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DNA breakage caused by hydrogen peroxide produced during the metabolism of 2-methyl-1,4-naphthoquinone (menadione) does not contribute to the cytotoxic action of the quinone

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Menadione is a redox active quinone that increases intracellular levels of superoxide anion free radical ($O_2^{\cdot-}$) and hydrogen peroxide [1]. Although a causal role for H_2O_2 and $O_2^{\cdot-}$ has been suggested [1, 2], the relative contribution of these reactive species in menadione-induced cell damage has not been definitely established. It is known that $O_2^{\cdot-}$ is characterized by a low reactivity, as compared to the high reactivity of hydroxyl radicals ($OH^{\cdot}$) formed by H_2O_2 and redox active metals and therefore it could be speculated that the toxicity of $O_2^{\cdot-}$ is mediated by its conversion (catalysed by the enzyme superoxide dismutase) to hydrogen peroxide. On the other hand, the extremely high reactivity of the $OH^{\cdot}$, and its very short half life, could be limiting factors, as far as toxicity is concerned, since this species may react with almost every biological macromolecule (critical and non-critical targets). The low reactivity of $O_2^{\cdot-}$ may be an important factor for allowing these species to reach critical targets, such as the DNA, where lethal lesions could be produced. Thus, a possibility exists that $O_2^{\cdot-}$ may mediate at least part of the cytotoxic response elicited by menadione. Indeed, recent evidence indicates that $O_2^{\cdot-}$ is capable of producing DNA damage and cytotoxicity [3].

In this study we have investigated the role played by hydrogen peroxide in the induction of DNA damage and cytotoxicity following treatment of cultured mammalian cells with low concentrations of menadione. Evidence is presented which suggests that most of the DNA breaks detectable following treatment with the quinone are produced by a Fenton reaction. These DNA lesions, however, do not significantly contribute to menadione cytotoxicity.

Materials and Methods

Materials. Radiolabelled compounds were purchased from New England Nuclear (Boston, MA, U.S.A.). Free acid EDTA, disodium EDTA, tetrasodium EDTA, sodium dodecyl sulfate, 1,10-phenanthroline and menadione (2-methyl-1,4-naphthoquinone) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Tetraethylammonium hydroxide was purchased from Merck-Schuchardt (Munchen, F.R.G.). Polycarbonate filters were from Nuclepore (Pleasanton, CA, U.S.A.). McCoy's 5a medium, foetal bovine serum and trypsin were from Gibco (Grand Island, NY, U.S.A.).

Cells and radioactive labelling. Wild type (AA8) Chinese Hamster Ovary (CHO) cells were routinely grown in McCoy's 5a medium supplemented with 15% foetal bovine serum, 2 mM L-glutamine and 1% penicillin–streptomycin in an atmosphere of 5% CO_2 in air, at 37°. All experiments were performed with log-phase cells (1×10^6/60 mm dish). Experimental cultures for alkaline elution were plated in 60 mm tissue culture dishes, labelled overnight with [*methyl*-^{14}C]thymidine (0.05 μCi/mL) and then chased for 6 hr in a label-free medium.

Alkaline elution assay. Cells containing [^{14}C]DNA were exposed for 20 min to increasing concentrations of menadione at 37°, in the absence or presence of 1,10-phenanthroline, and then analysed for DNA breakage. At the end of treatments, cells were removed from the dishes by trypsinization (1% trypsin for 5 min at ice temperature). The filter elution assay was carried out by a procedure virtually identical to that described by Kohn *et al.* [4] with minor modifications [5]. Briefly, 5×10^5 cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 mL of ice-cold Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose) containing 5 mM EDTA (disodium salt). Cells were then lysed with 5 mL of 2% sodium dodecyl sulfate, 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 overnight in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulfate (pH 12.1), at a flow rate of *ca.* 30 μL/min. Fractions of approximately 3 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 N 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.

Cytotoxicity assay. Cells were seeded at a density of 5×10^5 cells/60 mm dish and, after 24 hr, were treated for 20 min with various concentrations of menadione in complete medium either in the absence or presence of 1,10-phenanthroline. Cell 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 hemocytometer.

Superoxide assay. Superoxide production was measured as the reduction of acetylated cytochrome *c* using the wavelength pair 550–540 nm [6]. Addition of superoxide

dismutase (0.1 mg/mL) enabled the background reduction rate to be subtracted. Acetylated cytochrome *c* (0.2 mg/mL) was added prior to the addition of the substrate.

Results and Discussion

Exposure of cultured CHO cells for 20 min to menadione results in the induction of a concentration-dependent DNA single-strand breakage (Fig. 1). The level of DNA damage appears highly dependent on the incubation medium and follows the order McCoy's 5a > PBS + 5 mM glucose > PBS (Fig. 1). Thus, glucose seems to play a critical role for the production of DNA single-strand breaks, and this is not surprising since cellular reactions involved in the redox cycling of the quinone are strictly dependent on the availability of reducing equivalents from NAD(P)H [6]. The inclusion of glucose in the incubation medium would, in fact, lead to the generation of NAD(P)H by forcing the hexose monophosphate shunt. The supply of NAD(P)H to cytochrome *c* reductase, cytochrome b_5 reductase and DT-diaphorase, increases the rate of reduction of menadione to the semiquinone free radical and, in the presence of molecular oxygen, would increase also the rate of superoxide anion production.

The influence of glucose on metabolic events leading to the production of oxygen radicals in cells exposed to menadione can be appreciated from the data shown in Fig. 2, where the rate of O_2^- production is reported. The analysis of the results depicted in Fig. 2 indicates that near maximal production of O_2^- occurs at concentrations of menadione as low as 25 μM following incubation in either PBS or PBS plus glucose; in the latter case, however, the level of superoxide was at least three times higher as compared to the former one.

In order to investigate whether DNA breaks (as assayed by the alkaline elution technique) were dependent on a reaction of the Fenton type, increasing concentrations of the intracellular iron chelator 1,10-phenanthroline were added to the medium during exposure to a 50 μM concentration of the quinone. Results shown in Fig. 3 indicate that 1,10-phenanthroline was capable of reducing the extent of DNA lesions in a concentration-dependent fashion. It should be noted, however, that even at the

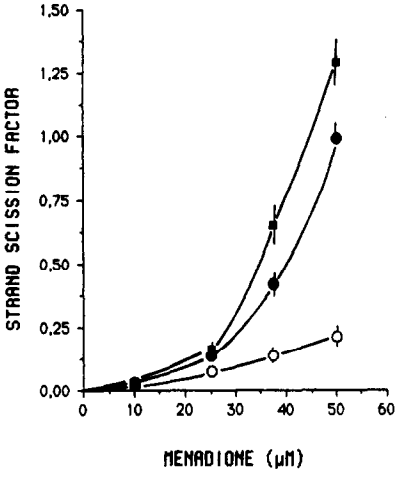

Fig. 1. Induction of DNA single strand breakage by menadione. CHO cells were exposed for 20 min to increasing concentrations of menadione in a complete medium (McCoy's 5a), (■) or PBS, (○) or PBS plus 5 mM glucose, (●). DNA single-strand breaks were measured by the alkaline elution technique (see Materials and Methods). Data are expressed as strand scission factor (SSF) that was calculated from the DNA elution profiles according to the following relationship: $SSF = -\log A/B$ where A = fraction of DNA retained on the filter after 9 hr of elution for treated cells, and B = fraction of DNA retained on the filter after 9 hr of elution for untreated cells. Values represent the mean ± SEM from at least three separate experiments.

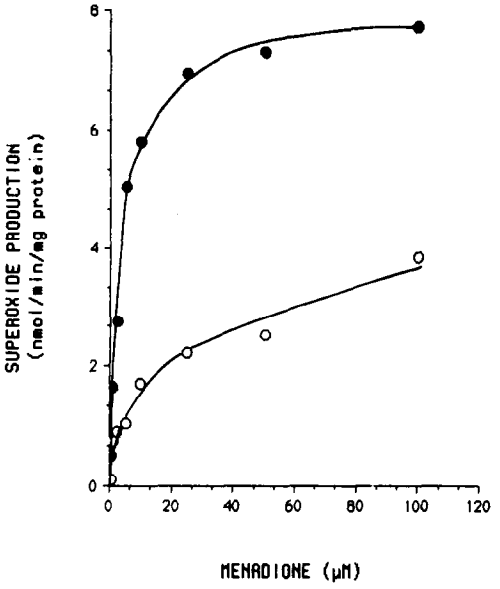

Fig. 2. Production of superoxide by CHO cells treated with menadione. Cells were exposed for 20 min to increasing concentrations of menadione in either PBS (○) or PBS plus 5 mM glucose (●). The release of superoxide to the extracellular milieu was assayed as detailed in Materials and Methods. Shown in the figure are the results from a representative experiment.

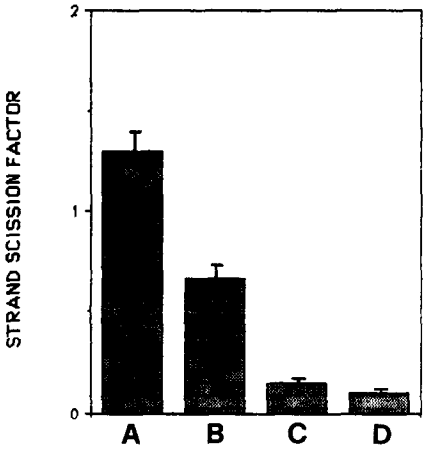

Fig. 3. Effect of 1,10-phenanthroline on menadione-induced DNA single-strand breakage. CHO cells were exposed for 20 min to 50 μM menadione in McCoy's 5a medium in the absence (A) or presence of 10 μM (B), 25 μM (C), 50 μM (D) 1,10 phenanthroline and assayed for DNA breakage by the filter elution assay. Results are expressed as detailed in legend to Fig. 1. Values represent the mean ± SEM from at least three separate experiments.

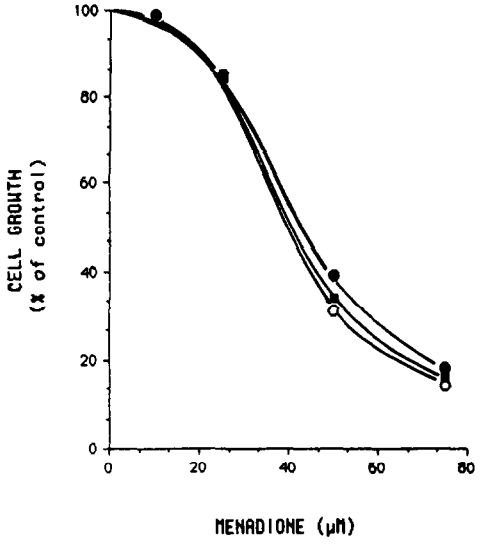

Fig. 4. Growth inhibitory effect of menadione in the absence or presence of 1,10-phenanthroline. CHO cells were exposed for 20 min to increasing concentrations of menadione in the absence (○) or presence of 10 (■) or 25 μM (●) 1,10-phenanthroline. The cell number was estimated following 48 hr of growth. Data points are the means of three independent experiments, each performed in duplicate. Standard errors are not shown for clarity and were less than 10%.

highest concentration tested, i.e. 50 μM, the iron chelator did not abolish DNA damage. These data may suggest that a proportion of DNA single-strand breaks was not produced by hydrogen peroxide since treatment with 10 μM 1,10-phenanthroline completely prevents DNA breakage induced by exogenously added H_2O_2 (not shown and Refs. 7 and 8). Neutral elutions were performed in cells treated with concentrations of menadione up to 75 μM and results have demonstrated that the quinone was not capable of producing DNA double-strand breakage (not shown). It should be noted that also hydrogen peroxide (tested at concentrations up to 1 mM) was unable to produce DNA double-strand breakage at physiologic temperature [9].

The effect of 1,10-phenanthroline was also tested on the cell growth inhibitory activity of menadione. Figure 4 indicates that the iron chelator, at concentrations as high as 25 μM, did not affect the toxicity of the quinone. It should be noted that, again, the toxicity of H_2O_2 was completely prevented by this iron chelator (not shown and Refs. 7–10).

Taken together, these data indicate that hydrogen peroxide produced during the metabolism of menadione is responsible for the induction of most of the DNA breakage detectable following exposure to the quinone and that this type of lesion does not result in cytotoxicity. It should be noted, however, that the results presented in this report do not rule out the possibility that DNA damage is the cause of, or at least contribute to, the induction of cell death following treatment with menadione, since a small proportion of DNA single-strand breaks was H_2O_2-independent. In addition, other types of DNA lesions (which cannot be detected by the filter elution assay) may be relevant in this regard.

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