Hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions

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In cells undergoing oxidative stress, DNA damage may result from attack by $\cdot\text{OH}$ radicals produced by the Fenton reaction, and/or by nucleases activated by nuclear calcium. In the present study, the participation of these two mechanisms was investigated in HeLa cells. Nuclear-targeted aequorin was used for selectively monitoring Ca^{2+} concentrations within the nuclei ($[\text{Ca}^{2+}]_n$), in conjunction with the cell-permeant calcium chelator bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester (BAPTA/AM), the lipid-soluble broad-spectrum metal chelator with low affinity for Ca^{2+} and Mg^{2+} N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), and the high-affinity iron/copper chelator 1,10-phenanthroline (PHE). In Ca^{2+} -containing medium, H_2O_2 induced extensive DNA strand breaks and an increase in $[\text{Ca}^{2+}]_n$ that was almost identical to that observed in the cytosol ($[\text{Ca}^{2+}]_c$). In cells bathed in Ca^{2+} -free/EGTA medium, in which the increases in $[\text{Ca}^{2+}]_n$ and $[\text{Ca}^{2+}]_c$ due to H_2O_2 were significantly reduced, similar levels of DNA fragmentation also occurred. In cells preloaded with BAPTA/AM or TPEN, the small increase of $[\text{Ca}^{2+}]_n$ normally elicited by H_2O_2 in Ca^{2+} -free medium was completely buffered, and DNA damage was largely prevented. On the other hand, pretreatment with PHE did not affect the calcium response in the nuclei, but

completely prevented DNA strand breakage induced by H_2O_2 . Re-addition of 100 μM CuSO_4 and 100 μM FeSO_4 to TPEN- and PHE-treated cells prior to H_2O_2 challenge reversed the effect of TPEN and PHE, whereas 1 mM was necessary to negate the effect of BAPTA/AM. The levels of DNA strand breakage observed, however, did not correlate with the amounts of 8-hydroxy 2'-deoxyguanosine (8-OHdG): H_2O_2 did not produce 8-OHdG, whereas PHE alone slightly increased 8-OHdG levels. CuSO_4 and FeSO_4 enhanced the effects of PHE, particularly in the presence of H_2O_2 . Exposure of cells to a mixture of $\text{CuSO}_4/\text{FeSO}_4$ also resulted in a significant increase in 8-OHdG levels, which was prevented in cells preloaded with BAPTA/AM. Similar results were obtained in a cell-free system using isolated calf thymus DNA exposed to $\text{CuSO}_4/\text{FeSO}_4$, regardless of whether H_2O_2 was present or not. These results suggest that BAPTA/AM prevents H_2O_2 -induced DNA damage by acting as an iron/copper chelator. These data also indicate that caution must be exercised in using Ca^{2+} chelating agents as evidence for a role in cellular Ca^{2+} levels in experimental conditions in which transition-metal-ion-mediated oxidant production is also occurring.

INTRODUCTION

Active oxygen species, such as H_2O_2 and $\text{O}_2^{\cdot-}$, cause a spectrum of DNA lesions, including single-strand breaks, double-strand breaks, crosslinking of DNA and damage to bases and the deoxyribose moiety. Because of the moderate reactivity of these species in aqueous solutions, it has been proposed that their conversion to the highly reactive $\cdot\text{OH}$ radical is responsible for DNA damage. The most likely mode of $\cdot\text{OH}$ radical production *in vivo* is via the Fenton chemistry, through reduction of H_2O_2 by ferrous ions: $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$ [1]. Cuprous ions are also an elemental producer of $\cdot\text{OH}$ radicals in the Fenton reaction [2]. Indeed, copper ions are much more efficient at promoting DNA damage by H_2O_2 than iron ions [3], and this is probably due to the high affinity of DNA for Cu^+ and the fact that Cu^{2+} ions are less extensively used in the human body than Fe^{3+} ions. On the other hand, a rapid and dramatic increase in cytosolic Ca^{2+} is one of the earliest and most prominent events that results from exposure to oxygen metabolites [4,5], and initial studies have suggested that a sustained rise in intracellular Ca^{2+} may be required for triggering the oxidant-induced cleavage of DNA by activating a Ca^{2+} -dependent endonuclease. The linkage between Ca^{2+} release from intracellular stores and the production

of DNA damage is grounded in the fact that extracellular Ca^{2+} omission and intracellular Ca^{2+} chelation by Quin2-acetoxymethyl ester almost completely suppressed DNA breakage by H_2O_2 and active oxygen species from xanthine/xanthine oxidase [6,7]. Thus, oxidant-induced DNA fragmentation may be brought about by both direct attack by $\cdot\text{OH}$ radicals and activation of Ca^{2+} -dependent endonucleases following a non-physiological increase in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$). However, recent evidence casts doubt on the Ca^{2+} hypothesis, as it has been shown that many divalent cations also bind strongly to Quin2 [8,9], and that bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) is capable of binding iron and prevents iron-dependent oxidative cell injury [10]. Moreover, it was also found that Quin2 can prevent H_2O_2 -induced DNA fragmentation in cells incubated at 4 °C, under which conditions endonuclease activation is not expected to occur. It is therefore plausible that the protective effect of Quin2 toward DNA in cells exposed to oxidants may be due to the chelation of metal ions rather than the prevention of Ca^{2+} -dependent endonuclease activation.

Although it is assumed widely that alterations in $[\text{Ca}^{2+}]_c$ can affect the activity of nuclear enzymes, or cause the modification of nuclear structure, it is still debated as to what extent fluctuations in $[\text{Ca}^{2+}]_c$ levels are reflected by changes in intra-

Abbreviations: BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester; CPA, cyclopiazonic acid; $[\text{Ca}^{2+}]_c$, cytosolic free calcium concentration; $[\text{Ca}^{2+}]_n$, nuclear free calcium concentration; PHE, 1,10-phenanthroline; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; 8-OHdG, 8-hydroxy 2'-deoxyguanosine; DMEM, Dulbecco's modified Eagle's medium; KH, Krebs Ringer phosphate buffer; cytAEQ, cytosolic aequorin; nuAEQ, nuclear-targeted aequorin.

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nuclear free Ca^{2+} concentration ($[\text{Ca}^{2+}]_n$). In fact, the data on this issue are largely contradictory. Thus $[\text{Ca}^{2+}]_n$ has been reported to be higher than $[\text{Ca}^{2+}]_o$ in sympathetic neurons [11,12], rat basophilic leukaemia cells [13] and human umbilical-vein endothelial cells [14], to be lower in smooth-muscle cells [15] and other cells [16], or very close, both at rest and upon stimulation, to those of the cytoplasm in HeLa and β -cells [17,18]. In many respects, it appears that the controversy arises primarily from methodological problems. Until recently, the estimation of $[\text{Ca}^{2+}]_n$ changes in intact cells was only possible by using digital imaging of fluorescent Ca^{2+} indicators by confocal microscopy, and these methods rested on the assumption that fluorescent dyes diffuse freely through the nuclear pores and that there are no nucleoplasmic factors capable of modifying the response of the dyes to Ca^{2+} . Both these assumptions have been challenged [19,20].

In this study, the relative significance of (i) the involvement of Ca^{2+} ions and thus Ca^{2+} -dependent endonucleases, and (ii) the participation of copper/iron ions, and thus of Fenton-like reactions, in the mediation of DNA strand breakage and base damage by reactive oxygen species was re-evaluated. To tackle this problem, $[\text{Ca}^{2+}]_n$ and DNA damage were measured in parallel cell cultures after intracellular Ca^{2+} and copper/iron ions were removed. A new strategy based on highly efficient targeting of the Ca^{2+} -sensitive photoprotein aequorin to the nucleus was used to selectively monitor Ca^{2+} concentration inside the nuclei of living cells [17] upon exposure to H_2O_2 . Chelators with significant binding to Ca^{2+} [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester (BAPTA/AM)] and to other divalent ions [N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and 1,10-phenanthroline (PHE)] were utilized to determine the participation of Ca^{2+} and copper/iron ions. The cell-permeant BAPTA/AM is already known in the chemical literature for its capacity to hold intracellular Ca^{2+} close to a baseline level [21]. TPEN, an uncharged polydentate ligand with nitrogens rather than oxygens as donor atoms, has an extraordinarily high affinity for a broad spectrum of transition-metal ions, including Cu^{2+} and Fe^{2+} , and a low affinity for Ca^{2+} [22]. PHE is a strong bivalent-metal-ion chelator that has been shown to be very effective in inhibiting oxidant-induced DNA damage in mammalian cells [23]. The results indicate that the DNA cleavage occurring upon addition of a bolus of H_2O_2 develops essentially as a consequence of an interaction between H_2O_2 and copper/iron ions, and that BAPTA/AM is an efficient chelator of metal ions. The protective effect of BAPTA/AM, due to its ability to chelate copper/iron, was confirmed with the measurement of 8-hydroxy 2'-deoxyguanosine (8-OHdG), one of the base products arising from $\cdot\text{OH}$ attack, produced in intact cells or isolated calf thymus DNA after treatment with $\text{CuSO}_4/\text{FeSO}_4$. Our data also demonstrate that H_2O_2 -induced increases in $[\text{Ca}^{2+}]_o$ are reflected by an equivalent increase in $[\text{Ca}^{2+}]_n$.

MATERIALS AND METHODS

Chemicals and reagents

H_2O_2 , dexamethasone, TPEN, PHE, cyclopiazonic acid (CPA) and 8-OHdG were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL (Basel, Switzerland). Fetal calf serum was from Seromed (Berlin, Germany). Coelenterazine was obtained from Molecular Probes Europe (Leiden, The Netherlands). BAPTA/AM and BAPTA were from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.) or Molecular Probes Europe. Calf thymus DNA was from Calbiochem-Novabiochem. All other reagents

were of analytical grade and came from Sigma or Fluka (Buchs, Switzerland).

Cell culture

HeLa cells (American Type Culture Collection CCL-2) were grown in DMEM supplemented with 25 mM Hepes/50 units/ml penicillin/50 $\mu\text{g}/\text{ml}$ streptomycin/10 % fetal calf serum.

Experimental conditions

Solutions

During experiments, cells were bathed with physiological Krebs Ringer phosphate buffer (KH), pH 7.4, of the following composition: 120 mM NaCl/5 mM KCl/1 mM MgSO_4 /25 mM NaHCO_3 /1.2 mM KH_2PO_4 /2.5 mM CaCl_2 /5 mM glucose/25 mM Hepes. Ca^{2+} -free/EGTA buffer contained 1 mM EGTA and the same salts as KH but without CaCl_2 .

Loading with BAPTA/AM, TPEN and PHE

To buffer intracellular $[\text{Ca}^{2+}]$ increase, cells were loaded with 20 μM BAPTA/AM in DMEM for 45 min before exposure to H_2O_2 for 10 min in Ca^{2+} -containing or Ca^{2+} free/EGTA buffer. To chelate intracellular metal ions, cells were treated with 20 μM TPEN in DMEM for 45 min prior exposure to H_2O_2 for 10 min in Ca^{2+} -containing or Ca^{2+} -free/EGTA buffer. To chelate specifically iron and copper ions, cells were treated with 20 μM PHE in DMEM for 45 min before addition of H_2O_2 for an additional 10 min. The chelators were present throughout the 10 min incubation with H_2O_2 .

Re-addition of transition-metal ions

The role of metal ions was examined by readdition of 100 μM CuSO_4 and 100 μM FeSO_4 to the cells that had been pretreated with BAPTA/AM, TPEN or PHE. After preincubation with 20 μM BAPTA/AM or TPEN in DMEM for 45 min, the cell monolayers were washed with Ca^{2+} -free buffer, incubated in Ca^{2+} -free/EGTA medium containing BAPTA/AM or TPEN together with 100 μM CuSO_4 and 100 μM FeSO_4 for 10 min, and then challenged with H_2O_2 for another 10 min. For PHE-treated cells, incubation with H_2O_2 in the presence of metal ions was performed in Ca^{2+} -containing medium.

Cytosolic and nuclear-targeted aequorin

The plasmid containing the cDNA encoding the HA1 haemagglutinin epitope and apoaequorin (cytosolic aequorin, cytAEQ), and the plasmid containing the cDNA encoding the glucocorticoid receptor including both the nuclear-localization signals and the steroid-binding domain, the cDNA encoding the HA1 haemagglutinin epitope and the cDNA of the whole photoprotein (nuclear-targeted aequorin, nuAEQ) were a generous gift from Dr. T. Pozzan and Dr. R. Rizzuto (University of Padova, Italy). These constructions have been described in detail elsewhere [17].

Transfection, aequorin reconstitution and measurement

The cells were seeded on to 13 mm diameter glass coverslips. When the cells reached $\approx 70\%$ density, transfection was performed with a standard calcium phosphate procedure in DMEM

with 10% fetal calf serum, utilizing 2.5 μg of DNA/coverslip. The cells were incubated with the DNA/calcium phosphate precipitate for 16–20 h, then the medium was removed and replaced with fresh DMEM with 10% fetal calf serum. The cells were used 24 h later. In order to induce the translocation of the transfected protein to the nucleus, the cells were treated with 10 μM dexamethasone for 2 h before the experiment. The functional photoprotein was reconstituted *in situ* by incubating the cells with 2.5 μM coelenterazine for at least 2 h at 37 °C in DMEM before the experiment. The excess of coelenterazine was washed away by perfusing the cells for 2–4 min prior to recording.

[Ca^{2+}] measurements

During the experiments, cells were superfused continuously with KH buffer containing either 2.5 mM CaCl_2 or no calcium with 1 mM EGTA. Exposure to H_2O_2 was done by perfusing in KH buffer and by stopped flow. Light emission was measured in a purpose-built luminometer and calibrated in terms of [Ca^{2+}], as described in [17]. At the end of each experiment, the cells were lysed by perfusing them with a hypo-osmotic medium containing 10 mM CaCl_2 , to expose all the cellular aequorin to a high [Ca^{2+}].

Immunolocalization of epitope-tagged aequorin

Transfected cells were washed 3–4 times with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and washed 3–4 times with PBS. Permeabilization of cell membranes was obtained with a 15 min incubation with 0.5% Triton X-100 in PBS, followed by a 15 min incubation with 1% BSA in PBS. The cells were then incubated for 2 h at room temperature with a rabbit polyclonal anti-aequorin serum in PBS (1:100), washed 3–4 times with PBS, and then stained with a sheep rhodamine-labelled anti-rabbit IgG antibody. Fluorescence was then analysed with a microscope and photographed using Kodak Ektachrome 400 ASA film.

Measurement of DNA single-strand breaks

A DNA precipitation assay, developed by Olive [24], was used for DNA-strand-breaks detection. Confluent cells in 35 mm diameter dishes were labelled in the presence of 0.25 $\mu\text{Ci/ml}$ [^3H]methylthymidine for 24 h. The cells were thoroughly washed with KH, and supplied with 2 ml of KH with the indicated additions. After treatment, the cells were washed with KH and lysed in a 10 ml disposable tube with 1 ml of lysis buffer (10 mM Tris/HCl/10 mM EDTA/50 mM NaOH/2% SDS, pH 12.4) followed by addition of 1 ml of 0.12 M KCl. The lysate was incubated for 10 min at 65 °C followed by a 5 min cooling-and-precipitation period on ice. A DNA–protein K–SDS precipitate was formed under these conditions, from which low-molecular-mass broken DNA was released. This DNA was recovered in the supernatant from a 10 min centrifugation at 200 g , 10 °C, and transferred into a liquid scintillation vial containing 1 ml of 50 mM HCl. The precipitated pellet (intact double-stranded DNA) was solubilized in 1 ml of water at 65 °C, the tube was rinsed with 1 ml of water, and 8 ml of scintillation fluid was added to each vial. The amount of double-stranded DNA remaining was calculated for each sample by dividing the d.p.m. value of the pellet by the total d.p.m. value of the pellet + supernatant and multiplying by 100. The results representing the extent of DNA damage were calculated as $(Dt/Dc) \times 100$, where Dt represents double-stranded DNA in treated cells and Dc represents double-stranded DNA in the respective control cells. In control cells (cells incubated in Ca^{2+} -containing or Ca^{2+} -free/EGTA), the level of total double-stranded DNA was around

75%. Pretreatment with the various chelators did not affect this level.

Preparation of DNA samples for agarose-gel electrophoresis and HPLC

DNA was isolated from cells by the procedure described by Shigenaga et al. [25]. Cells were lysed in hypotonic buffer (10 mM Tris/10 mM NaCl/2 mM EDTA/1 mM deferoxamine mesylate/0.2% Triton X-100, pH 8). Lysates were treated with 100 $\mu\text{g/ml}$ RNase A for 2 h at 50 °C, followed by addition of 1/10 vol. of sarkosyl 10%/100 $\mu\text{g/ml}$ proteinase K and incubation for 2 h at 50 °C. DNA was then extracted using the standard phenol/chloroform/isoamylalcohol procedure and ethanol precipitated.

Agarose-gel electrophoresis

The extracted DNA was dissolved in 10 mM Tris/1.0 mM EDTA (pH 7.4), and incubated for 30 min at 55 °C. The concentration of DNA was then estimated from the absorbance at 260 nm ($A_{260} = 1$ corresponds to 50 $\mu\text{g/ml}$ of double-stranded DNA). DNA (30 μg) was resolved on 1.8% (w/v) agarose gels in Tris/borate/EDTA buffer (89 mM Tris/boric acid/2 mM EDTA, pH 8), and revealed with ethidium bromide (0.5 $\mu\text{g/ml}$).

Enzymic hydrolysis of DNA, HPLC and electrochemical detection

Enzymic hydrolysis of DNA was carried out as described in [25]. DNA samples (200 μg) in 100 μl of 20 mM sodium acetate (pH 5) containing 1 mM deferoxamine mesylate were incubated with 3.3 μg of nuclease P1 for 10 min at 65 °C. The mixture was then adjusted to pH 8.5 by adding 10 μl of 1 M Tris/HCl, pH 8.5, and hydrolysed to the corresponding nucleosides with 1 unit of calf intestine alkaline phosphatase for 1 h at 37 °C. Sodium acetate (10 μl of 3 M at pH 5) was added, and the sample was injected into a HPLC system that consisted of an IsoChrom LC pump and a Waters 464 pulsed electrochemical detector. Separation of 8-OHdG was achieved using a Supelco Supelcosil[®] LC-18S 5 μM column (25 $\times$ 4.6 cm) equipped with a precolumn cartridge under isocratic conditions. The mobile phase consisted of filtered and vacuum-degassed 20 mM sodium acetate/17 mM sodium citrate, pH 3.01, containing 15% methanol with a flow rate of 1 ml/min [26]. The detector potential was set at +0.75 V. The amounts of 8-OHdG were calculated by comparison to peak values obtained with commercially available standards.

Treatment of calf thymus DNA *in vitro*

Reaction mixtures contained, in a final volume of 2 ml: 200 μg of calf thymus DNA/20 mM KH_2PO_4 /KOH buffer (pH 7.4) and, where indicated, 100 μM CuSO_4 /100 μM FeSO_4 /1 mM EGTA/1 mM BAPTA/500 μM H_2O_2 . Metal ions and chelating agents were pre-mixed, where appropriate, and incubated for 10 min at room temperature before addition to the reaction mixtures. Reaction mixtures were incubated for 1 h at 37 °C. Catalase (200 units/ml) was then added, and the DNA was precipitated with cold ethanol after addition of 200 μl of 3 M sodium acetate. The DNA was resuspended, digested to nucleosides and the concentration of 8-OHdG was quantified by HPLC as described above.

RESULTS

Immunolocalization of cytAEQ and nuAEQ

The subcellular localization of cytAEQ and nuAEQ was verified by immunofluorescence using a rabbit polyclonal antibody to

Figure 1 Immunolocalization of cytaEQ (B) and nuAEQ (D) in transiently transfected cells

Transfected cells were probed with preimmune sera (A, C) or a rabbit polyclonal antibody raised against the full-length protein aequorin (B, D), and visualized with rhodamine-labelled anti-rabbit antibodies.

aequorin. As shown in Figure 1, in transfected cells, the cytaEQ was confined to the cytosol and the nuAEQ appeared localized exclusively in the nucleus.

Effects of H_2O_2 on nuclear and cytosolic $[Ca^{2+}]$

As shown in Figure 2 (upper panels), in the presence of external calcium, H_2O_2 (0.5 mM) caused an immediate rise in $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ to a peak, which was followed by a plateau phase characterized by a sustained elevation of $[Ca^{2+}]$. Basal $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ averaged 146 ± 9 nM and 90 ± 6 nM, respectively ($n = 7$). $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ increased rapidly to 294 ± 21 nM [a $2.0 (\pm 0.12)$ -fold increase compared with basal levels] and 225 ± 21 nM, respectively [a $2.5 (\pm 0.2)$ -fold increase]. After this initial rapid rise, $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ decreased to 225 ± 16 nM [$1.5 (\pm 0.04)$ -fold increase] and 152 ± 15 nM [a $1.7 (\pm 0.1)$ -fold increase], respectively, after 5 min of stimulation. There were no obvious quantitative or qualitative differences between the nuclear and cytosolic signals.

In the absence of extracellular calcium, the initial transient Ca^{2+} rise was significantly smaller than the response in its presence [$1.55 (\pm 0.1)$ and $1.61 (\pm 0.1)$ -fold increases in the nucleus and cytosol, respectively, $n = 6$], and the secondary elevated plateau phase at 5 min after stimulation was no longer observed (Figure 2, lower panels). These findings provide evidence that H_2O_2 releases Ca^{2+} from internal stores and activates Ca^{2+} entry across the cell membrane.

Figure 3 depicts the typical Ca^{2+} responses in the nucleus and cytosol seen when H_2O_2 was applied to HeLa cells that had been pretreated with BAPTA/AM, TPEN or PHE. When stimulation of cells was performed in Ca^{2+} -free/EGTA buffer, the Ca^{2+}

response in both compartments was completely abolished in cells that had been pretreated with $20 \mu M$ BAPTA/AM or $20 \mu M$ TPEN. In Ca^{2+} -containing buffer, the Ca^{2+} increase elicited by H_2O_2 in BAPTA/AM-treated cells was also completely buffered, whereas the Ca^{2+} responses remained unchanged in TPEN- and PHE-treated cells when compared with those observed in the absence of the chelators. Quantitative data are summarized in Table 1.

Effects of H_2O_2 on DNA single-strand breaks

As shown in Table 2, substantial DNA strand breaks occurred after a 10 min incubation of cells with 0.5 mM H_2O_2 in Ca^{2+} -containing buffer. When compared with untreated cells, $29 \pm 1.7\%$ of the total DNA remained double-stranded ($n = 10$). In Ca^{2+} -free/EGTA buffer (conditions under which a small rise in $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ was observed), H_2O_2 was still able to induce DNA damage, the extent of which was similar to that observed in the presence of external calcium ($29 \pm 1.7\%$ double-stranded DNA remaining, $n = 10$).

The effects of Ca^{2+} and metal ion chelators on DNA strand breakage induced by H_2O_2 were then investigated. When the stimulation was performed in Ca^{2+} -containing buffer, the cleavage was largely prevented in cells that had been pretreated with $20 \mu M$ BAPTA/AM or $20 \mu M$ TPEN. Similar results were obtained with $100 \mu M$ BAPTA/AM or $100 \mu M$ TPEN (results not shown). The extent of the protective effect was somewhat less in the presence of external calcium. Pretreatment with $10 \mu M$ PHE was sufficient to significantly reduce DNA breakage by H_2O_2 : $74 \pm 3\%$ and $70 \pm 4\%$ ($n = 6$) of total DNA remained intact in cells stimulated in Ca^{2+} -containing and Ca^{2+} -free/EGTA

Figure 2 Effects of 0.5 mM H_2O_2 on $[\text{Ca}^{2+}]_n$ and $[\text{Ca}^{2+}]_c$ (in nM) in Ca^{2+} -containing (upper panels) and Ca^{2+} -free/EGTA (lower panels) KH

Tracings are representative of 5–6 independent experiments.

medium, respectively. When stimulation was performed in Ca^{2+} -containing medium and in the presence of 20 μM PHE, damage to DNA was eliminated totally ($n = 8$).

All these data are consistent with the idea that a $[\text{Ca}^{2+}]_n$ rise and, thus, a Ca^{2+} -dependent step, is by itself neither sufficient nor required for DNA strand breakage.

Effects of addition of copper and iron in DNA strand breaks induced by H_2O_2

The possibility that TPEN, PHE and BAPTA/AM were functioning as copper/iron chelators was tested further by examining the reversibility of their effects by re-addition of copper and iron ions. As shown in Table 2, the addition of 100 μM CuSO_4 and 100 μM FeSO_4 to the TPEN- and PHE-treated cultures 10 min before challenge with H_2O_2 in Ca^{2+} -free/EGTA (for TPEN) and Ca^{2+} -containing medium (for PHE) was sufficient to cancel completely the protective effect of TPEN and PHE pretreatment. Surprisingly, 100 μM CuSO_4 and 100 μM FeSO_4 were not able to negate the effect of BAPTA/AM, whereas addition of 1 mM each of these ions did reverse the BAPTA/AM effect.

Electrophoretic properties of DNA extracted from cells exposed to H_2O_2 , CuSO_4 , FeSO_4 and chelators

Examination by agarose-gel electrophoresis of the DNA extracted from treated cells revealed that H_2O_2 induced detectable

DNA fragmentation, regardless of whether calcium was present or not in the medium (Figure 4, lanes 1–4). The DNA degradation was completely prevented in cells pretreated with BAPTA/AM (lanes 5 and 6), PHE (lanes 11 and 12) and TPEN (lanes 15 and 16). As demonstrated previously with the DNA precipitation assay, addition of 100 μM CuSO_4 and 100 μM FeSO_4 to the TPEN- and PHE-treated cultures 10 min before challenge with H_2O_2 was sufficient to cancel completely the protective effect of TPEN (lane 18) and PHE (lane 14), whereas addition of 1 mM each of these ions was necessary to reverse the BAPTA/AM effect (lane 10). CuSO_4 and FeSO_4 caused some DNA damage even in the absence of added H_2O_2 (lanes 9 and 13).

Taken altogether, our results suggest that DNA damage by H_2O_2 is induced by Ca^{2+} -independent Fenton-type reactions directly at the DNA target, and that the effect of BAPTA/AM is most probably due to its function as a metal-ion chelator and not due to specific buffering of Ca^{2+} .

Effects of H_2O_2 , CuSO_4 , FeSO_4 and chelators on 8-OHdG levels in intact cells

Table 3 shows the effects of H_2O_2 and various chelators on the levels of 8-OHdG. In cells exposed to 500 μM H_2O_2 for 10 min either in Ca^{2+} -containing or Ca^{2+} -free/EGTA medium, no detectable increase in 8-OHdG levels could be detected. TPEN had no effect, whereas treatment with PHE resulted in a small increase in 8-OHdG levels. Addition of 100 μM CuSO_4 and

Figure 3 Effects of pretreatment with 20 μ M BAPTA/AM, TPEN or PHE on $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ (in nM) changes induced by 0.5 mM H_2O_2

Cells were incubated in either Ca^{2+} -containing (A, B, E, F, I, J) or Ca^{2+} -free/EGTA (C, D, G, H) buffer. (A–D) Cells treated with BAPTA/AM; (E–H) cells treated with TPEN; (I, J) cells treated with PHE. H_2O_2 was added at the times indicated by the arrows. Tracings are representative of 4–5 independent experiments.

100 μ M $FeSO_4$, particularly in the presence of H_2O_2 , enhanced the effects of PHE. Treatment of cells with BAPTA/AM did not affect the basal levels of 8-OHdG. Whereas addition of 100 μ M $CuSO_4$ and 100 μ M $FeSO_4$ was ineffective, 1 mM $CuSO_4$ and 1 mM $FeSO_4$ produced a small increase in the levels of 8-OHdG, regardless of whether H_2O_2 was present or not. The latter observation prompted us to measure the amounts of 8-OHdG in cells exposed to copper and iron. As shown in Table 4, a 20 min exposure to a mixture of $CuSO_4/FeSO_4$ (100 μ M each), in Ca^{2+} -containing or Ca^{2+} -free/EGTA medium, led to a 90 % and 60 %

increase in the levels of 8-OHdG, respectively, which was prevented in cells that have been pretreated with BAPTA/AM.

Effects of H_2O_2 , $CuSO_4$, $FeSO_4$ and BAPTA on the formation of 8-OHdG *in vitro*

The effects of $CuSO_4/FeSO_4$ and BAPTA on the production of 8-OHdG were confirmed *in vitro* with the use of isolated calf thymus DNA. As shown in Table 5, neither $FeSO_4$ alone

Table 1 Effects of pretreatment with TPEN and PHE on [Ca²⁺]_n and [Ca²⁺]_c.

Cells were loaded with 20 μ M TPEN or 20 μ M PHE for 45 min before challenge with 0.5 mM H₂O₂ in KH containing 2.5 mM CaCl₂. [Ca²⁺]_n values at rest (baseline), and at peak and plateau 5 min after stimulation (nM) are shown ($n = 5-7$). Values in parentheses indicate the fold increase with respect to the baseline value.

	[Ca ²⁺] _n (nM)			[Ca ²⁺] _c (nM)		
	Baseline	H ₂ O ₂ peak	H ₂ O ₂ plateau	Baseline	H ₂ O ₂ peak	H ₂ O ₂ plateau
Control	146 ± 9	294 ± 21 (2.0 ± 0.12)	225 ± 16 (1.5 ± 0.04)	90 ± 7	225 ± 21 (2.5 ± 0.16)	152 ± 15 (1.7 ± 0.1)
TPEN	149 ± 10	323 ± 67 (2.1 ± 0.3)	231 ± 49 (1.5 ± 0.24)	122 ± 14	234 ± 18 (2.0 ± 0.16)	167 ± 23 (1.4 ± 0.03)
PHE	139 ± 14	280 ± 35 (2.0 ± 0.05)	213 ± 35 (1.5 ± 0.1)	120 ± 7	248 ± 11 (2.1 ± 0.07)	175 ± 14 (1.5 ± 0.09)

Table 2 Effects of pretreatment with various chelators and of re-addition of 100 μ M (FeCu100) or 1000 μ M (FeCu1000) CuSO₄ and FeSO₄ on DNA strand breakage induced by a 10 min exposure to 500 μ M H₂O₂

Pretreatment of cells with chelators (BAPTA/AM, PHE20 and TPEN, 20 μ M, and PHE10, 10 μ M) was performed in DMEM for 45 min at 37 °C. The 10 min exposure to 500 μ M H₂O₂ was done in Ca²⁺-containing or Ca²⁺-free/EGTA buffer in the presence of chelators. Double-stranded DNA remaining in cells after the treatment is shown, expressed as a percentage of the respective controls (means ± S.E.M., $n = 6-10$).

Treatment	Re-addition of Cu ²⁺ /Fe ²⁺	Medium	Double-stranded DNA (%)
H ₂ O ₂		+ Ca ²⁺	27.5 ± 1.6
H ₂ O ₂		Ca ²⁺ free	29.0 ± 1.3
BAPTA/AM + H ₂ O ₂		+ Ca ²⁺	66.3 ± 2.6
BAPTA/AM + H ₂ O ₂		Ca ²⁺ free	82.3 ± 6.2
BAPTA/AM + H ₂ O ₂	FeCu100	Ca ²⁺ free	84.5 ± 6.5
BAPTA/AM + H ₂ O ₂	FeCu1000	Ca ²⁺ free	32.0 ± 3.6
PHE10 + H ₂ O ₂		Ca ²⁺ free	70.0 ± 4.2
PHE10 + H ₂ O ₂		+ Ca ²⁺	74.2 ± 3.2
PHE20 + H ₂ O ₂		+ Ca ²⁺	108.4 ± 3.2
PHE20 + H ₂ O ₂	FeCu100	+ Ca ²⁺	32.6 ± 3.1
TPEN + H ₂ O ₂		+ Ca ²⁺	45.1 ± 2.8
TPEN + H ₂ O ₂		Ca ²⁺ free	66.8 ± 2.3
TPEN + H ₂ O ₂	FeCu100	Ca ²⁺ free	33.6 ± 3.0

(100 μ M), nor H₂O₂ alone (500 μ M) induced hydroxylation of deoxyguanosine, whereas treating calf thymus DNA with 100 μ M CuSO₄ resulted in a substantial increase in the amount of 8-OHdG. A mixture of CuSO₄/FeSO₄/H₂O₂ caused a very large increase in 8-OHdG, much larger than that produced by CuSO₄ alone. These increases were inhibited by preincubating CuSO₄/FeSO₄ with BAPTA or EGTA before addition to the reaction mixtures.

Effects of CPA on [Ca²⁺]_n, [Ca²⁺]_c and DNA

As shown in Figure 5, in both the presence and absence of extracellular Ca²⁺, 20 μ M CPA, a potent inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, induced an increase in [Ca²⁺]_n and [Ca²⁺]_c. The response in the presence of external Ca²⁺ was much larger than the response in its absence, consistent with a CPA-induced release of internally stored Ca²⁺ and influx of extracellular Ca²⁺. However, at no stage did [Ca²⁺]_n rise to the level seen in the cytosol. No DNA damage was observed under these conditions: 98 ± 5% ($n = 7$) of total cellular DNA remained double-stranded after a 10 min exposure to 20 μ M CPA in Ca²⁺-containing medium when compared with normal cells.

Figure 4 Agarose-gel electrophoresis of DNA isolated from cells after various treatments

Cells were treated in the following way. Lane 1, control cells in Ca²⁺-containing medium; lane 2, H₂O₂ in Ca²⁺-containing medium; lane 3, control cells in Ca²⁺-free/EGTA medium; lane 4, H₂O₂ in Ca²⁺-free/EGTA medium; lanes 5–10, cells treated with BAPTA/AM and then exposed to H₂O₂ (lane 6), 100 μ M CuSO₄/100 μ M FeSO₄ (lane 7), 100 μ M CuSO₄/FeSO₄ + H₂O₂ (lane 8), 1 mM CuSO₄/FeSO₄ (lane 9), and 1 mM CuSO₄/FeSO₄ + H₂O₂ (lane 10); lanes 11–14, cells treated with PHE and then exposed to H₂O₂ (lane 12), 100 μ M CuSO₄/FeSO₄ (lane 13), and 100 μ M CuSO₄/FeSO₄ + H₂O₂ (lane 14); lanes 15–18, cells treated with TPEN and then exposed to H₂O₂ (lane 16), 100 μ M CuSO₄/FeSO₄ (lane 17), and 100 μ M CuSO₄/FeSO₄ + H₂O₂ (lane 18). Pretreatment with the various chelators was done in RPMI medium, and exposure to H₂O₂ was done in Ca²⁺/EGTA medium for BAPTA/AM and TPEN, and Ca²⁺-containing medium for PHE in the presence of the chelators.

DISCUSSION

In this study, the relative importance of Ca²⁺ ions, and thus Ca²⁺-dependent endonuclease activation, and redox-active metal ions, and thus Fenton-type reactions, in DNA damage (DNA strand breaks and base modification), promoted by a bolus of H₂O₂, was re-evaluated. For this purpose, the recombinant Ca²⁺-dependent luminescent photoprotein aequorin, targeted specifically to the nucleus, was utilized to monitor free Ca²⁺ changes within this compartment during exposure to H₂O₂. The question of Ca²⁺ and copper/iron ion involvement was addressed using three cell-permeant chelators with different specific affinities for Ca²⁺ and other metal ions, BAPTA/AM, TPEN and PHE.

It appears from our data that Ca²⁺ signals may not be a

Table 3 8-OHdG levels in cells after treatment with H_2O_2 (500 μM), FeSO_4 and CuSO_4 (100 or 1000 μM each, FeCu) and various chelators in Ca^{2+} -containing or Ca^{2+} -free/EGTA mediumValues represent the means $\pm$ S.E.M. of 4 separate experiments.

Treatment	Medium	8-OHdG (fmol/ μg of DNA)
No treatment	Ca^{2+} free	32.0 ± 18.9
H_2O_2	Ca^{2+} free	34.3 ± 10.5
No treatment	+ Ca^{2+}	36.3 ± 11.7
H_2O_2	+ Ca^{2+}	38.3 ± 10.8
BAPTA/AM	Ca^{2+} free	33.7 ± 5.7
BAPTA/AM + H_2O_2	Ca^{2+} free	39.3 ± 14.0
BAPTA/AM + FeCu100	Ca^{2+} free	38.4 ± 4.8
BAPTA/AM + H_2O_2 + FeCu100	Ca^{2+} free	42.0 ± 9.0
BAPTA/AM + FeCu1000	Ca^{2+} free	53.3 ± 5.3
BAPTA/AM + H_2O_2 + FeCu1000	Ca^{2+} free	52.1 ± 5.0
PHE	+ Ca^{2+}	54.8 ± 2.2
PHE + H_2O_2	+ Ca^{2+}	60.7 ± 14.5
PHE + FeCu100	+ Ca^{2+}	77.5 ± 12.9
PHE + H_2O_2 + FeCu100	+ Ca^{2+}	87.3 ± 14.0
TPEN	Ca^{2+} free	39.4 ± 12.6
TPEN + H_2O_2	Ca^{2+} free	41.3 ± 9.5
TPEN + FeCu100	Ca^{2+} free	42.7 ± 9.0
TPEN + H_2O_2 + FeCu100	Ca^{2+} free	33.0 ± 5.4

Table 4 8-OHdG levels detected in DNA from cells incubated with FeSO_4 and CuSO_4 (100 μM each, FeCu) in Ca^{2+} -containing or Ca^{2+} -free/EGTA medium, without or with a pretreatment with 20 μM BAPTA/AM (means $\pm$ S.E.M.; $n = 4$)

Treatment	Medium	8-OHdG (fmol/ μg of DNA)
No treatment	+ Ca^{2+}	38.0 ± 6.0
FeCu	+ Ca^{2+}	73.5 ± 10.1
No treatment	Ca^{2+} free	50.0 ± 6.3
FeCu	Ca^{2+} free	80.7 ± 10.4
BAPTA/AM	Ca^{2+} free	50.6 ± 4.4
BAPTA/AM + FeCu	Ca^{2+} free	50.4 ± 9.7

Table 5 Yields of 8-OHdG formed in calf thymus DNA by treatment with CuSO_4 and/or FeSO_4 (100 μM each) and H_2O_2 in the absence or presence of BAPTA or EGTAValues represent the means $\pm$ S.E.M. of results from 3 separate reaction mixtures.

Treatment	8-OHdG (fmol/ μg of DNA)
No treatment	362.6 ± 33.6
BAPTA	364.4 ± 57.4
EGTA	412.0 ± 57.1
FeSO_4	391.3 ± 127.0
CuSO_4	3808.8 ± 562.8
BAPTA + CuSO_4	336.8 ± 31.0
EGTA + CuSO_4	409.7 ± 10.1
CuSO_4 + FeSO_4	3971.8 ± 784.6
CuSO_4 + FeSO_4 + BAPTA	346.5 ± 59.4
CuSO_4 + FeSO_4 + EGTA	398.4 ± 39.4
H_2O_2	361.0 ± 38.9
H_2O_2 + CuSO_4 + FeSO_4	22104.6 ± 4275.0
H_2O_2 + CuSO_4 + FeSO_4 + BAPTA	528.4 ± 58.2
H_2O_2 + CuSO_4 + FeSO_4 + EGTA	1365.8 ± 183.6

prerequisite for DNA degradation following H_2O_2 challenge, since the extent of DNA damage does not parallel $[\text{Ca}^{2+}]_i$. Instead, our results suggest that oxygen radicals generated by the Fenton reaction are responsible for DNA strand breakage. Several lines of evidence support this concept: (i) DNA fragmentation can occur in cells bathed in Ca^{2+} -free/EGTA buffer; (ii) in Ca^{2+} -containing buffer, complete inhibition of DNA strand breakage was observed in cells pretreated with PHE, a chelator that binds copper and iron without disturbing intracellular Ca^{2+} concentrations, which can be reversed by readdition of an excess of copper and iron ions; and (iii) CPA, which produced a sustained increase in $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_e$ in the presence of extracellular calcium, did not promote DNA strand breakage.

The use of BAPTA/AM, TPEN and PHE offers a means for probing the importance of calcium and redox-active metal ions in DNA damage induced by oxidants. The BAPTA buffers of Tsien [21] are more selective for Ca^{2+} than EDTA and EGTA, and their metal binding is also much less pH sensitive. BAPTA binds and releases Ca^{2+} ions about 50–400 times faster than EGTA, but also binds other metals besides Ca^{2+} . The membrane-permeant AM ester of BAPTA is extensively used to clamp intracellular Ca^{2+} concentrations, providing insights on the role of free cytosolic Ca^{2+} in a number of important cell systems. TPEN, a structural analogue of EDTA in which the four carboxylates have been replaced by pyridine groups of similar steric placement, readily crosses membranes and has a remarkably high affinity for a range of transition-metal ions and a low affinity for Ca^{2+} and Mg^{2+} . Because TPEN binds metal ions without disturbing Ca^{2+} and Mg^{2+} concentrations, it is ideal for identifying artifacts that may arise from metal binding by BAPTA buffers. Its stoichiometry is simpler than that of PHE, a strong bivalent-metal ion chelator that has been shown to be very effective in inhibiting oxidant-induced DNA damage in endothelial cells [23]. We found that BAPTA/AM, TPEN and PHE were effective in inhibiting H_2O_2 -induced DNA damage, with PHE being the most efficient. However, whereas the protective effect of PHE was unrelated to external Ca^{2+} , the effects of BAPTA/AM and TPEN appeared to be affected by an excess of external Ca^{2+} . This can so far be explained by the affinity of these chelators for Ca^{2+} relative to that for other metal ions. In fact, BAPTA/AM has a very high affinity for Ca^{2+} , since it is extremely efficient at holding intracellular Ca^{2+} close to a baseline level, even in cells stimulated in Ca^{2+} -containing medium. Thus one would predict that an excess of Ca^{2+} might saturate the BAPTA molecules within the cells, which are no longer available for the sequestration of other divalent cations. On the contrary, the fact that externally added Ca^{2+} did not alter the PHE effect suggests that Ca^{2+} ions must not compete efficiently for Cu^{2+} and Fe^{2+} binding to PHE. This is indeed the case, since we found that PHE binds Ca^{2+} negligibly in cells at rest and after stimulation. The odd point in our experiments is the lesser efficacy of TPEN, known to be a high-affinity metal-ion chelator, for preventing DNA damage compared with PHE. One possibility is that free TPEN is not able to cross nuclear membranes readily and/or to accumulate within the nucleus. Indeed, unlike BAPTA/AM, TPEN must be present throughout the stimulation period to maintain its protective effect (L. Jornot, unpublished work). Alternatively, a fraction of administered TPEN is already present in preformed combination with divalent cations, thus preventing it from removing intracellular copper/iron ions.

The protective effect of the three chelators was eliminated by adding back Cu^{2+} and Fe^{2+} prior to H_2O_2 exposure. However, the concentrations of CuSO_4 and FeSO_4 necessary to reverse the effect of BAPTA/AM were 10 times higher than that needed to negate the TPEN and PHE effects. These findings implicate

Figure 5 Effects of 20 μM CPA on $[\text{Ca}^{2+}]_n$ (dotted lines) and $[\text{Ca}^{2+}]_c$ (solid lines) in the presence (A) and absence (B) of extracellular calcium

Tracings are representative of 3–4 experiments.

chelation of metal ions as the mechanism of action of the three chelators, but with different affinities. Taken altogether, our data suggest that the protective effect of BAPTA/AM is most likely due to the removal of metal ions instead of Ca^{2+} complexation. In agreement with our observation is the recent report of Britigan et al. [10], demonstrating that BAPTA binds iron and prevents iron-dependent oxidative cell injury. These results are also in keeping with the published data from Burkitt et al. [8] and Sandström [9], demonstrating that the protective effect of another Ca^{2+} chelator, Quin2, towards DNA in cells exposed to oxidants is due to the chelation of iron ions rather than the prevention of Ca^{2+} -dependent endonuclease activation. It could be argued that the nuclease responsible for DNA strand break production may be active in the absence of divalent cations, as suggested by Chaudun et al. [27]. However, our findings from experiments using redox-active copper/iron ions led us to believe that this might not be the case. Alternatively, HeLa cells may lack $\text{Ca}^{2+}/\text{Mg}^{2+}$ endonuclease activity, and thus differ from cells in which these enzymes are present, with regard to Ca^{2+} dependency for DNA degradation. In fact, there are a number of cases where extensive internucleosomal DNA fragmentation occurs upon incubation of nuclei with submicromolar Ca^{2+} and Mg^{2+} concentrations [28,29]. Our data by no means exclude the notion that, under some experimental conditions, or in some cell types, Ca^{2+} -activated endonucleases can be of central importance in DNA degradation.

In our cell system, the levels of 8-OHdG detected did not reflect the levels of DNA fragmentation observed. Thus, the amount of 8-OHdG was not increased in cells exposed to H_2O_2 . Although unexpected, this finding is in fairly good agreement with the data from Higuchi and Linn [30], who also found no significant increase in 8-OHdG in HeLa cells treated with H_2O_2 ,

even under conditions for which no viable cells remained and the DNA was extremely fragmented. A lack of correlation between the levels of 8-OHdG and the levels of DNA fragmentation was also observed by Burkitt et al. [31] in their studies of DNA lesions produced by H_2O_2 and ascorbate or PHE and ascorbate in isolated liver nuclei. On the other hand, it is noteworthy that in studies that report DNA base damage in cells exposed to oxidative stress, the extent of the increase in 8-OHdG is often small, by comparison with the increases in FAPy-adenine (4,6-diamino-5-formamidopyrimidine) and FAPy-guanine (2,6-diamino-4-hydroxy-5-formamidopyrimidine), suggesting that $\cdot\text{OH}$ attack led to imidazole ring-opening rather than hydroxylation [32,33]. From the results of these studies and our present results, it appears that the DNA fragmentation observed might result from the intramolecular transfer of damage from oxidatively modified bases other than 8-OHdG to the sugar-phosphate backbone.

Our finding that exposure of cells to PHE along with H_2O_2 and copper/iron resulted in an increase in 8-OHdG levels is consistent with the data of Burkitt et al. [31] and Tsang et al. [34] showing by the ESR spin-trapping technique that PHE markedly enhanced the efficiency of Cu^{2+} -dependent $\cdot\text{OH}$ formation in the presence of H_2O_2 . Quite intriguingly, we found that mere incubation of both intact cells and purified calf thymus DNA with CuSO_4 alone sufficed to induce production of 8-OHdG. Those results implicate autooxidation of copper with O_2 as the oxidant under our experimental conditions. In support of this proposal are the data of Hanna and Mason [35] and Gunther et al. [36], which showed $\cdot\text{OH}$ radical formation from copper oxidation by O_2 by ESR. The effect of CuSO_4 is enhanced, as expected, by H_2O_2 . Interestingly, pretreatment of cells with BAPTA/AM or, in the case of experiments *in vitro* using purified

calf thymus DNA, pre-mixing CuSO_4 with BAPTA before addition to the reaction mixture, completely prevented the production of 8-OHdG induced by $\text{CuSO}_4/\text{H}_2\text{O}_2$. Thus, our data clearly indicate that BAPTA is capable of chelating copper, and reinforce our hypothesis that the effect of BAPTA/AM in preventing H_2O_2 -induced DNA strand breakage in intact cells is related to its ability to prevent $\cdot\text{OH}$ radical formation by chelating metal ions. This is also in line with a recent report showing that BAPTA binds iron and inhibits iron-dependent oxidative cell injury [10]. Although EGTA is also capable of chelating copper and thus prevents the formation of $\cdot\text{OH}$ radicals, we consider this possibility unlikely in intact cells because EGTA is membrane-impermeant.

The use of the Ca^{2+} -sensitive photoprotein aequorin, which can be localized to specific organelles, enables changes in nuclear free Ca^{2+} to be monitored in living cells. So far, this is the first report demonstrating that increases in $[\text{Ca}^{2+}]_c$ are reflected by an equivalent increase in $[\text{Ca}^{2+}]_n$ in HeLa cells stimulated by H_2O_2 . Interestingly, this Ca^{2+} response contrasts with that observed upon inhibition of the endoplasmic reticulum Ca^{2+} -ATPase by CPA. Consistent with the report of Badminton et al. [16], we observed that treatment with CPA (and also inositol 1,4,5-trisphosphate-generating agonists, such as histamine; L. Jornot, unpublished work) increased $[\text{Ca}^{2+}]_n$ quantitatively less than $[\text{Ca}^{2+}]_c$. A key issue now is whether the source of Ca^{2+} determines whether Ca^{2+} signals are transmitted to the nucleus. Also, it is plausible that small increases in cytosolic Ca^{2+} are transmitted into the nucleus, but large increases in $[\text{Ca}^{2+}]_c$ are attenuated, resulting in only a small increase in nuclear Ca^{2+} . It is noteworthy that, unlike DNA strand breakage, the Ca^{2+} rise observed in the nucleus upon exposure to H_2O_2 is not due to $\cdot\text{OH}$ radicals since it is not affected by PHE or TPEN pretreatment.

In summary, our experiments could not support a role for Ca^{2+} -dependent endonucleases in H_2O_2 -induced cellular DNA damage. Instead, in line with extensive work done elsewhere, our results favour the hypothesis that an oxidative agent generated by the Fenton reaction is responsible for DNA strand breakage in cells treated with H_2O_2 , and point to a serious drawback to the use of BAPTA/AM as a specific Ca^{2+} chelator in experiments aimed at buffering intracellular Ca^{2+} . BAPTA/AM will undoubtedly have many unexpected effects on oxygen-radical reactions mediated by copper and iron, and one must be cautious in concluding a role for cellular Ca^{2+} levels on the basis of the use of BAPTA/AM in experimental conditions in which transition-metal-ion-mediated oxidant production is also occurring.

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