efficiency in the presence of pyruvate after addition of catalase. As shown in Figure 5, inhibition of mitochondrial biosynthesis enhanced the H_2O_2-sensitivity of the parental cells, slightly but reproducibly, towards the level of cells cultured without pyruvate. Therefore, the effect of these inhibitors on H_2O_2-sensitivity was consistent in both the parental and Hpr-4 cells.

DISCUSSION

The results obtained in the present work can be summarized as follows: 1) a slightly impaired mitochondrial functional integrity of Hpr-4 cells recovered in a few hours after exposure to 1 mM H_2O_2, while that of the parental cells remained impaired by the same treatment (Figure 1). 2) Hpr-4 cells became more sensitive to H_2O_2 by inhibition of mitochondrial biosynthesis and restored their initial resistance by removing the inhibitors (Figure 4). 3) The parental cells were auxotrophic for pyruvate after exposure to H_2O_2 (Figure 5). 4) The parental cells showed slight but reproducible enhancement of H_2O_2-sensitivity by inhibiting mitochondria biosynthesis (Figure 5).

Mitochondria have been reported to be damaged by oxidative stress,[15] yet, mitochondria have not been proved as a critical target of cell death. There is circumstantial evidence that mitochondria may be involved in H_2O_2-induced cell death. In cells treated with H_2O_2, a reduction of the ATP level and a parallel reduction of oxygen consumption have been reported to be correlated with cell death.[15] Furthermore, a large number of mitochondrial proteins has been reported to be inactivated by H_2O_2-exposure,[17] i.e. NADH dehydrogenase, NADH oxidase and ATPase. Among these enzymes, NADH dehydrogenase and ATPase contain proteins encoded by mitochondrial DNA.[16] In spite of this information, we do not know whether mitochondria inactivation in cells was due to damage to proteins, membrane lipids or DNA in mitochondrion. After H_2O_2-treatment, mitochondrial functional integrity was impaired in the parental and slightly in Hpr-4 cells. The mitochondrial function in the parental cells decreased during post-incubation period with no change of viability, while that in Hpr-4 cells recovered in a few hours. On the other hand, ATP levels in both the parental and Hpr-4 cells dropped to 20% of the control levels. Those in the parental cells remained at the reduced levels, while those in Hpr-4 cells gradually increased to the control levels (Figure 2). Although it was reported that the decreased ATP levels following exposure to mM concentrations of H_2O_2 were due to a decreased formation of ATP by both glycolytic and mitochondrial syntheses in P388D_1 murine cell line,[10] we had no information about it. Since, the mitochondrial inner membranes in the parental cells shrank after 20 hr incubation as shown in Figure 3, the decreased synthesis of ATP by the mitochondria might be attributed to the decreased levels of ATP in the parental cells after 18 hr post-incubation.

Since the respiratory deficient cells are auxotrophic for pyruvate,[14,15] we used the pyruvate requirement of H_2O_2-treated cells as a probe for

mitochondria damage. Previously, the protective effect of pyruvate on cell toxicity by H_2O_2 was explained by antioxidative effect of pyruvate.[16] To avoid the possible antioxidative effect of pyruvate, in the present work, pyruvate was added after the treatment with H_2O_2. Cells cultured in the absence of pyruvate were more sensitive to H_2O_2 than those in the presence of pyruvate even in the presence of catalase in the culture medium (data not shown). Therefore, the antioxidative effect could not explain the protective effect of pyruvate in the medium after H_2O_2-exposure.

As shown in Figure 4, the presence of inhibitors of mitochondrial biosynthesis reduced the survival of Hpr-4 cells to the level of the parent cells below 1 mM H_2O_2. At concentrations above 5 mM H_2O_2, inhibition of mitochondrial biosynthesis had no effect on the survival of Hpr-4 cells. The mechanism of toxicity of H_2O_2 beyond 5 mM would be different from that below 1 mM. The former might be independent of mitochondria. Inhibition of mitochondrial biosynthesis enhanced H_2O_2-sensitivity both in the parental cells and Hpr-4 cells. Mitochondrial function might reduce or repair lethal cellular damage by H_2O_2. This is the first observation that H_2O_2-resistance of Hpr-4 cells was reversibly controlled by modulation of mitochondrial function. Based on these observations, we propose the following model of two lethal targets for H_2O_2-induced cytotoxicity of Chinese hamster V79 cells.

In our model, critical targets for cell death by H_2O_2 consist of mitochondria and another unknown target X. Cells with damaged mitochondria but with intact X would be killed in the absence of pyruvate. With the supplement of pyruvate, cells with damaged mitochondria but with intact X could survive without mitochondrial function. Cells with both damaged mitochondria and damaged X would be killed, whereas cells with damaged X but with intact mitochondria would be rescued by the help of mitochondrial function. In the parental cells, functional mitochondria were easily damaged by the treatment with H_2O_2. Consequently, damaged X would be fatal to the parent cells even in the presence of pyruvate, due to the fact that mitochondria were damaged. In the case of Hpr-4 cells, functional mitochondria recovered from the initial damage. Therefore, Hpr-4 cells with damaged X would be able to survive. The unknown lethal target X could be a glycolytic pathway which is a target of H_2O_2 and contributes to the synthesis of ATP.[10]

The bimodal pattern of killing by H_2O_2 was originally observed in E. coli by the group of Linn.[19] Recently, they observed the effect of iron-chelators on cell killing by H_2O_2 to investigate the mechanism of two modes of killing and interpreted the mechanism based on the site of Fe^{2+} associated with DNA.[20] They showed that mode I killing (observed at low concentrations of H_2O_2 up to 2 mM) was blocked by 1,10-phenanthroline and mode II killing (observed at high concentrations of H_2O_2 up to 20 mM) was enhanced by the iron-chelator, and it was considered that 1,10-phenanthroline might enhance mode II killing by serving as a shuttle for delivering Fe^{2+} to DNA. In contrast to the observation in E. coli, both modes of killing by H_2O_2 in parental and Hpr-4 cells (one; less than 300 μM, the other; less than 3 mM H_2O_2, apparently corresponding to two modes of killing in E. coli) were protected by the presence of 1,10 phenanthroline.[6] Therefore, the different mode of killing in V79 cells may not be due to different association of Fe^{2+} to DNA.

It has been proposed that active oxygen species are involved in tumorigenesis[21] and that tumorigenesis is due to escape from apoptosis.[22] Several human tumor cells have been reported to exhibit a resistance to H_2O_2 independent of the ability to degrade H_2O_2.[23] If the resistance was expressed before neoplastic transformation, contribution of oxidative stress on tumorgenesis could be strengthened by H_2O_2-resistance. Those cells with elevated H_2O_2-resistance could have a better chance toward malignant conversion by oxidative stress. Since contribution of mitochondria on ATP synthesis in normal human tissue is high, there is some possibility that H_2O_2-resistance due to the alteration of mitochondria plays a substantial role in the carcinogenic process.

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