Effects of quin2 acetoxymethyl ester on H_2O_2-induced DNA single-strand breakage in mammalian cells: H_2O_2-concentration-dependent inhibition of damage and additive protective effect with the hydroxyl-radical scavenger dimethyl sulphoxide

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The cell-membrane-permeable calcium probe quin2 acetoxymethyl ester (quin2 AM) was ineffective, in comparison with *o*-phenanthroline, in protecting cells against H_2O_2-induced DNA single-strand breakage at H_2O_2 concentrations of about, and higher than, 0.5 mM. The present study shows that quin2 actually potentiated intracellular DNA damage at high H_2O_2 concentrations. H_2O_2-induced DNA breakage appeared within 5 min after exposure, and quin2 affected the induction of DNA breaks at both 0 °C and 37 °C. Aurintricarboxylic acid, an endonuclease inhibitor, or a decrease in extracellular Ca^{2+}, did not reduce DNA damage. These facts strongly suggest that the breaks were not produced by a Ca^{2+}-dependent nuclease. We showed previously that, in the presence of Fe^{3+} and H_2O_2, quin2 strongly potentiated the formation of oxidizing species as well as plasmid DNA breakage, and, as could be expected for a transition-metal chelator, quin2 inhibited the Fenton reaction when Cu^{2+} was tested instead of Fe^{3+} [Sandström, Granström and Marklund (1994) Free Radicals Biol. Med. **16**, 177–185]. In the present work with cultured cells, titration with quin2 AM showed that, despite the fact that Cu^{2+} has a three-to-four-orders-of-magnitude higher affinity for quin2 than has Fe^{3+}, both inhibition and potentiation of H_2O_2-induced DNA damage occurred at quin2 AM concentrations of about 100 nM. Thus inhibition appeared not to involve Cu^{2+}. The combination of quin2 AM and dimethyl sulphoxide (DMSO) gave an additive effect on H_2O_2-induced DNA damage compared with the effect of quin2 AM or DMSO alone, whereas the combination of *o*-phenanthroline and DMSO gave about the same effect as *o*-phenanthroline alone. In conclusion, our results do not support a role for Ca^{2+} in the inhibiting effect of quin2 on H_2O_2-induced DNA damage. Instead, it is likely that inhibition and potentiation by quin2 involves interaction with Fe ions.

INTRODUCTION

Mammalian tissues produce H_2O_2 during normal metabolism partly as a consequence of superoxide-radical ($O_2^{-\cdot}$) dismutation, either spontaneously or catalysed by superoxide dismutases. H_2O_2 can also be produced by different oxidases, u.v. radiation or ionizing radiation. The major H_2O_2-detoxification enzymes in mammalian cells are catalase and glutathione peroxidase. The uncharged H_2O_2 molecules pass easily across membranes and have been proposed to cause intracellular damage to, for example, DNA, by reacting with transition-metal ions [1]. This reaction is thought to generate the highly reactive hydroxyl radical ($HO^{\cdot}$)[2] or another oxidizing species with equal reactivity [3] that will indiscriminately hit targets at the site of reaction. Depending on the targets, the damage may lead to mutations or cell death. An alternative hypothesis concerning H_2O_2 cytotoxicity views alterations in intracellular Ca^{2+} homoeostasis following glutathione and protein thiol depletion as more critical [4]. Recently Cantoni and his colleagues [6], after performing experiments with the fluorescent calcium probe quin2 [5], proposed, in line with the latter hypothesis, that H_2O_2-induced DNA strand breakage and cytotoxicity could be caused by disturbance of Ca^{2+} homoeostasis. They suggested that secondary reactions could ultimately lead to DNA strand breakage and pointed to the possibility that a Ca^{2+}-dependent endonuclease [7] might be directly responsible for most of the H_2O_2-induced DNA damage. The protective effect of quin2 on t-butyl hydroperoxide- [8], methyl linoleate hydroperoxide- [8] or quinone-induced cytotoxicity [9] are other examples of studies potentially involving oxygen-radical formation, where preferentially Ca^{2+}-dependent mechanisms of protection have been proposed.

We have recently shown [10] that quin2 in a mixture with Fe^{3+} and H_2O_2 potentiated reactive-oxygen-species formation and plasmid DNA strand breakage. Hydroxyl radical appeared to be the major oxidizing species produced in the reaction and, surprisingly, superoxide dismutase was ineffective as inhibitor of the reaction. This is in complete contrast with the effect of superoxide dismutase on Fenton-type reactions with other Fe^{3+} chelators such as EDTA and nitrilotriacetic acid [11] and indicates that superoxide reduction of Fe^{3+}-quin2 may not be necessary for reaction. When we mixed quin2 with Cu^{2+} and H_2O_2, quin2 inhibited both reactive-oxygen-species production and plasmid DNA strand breakage [10]. The present study was undertaken, on the basis of the new effects of quin2 found by us, to gain further insight into the mechanisms behind quin2 inhibition of H_2O_2-induced DNA damage in mammalian cells.

MATERIALS AND METHODS

Cell culture

P31 is a human cell line, derived from a mesothelioma of probable asbestos-induced origin, that has low activities of the H_2O_2-reducing enzymes catalase and glutathione peroxidase [12]. U-937 originates from a histiocytic lymphoma and is a cell line with very high activities of both catalase and glutathione peroxidase [12]. The difference between the cell lines in respect of

Abbreviations used: AM, acetoxymethyl ester; DMSO, dimethyl sulphoxide.

catalase activity has been shown to be more than 100-fold and that of glutathione peroxidase activity to be about 30-fold [12]. Chinese hamster V79-379A cells, with an intermediate sensitivity to H_2O_2, were also used in the study. Stock cultures of P31 and V79-379A cells were maintained in Ham's F-10 medium (NordCell, Bromma, Sweden) and U-937 cells in RPMI 1640 medium (NordCell) at 37 °C in CO_2/humidified air (1:19). The media were supplemented with 10% foetal-calf serum, antibiotics and L-glutamine (NordCell) as previously described [13]. We changed the medium two or three times a week, and the cells were subcultured once (P31 and U-937 cells) or twice (V79-379A cells) a week to maintain exponentially growing cultures.

DNA-single-strand-break assay

We detected DNA single-strand breakage with the DNA precipitation assay [14]. Exponentially growing cells were labelled with [^{3}H]thymidine [3.7 kBq (0.1 μCi)/ml; sp. radioactivity 74 GBq (2 Ci)/mmol (Amersham International)]. The labelling medium was removed after 18 h (V79-379A cells) or 42 h (P31 and U-937 cells) and replaced with non-radioactive medium for ~ 1 h. The anchorage-dependent P31 and V79-379A cells were then trypsin-treated and diluted, in Hepes-buffered (37 °C) medium, to 4×10^4 cells/ml, before exposure to H_2O_2 as previously discussed [15].

We made a stock solution of the quin2 acetoxymethyl ester (quin2 AM) by dissolving it in dimethyl sulphoxide (DMSO). It was kept frozen at −20 °C between experiments. Quin2 AM (generally at a concentration of 150 nM) was added 40 min before, *o*-phenanthroline (25 μM) 30 min before, and DMSO (1 M) 10 min before, exposure to H_2O_2. As we dissolved quin2 AM in DMSO, control cells were given the same amount of DMSO (~ 5 mM) as quin2 AM-treated cells. We exposed the cells under repeated agitation to H_2O_2 for 10 min (Sigma Chemical Co.) in a water bath at 37 °C. In the experiments described in Figure 1 (0 °C), the cells were exposed on ice for 10 min. After centrifugation the medium was discarded and the cells were washed once with ice-cold Hepes-buffered medium. Thereafter, the protocol described by Olive for DNA precipitation [14] was essentially followed.

Thus individual samples of (3–4) $\times 10^5$ cells in 100 μl of medium were lysed in 0.5 ml of 2% SDS, pH ~ 12.2, for at least 1 min, after which the samples were moved to a heating block (65 °C) one by one, and 0.5 ml of KCl (0.12 M) was added as gently as possible. After 10 min at 65 °C, allowing damaged regions of DNA to 'melt' and dissociate from histones, the samples were transferred to an ice bath for about 10 min. The precipitate, consisting of K^+, SDS, proteins and intact DNA attached to histones, was pelleted by centrifugation at 2500 *g* at 4 °C for 10 min. Then the supernatant was separated from the pellet and the amount of DNA in the precipitate relative to the total amount of DNA (precipitate + supernatant) was estimated by liquid-scintillation counting (Tri-Carb 2500 TR/AB; Packard, Meriden, CT, U.S.A.). Our controls contained around 85–95% precipitated DNA. The DNA precipitation assay appears to be saturated when the amount of precipitated DNA has decreased to about 10% (B. E. Sandström, unpublished work).

RESULTS

Effects of quin2 AM on H_2O_2-induced DNA damage

The membrane-permeable AM ester form of quin2 has to be used in work with cells. Inside the cell, the hydrophobic quin2 AM is de-esterified by cytosolic esterases to the hydrophilic quin2 form. It has been shown that this process gives an intracellular quin2 concentration 10–20 times higher than the extracellular quin2 AM concentration [16,17].

Extending previous observations [6] concerning quin2 inhibition of H_2O_2-induced DNA damage, we found that quin2 at 37 °C was only efficient in protecting cells from H_2O_2-induced DNA damage at low H_2O_2 concentrations (Figures 1–3). On increasing the concentration of H_2O_2 we found a gradual loss of protection and, in the two more H_2O_2-resistant cell types, U-937

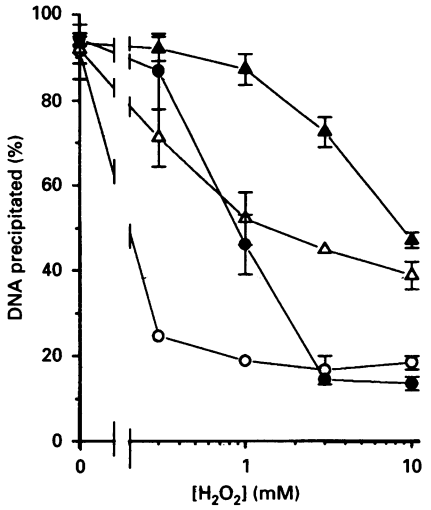

Figure 1 Effects of quin2 AM on H_2O_2-induced DNA strand breakage at 0 °C and 37 °C

Untreated (open symbols) and quin2 AM-treated (closed symbols) U-937 cells, at 4×10^4 cells/ml, were exposed to different concentrations of H_2O_2 at 0 °C (triangles) or 37 °C (circles) for 10 min. The induction of DNA single-strand breaks (the decrease in percentage of precipitated DNA) was measured after 10 min. Results are the means ± S.D. for duplicate determinations in three experiments.

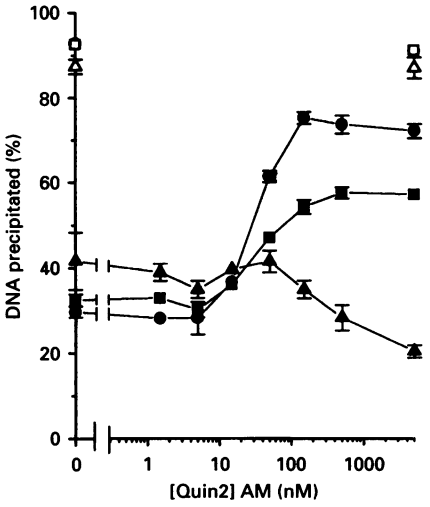

Figure 2 DNA single-strand breakage in relation to quin2 AM dosage

V79-379A cells, at 4×10^4 cells/ml, were treated with the indicated amounts of quin2 AM for 40 min and then exposed to 150 (circles), 300 (squares) or 400 (triangles) μM H_2O_2 for 10 min. Open symbols indicate control cells not exposed to H_2O_2. The induction of DNA single-strand breaks (the decrease in percentage of precipitated DNA) was measured after 10 min. Results are means ± S.D. for triplicate determinations in three experiments.

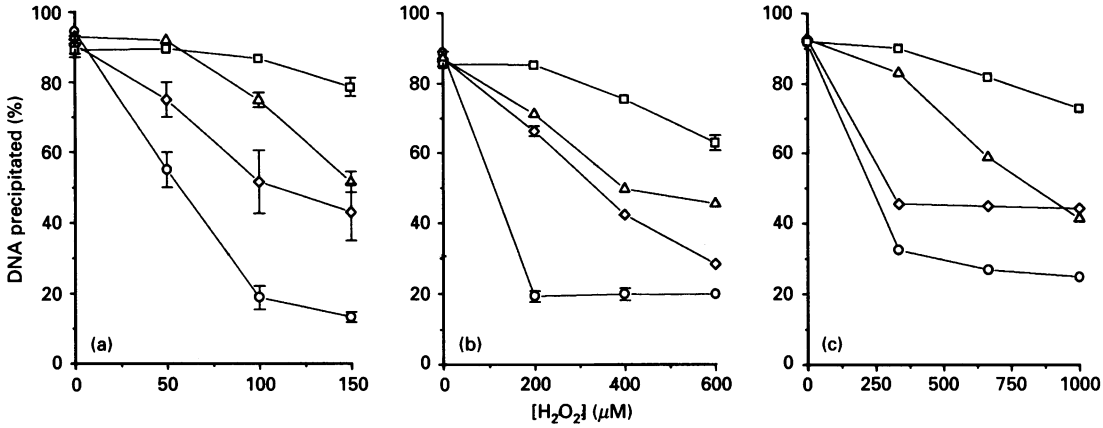

Figure 3 Effects of quin2 AM and DMSO on H_2O_2-induced DNA damage

P31 (**a**), V79-379A (**b**), and U-937 (**c**) cells, at 4×10^4 cells/ml, were exposed to different concentrations of H_2O_2. The different symbols indicate no treatment (circles), 150 nM quin2 AM (triangles), 1 M DMSO (diamonds) and the combination of quin2 AM and DMSO (squares). The induction of DNA single-strand breaks (determined as a decrease in percentage of precipitated DNA) was measured after 10 min. The results represent means ± S.D. for duplicate determinations in two experiments with P31 cells and the means ± variation in one experiment each with V79-379A and U-937 cells.

and V79-379A, even a potentiation of the damage in quin2 AM-treated cells (cf. Figures 1 and 2). With the very H_2O_2-sensitive P31 cells, saturation of our DNA damage assay became an analytical problem in establishing the potentiating effect (Figure 3a).

The possible contribution of Ca^{2+}-dependent mechanisms

The facts that strand breaks appeared within 5 min after exposure to H_2O_2 [18] and that quin2 was protective also at 0 °C (Figure 1) rather suggested that the strand breaks were formed by a direct transition-metal-mediated Fenton-type effect than through an indirect effect involving endonuclease activation. Aurintricarboxylic acid is a general inhibitor of nucleases [16]. We tested this compound at concentrations of 0.05 and 1 mM [17] on P31 cells and found no effect on the amount of H_2O_2-induced DNA strand breakage (results not shown). To control further the possible effect of Ca^{2+}-dependent mechanisms on the induction of strand breaks, we exposed V79-379A cells in medium lacking $CaCl_2$ and also used the Ca^{2+}-chelator EGTA to ensure a negligible level of extracellular Ca^{2+}. The experiments showed that EGTA had very little effect on strand breakage (Table 1). Thus low extracellular Ca^{2+} concentrations appeared not to decrease H_2O_2-induced DNA strand breakage.

Dosage of quin2 AM

In our previous *in vitro* study [10] we found quin2 to inhibit plasmid DNA strand breakage induced by Cu^{2+} and H_2O_2, but to potentiate strand breakage induced by Fe^{3+} and H_2O_2. Accordingly, one way to interpret the H_2O_2-concentration-dependent effects of quin2 on cellular DNA strand breakage is that Cu ions are the important mediators of damage at low concentrations of H_2O_2, whereas Fe ions would become increasingly important with rising concentration of H_2O_2. Furthermore, *in vitro* studies by us [10] and others [19–21] show, in line with this interpretation, that much less H_2O_2 is needed to damage isolated DNA in the presence of Cu^{2+} than in the presence of Fe^{3+}. Previously we also showed that the binding affinity of quin2 for Cu^{2+} is about 5×10^3 times greater than that of Fe^{3+} and 7×10^4 times greater than that of Ca^{2+} [10]. Thus, by varying the concentrations of both quin2 AM and H_2O_2, we attempted to find the quin2 AM concentrations at which inhibition and potentiation of DNA damage occurred and to determine whether these concentrations varied according to the obtained binding affinities of quin2 for Cu^{2+} and Fe^{3+}.

Table 1 Influence of quin2 AM and EGTA on DNA single-strand breakage in Ca^{2+}-poor and Ca^{2+}-sufficient medium

V79-379A cells were treated with quin2 AM for 40 min and EGTA was added 20 min before the cells were exposed to H_2O_2 for 10 min at 37 °C. Then DNA single-strand breakage was determined by the DNA precipitation assay. A decrease in the amount of precipitated DNA indicates an increase in DNA single-strand breakage. Results are means ± S.D. ($n = 6$). The Ca^{2+}-poor medium contained 1 μg of calcium D-pantothenate/ml, providing a maximal Ca^{2+} concentration of 2.1 μM. The Ca^{2+}-sufficient medium contained 300 μM $CaCl_2$ and 0.7 g of calcium D-pantothenate/ml.

	No treatment	EGTA (300 μM)	Quin2 AM (150 nM)	Quin2 AM + EGTA
Ca^{2+}-poor medium				
Control	90 ± 3.6	90 ± 3.8	90 ± 5.8	90 ± 6.8
0.2 mM H_2O_2	29 ± 9.4	28 ± 10	41 ± 11	43 ± 11
0.4 mM H_2O_2	31 ± 9.9	28 ± 9.6	28 ± 5.6	28 ± 4.2
Ca^{2+}-sufficient medium				
Control	88 ± 6.5	87 ± 5.2	87 ± 7.7	86 ± 8.9
0.2 mM H_2O_2	19 ± 5.2	21 ± 7.2	58 ± 16	57 ± 7.3

The experiments showed that, for V79-379A cells, maximal inhibition could be seen at about 150–500 nM quin2 AM (Figure 2). We subsequently chose 150 nM as the quin2 AM concentration in the other experiments performed in this study. This quin2 AM concentration is at least one order of magnitude lower than the concentration generally used to intracellularly buffer Ca^{2+} [22]. Furthermore, the minimal quin2 AM concentration at which inhibition or potentiation appeared varied only between 50 and 150 nM. Consequently, on the basis of the differences in binding affinity, both inhibition and potentiation appeared to involve one and the same metal ion. The experiments (Figure 2) also strongly underline the fact that other factors beside the H_2O_2 concentration are important for H_2O_2-induced DNA

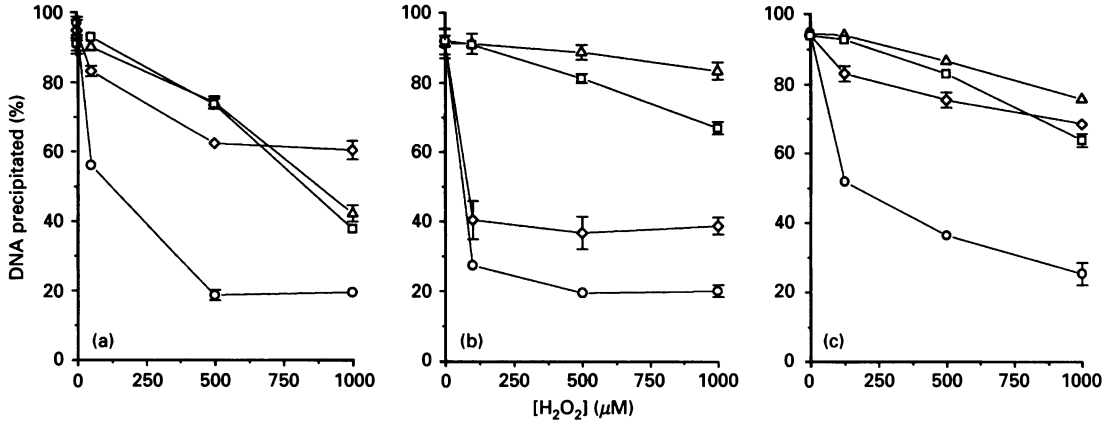

Figure 4 Effects of *o*-phenanthroline and DMSO on H_2O_2-induced DNA damage

P31 (**a**), V79-379A (**b**) and U-937 (**c**) cells, at 4×10^4 cells/ml, were exposed to different concentrations of H_2O_2. The different symbols indicate no treatment (circles), 25 μM *o*-phenanthroline (triangles), 1 M DMSO (diamonds) and the combination of *o*-phenanthroline and DMSO (squares). The induction of DNA single-strand breaks (determined as a decrease in percentage precipitated DNA) was measured after 10 min. The results represent means $\pm$ S.D. for duplicate determinations in two experiments with V79-379A cells and the means $\pm$ variation in one experiment each with P31 and U-937 cells.

damage in mammalian cells, as the highest H_2O_2 concentration (400 μM) in the absence, or at low doses, of quin2 AM resulted in somewhat less DNA damage than the two lower (150 and 300 μM) H_2O_2 concentrations (Figure 2 and cf. Figure 3b).

Effects of combining quin2 AM and DMSO

We compared previously [10] the effects of Fe^{3+}-quin2, in reaction with either H_2O_2 or a superoxide/H_2O_2 mixture produced by hypoxanthine/xanthine oxidase, in two assays detecting oxidant formation: the DMSO [23] and deoxyribose [24] assays. We found that Fe^{3+}-quin2 formed oxidants with H_2O_2 that, like ionizing radiation, reacted with both detector molecules, but, in comparison with ionizing radiation, reacted somewhat more strongly with DMSO. On the other hand, Fe^{3+}-EDTA in reaction with H_2O_2 in absence of reductants such as superoxide or ascorbate, formed oxidants strongly reacting with deoxyribose, but negligibly with DMSO. Interestingly, in the presence of the reductants, the oxidizing species in the Fe^{3+}-EDTA/H_2O_2 reaction could be detected in both assays in the same way as the oxidants produced with Fe^{3+}-quin2/H_2O_2 and by ionizing radiation [25].

As Fe^{3+}-quin2 *in vitro* preferentially reacted with DMSO, we hypothesized that the combination of quin2 and DMSO could have an increased protective effect compared with quin2 or DMSO alone. Indeed, we found that this combination gave a better protection than the chelator or the scavenger alone, and the protective effects seemed largely additive independently of the cell line used (Figure 3). The additive effect of quin2 and DMSO was independent of the Ca^{2+} concentration in the medium (results not shown). *o*-Phenanthroline is a strong bivalent-metal-ion chelator that has been shown to be effective in inhibiting H_2O_2-induced DNA strand breakage in mammalian cells [26]. In contrast with quin2, *o*-phenanthroline inhibits oxidant formation *in vitro* when mixed with Fe^{3+} and H_2O_2 [11]. We replaced quin2 AM with *o*-phenanthroline and repeated the combination experiment with DMSO. In this case, DMSO did not improve the protective effect of the chelator (Figure 4). Thus, as may be expected from a comparison of the *in vitro* results [10,11], the mechanisms behind the protective effects of *o*-phenanthroline and quin2 on H_2O_2-induced DNA strand breakage appear to be of different nature.

DISCUSSION

The genotoxicity of H_2O_2 has been proposed by many authors to be the result of a transition-metal-driven Haber–Weiss reaction (Fenton reaction). The prerequisites of this reaction are a transition-metal ion and H_2O_2. In the case of iron, Fe^{2+} can react directly with H_2O_2 to create either hydroxyl radical or possibly a ferryl species (FeO^{2+}), whereas Fe^{3+} has to be reduced first for reaction to take place. Superoxide and ascorbate are often used as Fe^{3+} reductants *in vitro*. Low-molecular-mass intracellular iron complexes, such as ATP-Fe [27], have been suggested to be the source of the catalysis of the Fenton reaction *in vivo*. Another hypothetical source is protein-bound iron, which, after reduction by superoxide, may be released in cells under oxidative stress to become available for Fenton reaction [28]. A problem of interpretation, when a chelator is added to a biological system, is that chelators bind both reduced and oxidized states of metal ions. The affinity, however, often differs severalfold. *o*-Phenanthroline, for example, has several-orders-of-magnitude stronger affinities for Fe^{2+} (10^{21} M^{-1}) and Fe^{3+} (10^{14} M^{-1}) [29] than those of quin2, namely $10^{9.8}$ and $10^{8.4}$ M^{-1} [10,30,31]. Thus it is difficult to determine if it is the reduced or oxidized form of Fe that *o*-phenanthroline and quin2 are chelating inside the cell.

It has been shown that the hydroxyl-radical scavenger DMSO does not protect against all induced DNA strand breaks when cells are exposed to H_2O_2 [32]. There are at least two possible explanations for this. One possibility is that H_2O_2 in reaction with Fe ions forms two types of oxidizing species, namely hydroxyl radical and also some kind of iron–oxo complex, possibly a ferryl species [33] that DMSO does not scavenge. The other explanation is that a large portion of the oxidants formed are site-specific, i.e. the metal ion producing the radical is attached to DNA in such a way that a scavenger has no possible chance of interfering before the radical has hit the target. In this scheme, DNA single-strand breaks induced in the presence of DMSO represent the relative portion of site-specific DNA damage.

Our finding that DMSO and quin2 give additive protective effects can so far be explained by both of these hypotheses. The role of quin2 according to the first hypothesis would be to form an iron–quin2 complex that, in reaction with H_2O_2, would predominantly produce hydroxyl radical, as supported by a comparison between the DMSO and the deoxyribose assays and discussed in [10]. Thus the majority of the oxidizing species produced could be scavenged by DMSO. The suggested role of quin2 in the hypothesis concerning prevention of site-specific damage is to displace iron from DNA in a manner similar to that previously discussed for EDTA, but then in relation to work on isolated DNA [34]. If quin2 displaces iron, it would give DMSO the chance to react with the radical, and the combination of quin2 and DMSO would result in an additive protective effect on DNA, despite the fact that Fe^{3+}-quin2 in the test tube and probably also inside a cell potentiates radical formation in the presence of H_2O_2. Both hypotheses seem plausible, and at this stage we cannot reject either of them.

The endonuclease inhibitor aurintricarboxylic acid was previously shown to inhibit different forms of what is believed to be DNA fragmentation by endonucleases [17]. Our experiments using this compound (results not shown) or Ca^{2+}-poor medium and EGTA (Table 1) could not support a role for endonucleases in H_2O_2-induced cellular DNA damage [6]. This is also in line with extensive work done elsewhere (reviewed in [35]). Instead, on the basis of this and our previous work with quin2 [10], we suggest that the effects of quin2 on H_2O_2-induced cellular DNA damage are due to chelation of transition-metal ions and of these, Fe ions in particular. We think that the apparent effect of quin2, which we have shown *in vitro*, to make Fe^{3+} less sensitive towards reduction by superoxide and instead possibly be reduced by H_2O_2 [10], might explain the inhibition of H_2O_2-induced cellular DNA damage at low doses of H_2O_2. Superoxide-dependent reduction of Fe^{3+} has been suggested to be an important primary step in H_2O_2-induced cellular DNA damage [35,36], and Fe^{3+} bound to quin2 appears not to critically depend on that step for further reaction [10]. It is possible that, when Fe^{3+} is chelated by quin2 in cells, there exists a H_2O_2-concentration-dependent competition between two processes: inhibition of superoxide-dependent reduction of Fe^{3+} and potentiation of H_2O_2-dependent reduction of Fe^{3+}. This would then explain both the inhibition by quin2 that we see at low concentrations of H_2O_2 and the potentiation at the high concentrations. However, if binding of Fe^{2+} by quin2 is more important, the role of quin2 may simply be to displace the Fe from DNA for catalysis of the reaction at a more favourable site in terms of cell survival, as suggested by the protective effect of quin2 on H_2O_2-induced cell killing [6].

In summary, we have found that the Ca^{2+} probe quin2 not only inhibits, but can also potentiate, H_2O_2-induced cellular DNA damage and that the transition-metal-ion-binding property of quin2 is likely to cause these effects. The results point to a serious drawback to the use of quin2 as a Ca^{2+} chelator in experiments aimed at buffering intracellular Ca^{2+}. Quin2 will, as we have shown in this and previous studies [10,25], undoubtedly have many unexpected effects on oxygen-radical reactions mediated by transition metals such as Fe and Cu, but also probably effects on other transition-metal ions such as Zn^{2+} and Mn^{2+}. Mapping of these effects may open up a new role for quin2 as a cell-membrane-permeable transition-metal-ion chelator.

I thank Mr. Micael Granström for expert technical assistance, Ms. Åsa Genborg for valuable discussions and Professsor Mats Harms-Ringdahl (University of Stockholm) for critical reading of the manuscript before its submission.

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Received 1 March 1994/12 July 1994; accepted 26 July 1994