Apoptosis induced by hydrogen peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milieu

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Abstract Hydrogen peroxide (H_2O_2) is considered to be a mediator of apoptotic cell death but the mechanism by which it induces apoptosis is unclear. Here, we show that cells undergoing apoptosis from exposure to H_2O_2 display a significant decrease in intracellular concentration of superoxide (O_2^-) which is associated with a reduction of the intracellular milieu, as measured by an increase in the GSH/GSSG ratio and a decrease in intracellular pH. The notion that a decrease in intracellular O_2^- concentration triggers apoptosis is supported by the observation that H_2O_2-mediated apoptosis could be retarded in cells in which the intracellular O_2^- concentration is maintained at or above the cellular baseline level by inhibition of the major O_2^- scavenger superoxide dismutase (Cu/Zn SOD). Taken together, our observations indicate that a decrease in the intracellular O_2^- concentration, reduction and acidification of the intracellular milieu constitute a signal for H_2O_2-mediated apoptosis, thereby inducing a reductive as opposed to an oxidative stress.

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Key words: Hydrogen peroxide; Superoxide anion; Apoptosis; Caspase; Cellular redox state

1. Introduction

The equilibrium between cell growth and cell death is crucial for the maintenance of tissue homeostasis and must therefore be tightly regulated [1,2]. Apoptosis or programmed cell death is a key component of this equilibrium and is subject to developmental and environmental control mechanisms [3]. A multitude of environmental signals can trigger apoptotic cell death, ranging from nutrient or growth factor depletion to engagement of specific cell surface receptors, such as CD95 and CD120 receptors [4–6]. Because many of the known triggers of apoptosis are oxidants or stimulators of intracellular generation of reactive oxygen intermediates (ROI), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$), ROI are commonly held to be mediators of apoptotic cell death [7,8]. However, several observations have challenged this view. First, studies on the effects of hypoxia on cell survival have suggested that ROI are not necessary to induce apoptosis [9,10]. Second, we have shown that an increase in the intracellular O_2^- concentration, induced by inhibition of the principal intracellular O_2^- scavenger superoxide dismutase (Cu/Zn SOD), or by stimulating cells with phorbol esters and chemicals which directly induce O_2^- production, can inhibit CD95-mediated apoptosis in mammalian cell lines. Conversely, inhibition of cellular O_2^- production was observed to induce sensitivity to CD95-mediated cytotoxicity in cells which were constitutively CD95-resistant [11]. These results have recently been further supported by reports from Hampton and Orrenius [12,13] which suggest that alteration of intracellular redox status may either trigger or block the apoptotic death program, depending on the severity of the oxidative stress.

In an attempt to better define the role and mechanism of ROI-mediated apoptosis, we exposed cells to varying concentration of H_2O_2 and determined the effect on the intracellular O_2^- concentration and the redox status of the cells as related to the induction of apoptotic cell death. Our results show that concentrations of H_2O_2 which trigger apoptosis induce a significant decrease in the intracellular concentration of O_2^-, reduction of the intracellular milieu, and a concomitant drop in the intracellular pH (acidification), thereby inducing a reductive as opposed to an oxidative stress.

2. Materials and methods

2.1. Chemicals

Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA) and the VAD-FK peptide from Kamiya Biochemical Company (Thousand Oaks, CA). Hydrogen peroxide (H_2O_2) used in the present work was a 30% stable solution (EM Science, Gibbstown, NJ, USA). Appropriate H_2O_2 dilutions were made immediately prior to use in serum-free Dulbecco's modified Eagle's (DME) medium. All other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). DPI and PMA were diluted in dimethylsulfoxide (DMSO). Stock solutions were at least 1000×. 1/1000 dilutions of DMSO did not alter cell viability or interfere with apoptotic cell death. Lucigenin stock solution was diluted in distilled water. All other chemicals were directly diluted in the culture medium.

2.2. Cells

The human melanoma M14 cell line has been described previously [11]. M14 cells were cultured in DME medium supplemented with 5% fetal bovine serum (FBS) (both from Hyclone, Irvine Scientific, CA) except in experiments with H_2O_2. All of the experiments involving exposure to H_2O_2 were performed in serum-free DME to avoid rapid H_2O_2 degradation by antioxidants present in FBS. Cell culture in serum-free medium did not result in cell death within the time frame of the experiments.

2.3. Development of a tetracycline-responsive transactivator protein expressing the M14 cell line

The M14 melanoma cell line was stably transfected with the pTet-off plasmid (Clontech Laboratories, Palo Alto, CA) and selected using 1 mg/ml G418 (Gibco-BRL, Gaithersburg, MD).

2.4. Cloning of Cu/Zn SOD cDNA in the antisense orientation in pBiG vector

Cu/Zn SOD cDNA was amplified using the Quick clone human

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cDNA library from human leukocytes as template with the advantage TM cDNA PCR Kit (Clontech Laboratories, Palo Alto, CA). The following oligonucleotide primers were used: oligonucleotide forward with a *Sal*I restriction enzyme site 5′-CAC GCG GTC GAC ATG GCG ACG AAG GCC GTG TGC GTG CTG AAG-3′ and reverse oligonucleotide with a *Mlu*I restriction enzyme site 5′-CAC GCG ACG CGT TTA TTG GGC GAT CCC AAT TAC ACC ACA AGC-3′. The PCR-amplified full-length SOD cDNA (464 bp) was subsequently cloned in the antisense orientation into the vector pBiG. The sequence of the insert was verified by the Core Sequencing facility at the National University of Singapore.

2.5. Development of Cu/Zn SOD antisense expressing the M14 cell line

The M14 Tet-off clone was transfected with pBiG response plasmid containing the SOD cDNA in the antisense orientation, and the pTK-Hyg plasmid (Clontech Laboratories, Palo Alto, CA) containing the gene for resistance to hygromycin. After selection stably transfected clones were screened for repression of Cu/Zn SOD by antisense mRNA expression. Transfections of all cell lines were performed using the SuperFect Transfection Reagents from Qiagen GmbH (Germany) according to the vendor's instructions.

2.6. Western Blot analysis

Lysates from the M14 AS-SOD3 clone (5×10^6 cells) were prepared following incubation in the presence and absence of 2 μg/ml tetracycline for 48 h using 0.5 ml of RIPA/NP-40 lysis buffer (5 mM Tris pH 7.4, 30 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, and 0.5% NP-40). 30 μg of total protein per sample was then subjected to 10% PAGE, transferred to nitrocellulose, blocked overnight with 5% dried milk in Tris-buffered saline/0.1% Tween 20 (TBST), blotted with 2 μg/ml of a polyclonal sheep anti-human Cu/Zn SOD (UBI, Lake Placid, NY) for 1 h at room temperature. After three washes with TBST, the Cu/Zn SOD protein was detected using 1:5000 dilution of an anti-sheep HRP-conjugated IgG (Pierce, Rockford, IL). Poly(ADP-ribose) polymerase (PARP) cleavage was detected as described previously [14]. SuperSignal Substrate Western Blotting Kit (Pierce, Rockford, IL) was used for signal detection.

2.7. Chemiluminescence assay

The lucigenin chemiluminescence assay was performed as previously described [11]. Chemiluminescence was monitored for 20 s in a Lumat LB 9501 luminometer (Wallac, Gaithersburg, MD). Data are shown in relative light units per 20 s (RLU/20 s) ±S.E.M. from two independent measurements.

2.8. Determination of the cellular redox state (GSH/GSSG)

Determination of the cellular redox state was determined according to the method described by Anderson [16]. Briefly, 30×10^6 cells were used for each determination. Following incubation with different concentrations of H_2O_2, cells were trypsinized, pelleted at 4°C and washed twice with phosphate-buffered saline (PBS). The pellet was immediately frozen at −80°C or used fresh. The cell pellet was resuspended in buffer containing 0.2% Triton X-100 and 2.5% sulfosalicylic acid (Sigma Chemical Co., St. Louis, MO). After centrifugation at $15\,000\times g$ for 10 min, the supernatant was used for the determination of total glutathione (GSH+GSSG) and oxidized glutathione (GSSG). The ratio GSH/GSSG was calculated using the formula [(GSH+GSSG)−(GSSG)]/(GSSG)].

2.9. Measurement of intracellular pH

Intracellular pH (pH_i) of M14 cells (2×10^6/ml) with or without H_2O_2 treatment was measured by loading cells with the membrane-impermeant dye BCECF (2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein; Sigma Chemical Co., St. Louis, MO), and pH_i values were obtained from calibration curves obtained from cells loaded with 20 μM nigericin as described elsewhere [15].

2.10. Crystal violet assay

Crystal violet assays were performed in 96-well microtiter plates as described previously [11]. Cell viability was assessed by dye absorbance at 595 nm on an automated ELISA reader. Percentage cell death was calculated as 100−% cell survival. Cell survival was calculated as the mean of triplicate OD values of cells incubated with the specific apoptotic inducer divided by the mean of triplicate OD values of cells incubated in control medium, and expressed as a percentage. In each case, the control medium included exactly the same ingredients as the test medium, with the exception of H_2O_2. The S.E.M. of triplicate OD values never exceeded 5%. The data presented show one representative experiment from a minimum of three performed in triplicate.

3. Results

3.1. Induction of apoptosis by H_2O_2 is associated with caspase activation

It has previously been shown that exposure of certain cell types to low concentrations of H_2O_2 can induce morphological changes consistent with apoptosis, while higher concentrations of H_2O_2 invariably induce necrosis [12,17]. However, to precisely understand the mechanism by which H_2O_2 induces apoptotic cell death, we first addressed the status of caspase activity following exposure of M14 melanoma cells to increasing concentrations of H_2O_2. Caspase activity was assessed by the proteolytic cleavage of the nuclear protein PARP [18], a substrate for certain members of the caspase family including caspase 3, the key protease in the apoptotic machinery [19]. As expected, incubation of M14 cells with increasing concentrations of H_2O_2 resulted in both apoptotic and necrotic cell death depending upon the concentration of H_2O_2 used (Fig. 1a,b). At concentrations in excess of 1 mM in the cell culture medium, H_2O_2 induced necrosis of M14 cells, characterized by cell swelling, rupture of the plasma membrane, conserved nuclear structure (data not shown) and absence of PARP cleavage (Fig. 1b). However, at concentrations

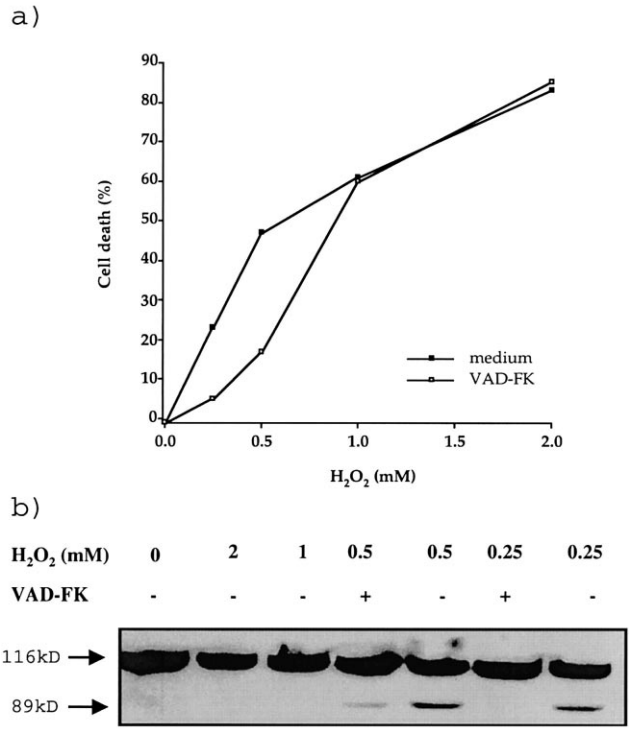

Fig. 1. H_2O_2 induces M14 cell death and PARP cleavage. M14 cells (2×10^6) were incubated for 18 h in DME without serum supplemented with increasing concentrations of H_2O_2 in the presence or absence of 200 μM VAD-FK, a specific ICE-like protease inhibitor peptide, prior to determination of (a) cell death and (b) PARP cleavage. Determination of the percentage of cell death was performed using crystal violet staining, and detection of the 85 kDa cleavage product of PARP was achieved by Western blot analysis using specific anti-PARP antibody.

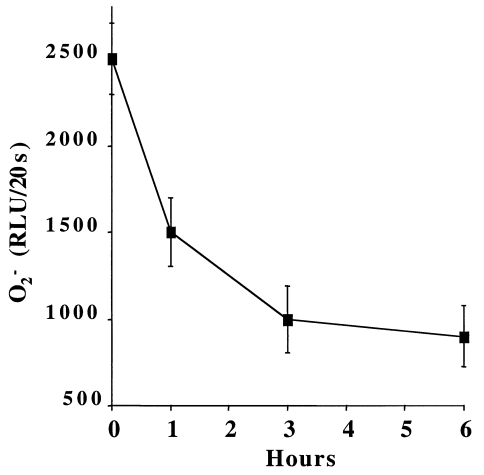

Fig. 2. H_2O_2-mediated apoptosis is associated with a decrease in the intracellular O_2^- concentration. Intracellular O_2^- concentration of M14 cells (2×10^6) was assessed following incubation with 0.5 mM H_2O_2 for 1, 3, and 6 h. Level of intracellular O_2^- was measured by a lucigenin-based chemiluminescence assay. Data are shown in relative light units per 20 s (RLU/20s) ± S.E.M from two independent measurements.

ranging between 0.25 mM and 0.5 mM, H_2O_2-induced cell death was characterized by the phenotypic features of apoptosis (data not shown) and accompanied by PARP cleavage (Fig. 1b). Moreover, H_2O_2-induced apoptosis and PARP cleavage were inhibited by a caspase-specific inhibitor, the VAD-FK peptide, while the same peptide had no effect on H_2O_2-induced necrosis (Fig. 1a,b).

3.2. *Apoptotic concentrations of H_2O_2 induce an intracellular decrease of O_2^-*

To determine if a relationship between H_2O_2-mediated apoptosis and intracellular O_2^- concentration could be found, we measured intracellular O_2^- concentration in M14 cells following incubation with 0.5 mM H_2O_2, which we found to be the optimal concentration for caspase activation and apoptosis in M14 cells. Intracellular O_2^- concentration was measured using a lucigenin-based chemiluminescence assay. Lucigenin has been widely used as a chemiluminescent detector of O_2^- production in biological systems [20], and despite a recent report questioning its validity [21], the work by Li et al. [22] has elegantly demonstrated that lucigenin is a reliable assay for detecting O_2^- production by enzymatic and cellular sources. Using this technique, a significant (almost 50%) time-dependent decrease in intracellular O_2^- concentration was detected upon exposure of M14 cells to 0.5 mM H_2O_2 which started as early as 1 h following the addition of H_2O_2 (Fig. 2). In order to be certain that the decrease in intracellular O_2^- upon exposure to an apoptotic concentration of H_2O_2 (0.5 mM) was not due to interference in the lucigenin-based detection of O_2^- by the presence of H_2O_2, we determined the effect of necrotic concentrations of H_2O_2 (1–2 mM) on intracellular O_2^- measurement. Our data showed that a decrease in intracellular O_2^- was only detected at concentrations of H_2O_2 which induce apoptosis, whereas necrotic concentrations resulted in no detectable change in O_2^- concentration over the untreated control cells (data not shown).

In order to correlate the decrease of intracellular O_2^- and activation of caspase following incubation of M14 cells with 0.5 mM H_2O_2, we thought to delay the H_2O_2-induced drop in intracellular O_2^- concentration by inhibiting Cu/Zn SOD, the major scavenger of intracellular O_2^-. M14 cells (2×10^6) were incubated for 1 h with 1 mM diethyldithiocarbamate (DDC), an inhibitor of Cu/Zn SOD [23] (M14/DDC) or with medium alone (M14). Cells were then washed once with PBS and incubated for another 3 and 7 h either with medium alone (medium) or with 0.5 mM H_2O_2 (H_2O_2) prior to the measurement of intracellular O_2^- and Western blot analysis for PARP cleavage.

Indeed, inhibition of Cu/Zn SOD by DDC effectively increased the intracellular O_2^- concentration when compared to untreated M14 cells (Fig. 3a). However, H_2O_2 was able to reduce the intracellular O_2^- concentration in M14 cells cultured in normal medium, as well as in DDC-treated M14 cells (M14 or M14/DDC) as shown in Fig. 3a. However, the time it took for H_2O_2 to decrease the intracellular O_2^- concentration in DDC-treated cells to levels lower than M14 cells (7 h as opposed to 3 h in M14 cells cultured in the absence of DDC) correlated well with the activation of caspases, as assayed by caspase-specific cleavage of PARP (Fig. 3b). These results show that maintenance of intracellular O_2^- concentration at or above that of M14 control cells prevents caspase activation, whereas a decrease in intracellular O_2^- below the natural

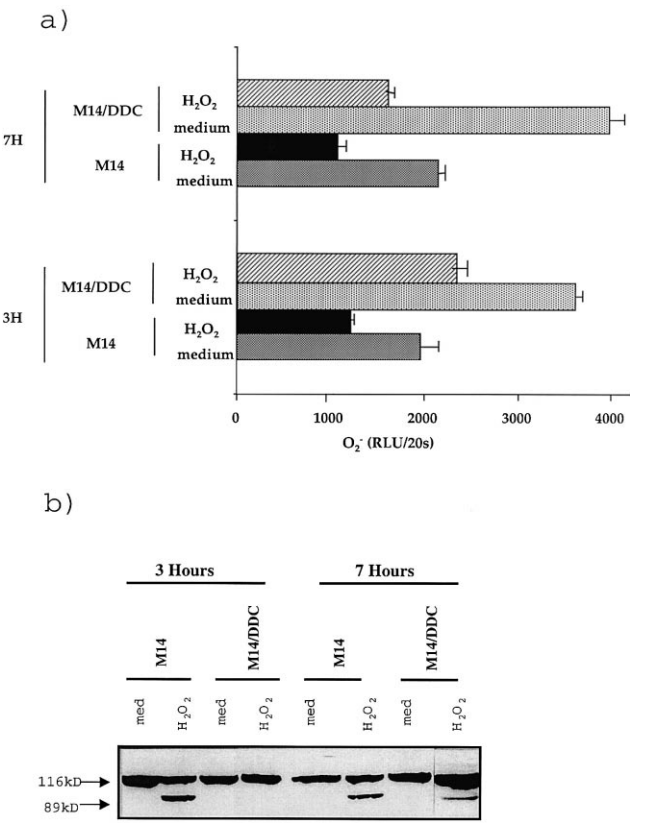

Fig. 3. Inhibition of intracellular Cu/Zn SOD retards H_2O_2-induced decrease in intracellular O_2^- concentration and caspase activity. a: Intracellular O_2^- concentration in M14 and M14/DDC cells (1 mM DDC) was evaluated following 3 and 7 h incubation of 2×10^6 cells with serum-free DME alone (medium) or supplemented with 0.5 mM H_2O_2 (H_2O_2). Intracellular O_2^- concentration was assessed as in Fig. 2. b: Western blot analysis of PARP cleavage following 3 and 7 h incubations of M14 and M14/DDC cells in serum-free medium alone (med) or supplemented with 0.5 mM H_2O_2 (H_2O_2). Western blot analysis was performed as described in Fig. 1.

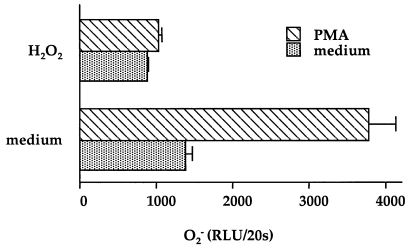

Fig. 4. Apoptotic concentration of H_2O_2 inhibits O_2^--producing enzymes. M14 cells (2×10^6) were incubated for 1 h in serum-free DME (medium) or serum-free DME containing 0.5 mM H_2O_2 (H_2O_2) prior to incubation with PMA (0.5 μg/ml). After 30 min exposure to PMA, intracellular O_2^- level was assessed by a lucigenin-based chemiluminescence assay as described in Fig. 2.

concentration induces activation of caspases. Further supporting these findings, exposure of M14 cells expressing the antisense SOD mRNA (Antisense SOD ON) to 0.5 mM H_2O_2 for 18 h resulted in a significant inhibition of cell death when compared to control M14 cells which did not express the antisense SOD mRNA (Antisense SOD OFF) (Fig. 4), highlighting the role of intracellular O_2^- concentration in H_2O_2-induced apoptosis. Taken together, these observations strongly support the notion that H_2O_2-induced apoptosis may in part be due to a direct or an indirect inhibition of intracellular O_2^- production that can induce activation of the apoptotic machinery.

3.3. H_2O_2 inhibits NADH/NADPH-dependent oxidases

Although PARP is one of the well described substrates for caspases, its activation has also been implicated in apoptosis induced by H_2O_2. PARP utilizes nicotinamide adenine dinucleotide (NAD) as substrate, and sustained activation of PARP by low doses of H_2O_2 has been shown to deplete the intracellular NAD^+ pool [17] which may have physiological implications, such as the inhibition of O_2^- producing NADH/NADPH-dependent oxidases. Because generation of O_2^- by these enzymes is typically stimulated by phorbol esters, we determined whether intracellular O_2^- production could be induced by PMA following exposure of cells to low concentrations of H_2O_2. When M14 cells (2×10^6), cultured for 1 h in medium supplemented with 0.5 mM H_2O_2, were further treated with PMA (0.5 μg/ml), no production of O_2^- was detected compared to control cells left for the same period of time in medium alone (Fig. 5). These data suggest that one mechanism by which H_2O_2 induces a decrease in intracellular O_2^- concentration could be through a direct or indirect inhibition of NADH/NADPH-dependent oxidases.

3.4. A decrease in intracellular O_2^- enhances the reducing state of the intracellular milieu

Since variation in the intracellular concentration of reactive oxygen species is a major regulator of the cellular redox state, we determined the effect of decreasing the intracellular O_2^- concentration with H_2O_2 on the redox state of M14 cells. Assessment of intracellular redox variations was performed by measuring the reduced (GSH)/oxidized (GSSG) ratio of glutathione. The enzyme glutathione disulfide reductase (GSSG reductase) catalyzes an equilibrium that greatly favors formation of reduced glutathione (GSH) [24]. Thus, the intracellular milieu is normally in a reducing state, with most of the glutathione present in the cell in the thiol form, and a

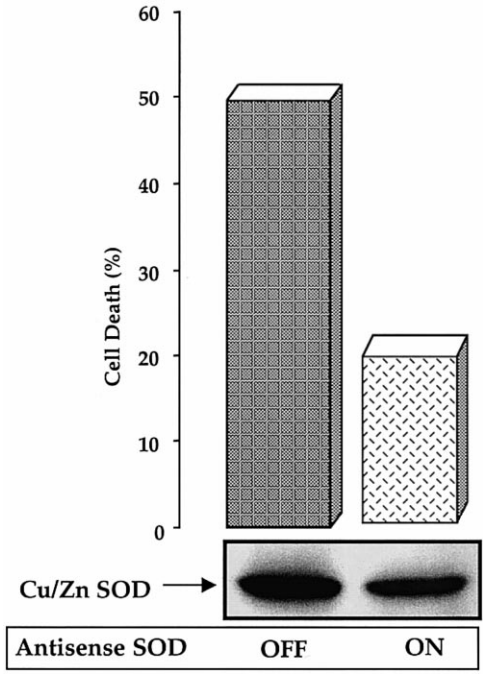

Fig. 5. Decrease in intracellular Cu/Zn SOD protein inhibits H_2O_2-induced apoptosis. Western blot analysis of Cu/Zn SOD expression in lysates of M14 AS-SOD3 clone (5×10^6 cells) cultured in the presence of 2 μg/ml of tetracycline (OFF) or 48 h after the removal of tetracycline (ON). 30 μg of total protein was subjected to 10% PAGE, transferred to nitrocellulose, blotted with 2 μg/ml of a polyclonal sheep anti-human Cu/Zn SOD for 1 h at room temperature. The Cu/Zn SOD protein was detected using a 1:5000 dilution of an anti-sheep HRP-conjugated IgG and visualized by the Super Signal Substrate Western Blotting Kit. Determination of the percentage cell death was performed using crystal violet staining after 18 h incubation of M14 cells in presence of 0.5 mM H_2O_2.

[GSH]/[GSSG] ratio in the 100–200:1 range [25]. However, metabolic changes which result in enhanced O_2^- production can lower the [GSH]/[GSSG] ratio to 10:1 and even 1:1 [25]. In contrast, any situation which leads to the depletion of intracellular ROI may be expected to augment the baseline cellular reducing state and therefore the [GSH]/[GSSG] ratio.

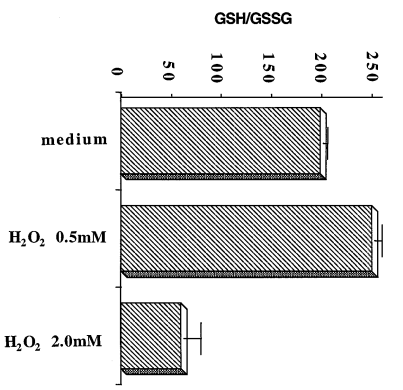

Fig. 6. Changes in the redox state of M14 cells following incubation with H_2O_2. M14 cells were incubated for 5 h in serum-free DME alone (medium) or supplemented with 0.5 mM (H_2O_2 0.5mM) or 2 mM (H_2O_2 2mM) H_2O_2 prior to determining the [GSH]/[GSSG] ratio. GSH/GSSG measurements were performed as described in Section 2. The baseline [GSH]/[GSSG] level at the time of the experiment is indicated (medium).

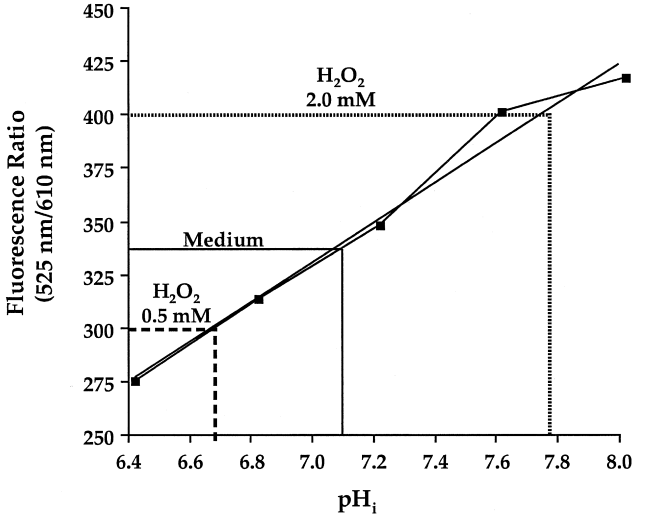

Fig. 7. Apoptotic concentration of H_2O_2 induces intracellular acidification. M14 cells (2×10^6) were incubated for 2 h in serum-free DME (medium) or serum-free DME containing 2.0 mM or 0.5 mM H_2O_2 before intracellular pH was measured by obtaining the fluorescence ratio (525/610 nm) of cells loaded with BCECF, and plotted against a standard curve generated by nigericin (20 μM) treatment of cells loaded with BCECF in a high K^+ buffer at various pH values (6.4–8.0), as described in Section 2.

As expected, a major decrease in the [GSH]/[GSSG] ratio, as well as in total cellular glutathione, reflecting oxidative stress, was observed in cells exposed to 2 mM H_2O_2 which underwent death by necrosis (Fig. 6 and data not shown). Conversely, a sustained decrease in the intracellular O_2^- concentration following incubation of M14 cells with 0.5 mM H_2O_2 resulted in an augmentation of the intracellular reducing state, as determined by an increase in the ratio of intracellular [GSH]/[GSSG] (Fig. 6). Since the total intracellular glutathione content was unchanged (data not shown), these data suggest that the observed increase in [GSH]/[GSSG] was due to an increase in [GSH] as a result of the reduction of GSSG, and therefore is reflective of a redox equilibrium which favors the reduced rather than the oxidized state of thiol containing proteins.

3.5. *H_2O_2-induced decrease in intracellular O_2^- is accompanied by acidification of the intracellular milieu*

Recent observations have demonstrated that apoptosis triggered by a variety of agents is preceded by acidification of the intracellular environment [26] and induction of apoptosis by hypoxia requires acidosis of the intracellular milieu [27]. Therefore, we asked the question if the decrease in intracellular O_2^- induced by exposure of M14 cells to apoptotic concentrations of H_2O_2 could, indeed, also be associated with intracellular acidification. In order to investigate that, we measured the pH_i of M14 cells using a cell-impermeant dye BCECF following 2 h incubation with increasing concentrations of H_2O_2. As shown in Fig. 7, incubation of M14 cells with apoptotic doses of H_2O_2 resulted in a decrease in the pH_i from 7.3 (control cells) to 6.7, while concentrations of H_2O_2 that resulted in necrotic death induced a significant increase in the pH_i. These data demonstrate that apoptotic concentrations of H_2O_2 induce acidification of the intracellular milieu by an increase in the concentration of H^+, and conversely, necrotic concentrations promote intracellular alkalinization.

4. Discussion

Numerous agents that induce apoptosis stimulate intracellular production of ROI, leading most frequently to an accumulation of H_2O_2. Moreover, many inhibitors of apoptosis have antioxidant properties, or enhance the cellular antioxidant defense mechanism. These observations have led to the suggestion that ROI are effectors for a variety of triggers of apoptosis, including tumor necrosis factor α, C2 ceramide, anti-IgM antibody, dexamethasone, irradiation and numerous anticancer drugs [7]. On the other hand, the observation that hypoxia can induce apoptosis supports the argument that ROI are not necessary for mediating apoptotic cell death [9,10]. Our present observations propose an explanation for such conflicting results. We show that a decrease in the intracellular O_2^- concentration, reduction and acidification of the intracellular milieu constitute three major characteristics of apoptotic cell death induced by H_2O_2. Thus, similar to apoptosis induced by hypoxia, H_2O_2-induced apoptotic cell death results from a sustained decrease in the intracellular O_2^- concentration, and augmentation of the intrinsic reducing state of the cells [27]. By contrast, cell death triggered by H_2O_2 concentrations that induce oxidative stress, as shown by measurement of the GSH/GSSG ratio, has a necrotic phenotype. Taken together, these observations suggest that a decrease in the O_2^- concentration and augmentation of the reducing state of the intracellular milieu may therefore represent a common mechanism for triggering apoptosis by stimuli which induce H_2O_2 production or directly inhibit intracellular O_2^- production. We therefore propose to refer to the mechanism of apoptosis induced by such stimuli as 'reductive stress-induced' (RSI) apoptosis as opposed to oxidative stress that should be reserved for necrotic cell death seen at higher concentrations of H_2O_2.

The precise mechanism by which H_2O_2 induces a decrease in intracellular O_2^- concentration leading to activation of the apoptotic machinery is far from understood. However, as addressed in this communication, inhibition of NADH/NADPH-dependent oxidases could be one mechanism. Moreover, the oxidation-reduction state of the cell, better known as the redox state, has been shown to influence the physiology of the cell. For example, changes in redox state influence transcription factor activation [28], protein conformation and phosphorylation states, as well as cytosolic Ca^{2+} metabolism [29]. Any of these events may potentially induce activation of the ICE-like protease cascade. In addition, recent findings have shown that intracellular acidification can induce apoptosis by directly stimulating caspase activity [30], however, the molecular mechanisms of this activation are still not well understood. Finally, recent observations suggest that cells may need to maintain a reducing environment to effectively carry out the apoptotic program [12,13].

These findings in conjunction with our previous observations on the inhibitory role of O_2^- in CD95-mediated cell death [11] underscore the importance of distinguishing between the role of ROI in necrotic versus apoptotic cell death. Whereas ROI provide a direct effector mechanism for necrotic cell death, they rather seem to function as regulators of cell sensitivity to apoptosis. In order to survive, all cells depend on a constant repression of their intrinsic suicide program by signals from surrounding cells and the extracellular matrix. An important effect of extracellular survival signals may

therefore be to maintain a critical cellular redox equilibrium, based, at least in part, on adequate intracellular O_2^- production.

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