Prevention of Mitochondrial Injury by Manganese Superoxide Dismutase Reveals a Primary Mechanism for Alkaline-induced Cell Death*

(Received for publication, November 3, 1997, and in revised form, January 14, 1998)

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Alkalosis is a clinical complication resulting from various pathological and physiological conditions. Although it is well established that reducing the cellular proton concentration is lethal, the mechanism leading to cell death is unknown. Mitochondrial respiration generates a proton gradient and superoxide radicals, suggesting a possible link between oxidative stress, mitochondrial integrity, and alkaline-induced cell death. Manganese superoxide dismutase removes superoxide radicals in mitochondria, and thus protects mitochondria from oxidative injury. Cells cultured under alkaline conditions were found to exhibit elevated levels of mitochondrial membrane potential, reactive oxygen species, and calcium which was accompanied by mitochondrial damage, DNA fragmentation, and cell death. Overexpression of manganese superoxide dismutase reduced the levels of intracellular reactive oxygen species and calcium, restored mitochondrial transmembrane potential, and prevented cell death. The results suggest that mitochondria are the primary target for alkaline-induced cell death and that free radical generation is an important and early event conveying cell death signals under alkaline conditions.

Alkalosis, a process which raises the cellular pH, is associated with various clinical conditions. Alkalosis induced by alterations in the FCO2 is considered respiratory alkalosis since FCO2 is regulated by respiration. The general symptoms produced by respiratory alkalosis are increased irritability of the central and peripheral nervous systems which are thought to be related to the ability of alkalosis to impair cerebral function (reviewed in Ref. 1). Although it is well known that alkalosis, a process which raises the cellular pH, is associated with various clinical conditions, alkalosis induced by alterations in the FCO2 is considered respiratory alkalosis since FCO2 is regulated by respiration. The general symptoms produced by respiratory alkalosis are increased irritability of the central and peripheral nervous systems which are thought to be related to the ability of alkalosis to impair cerebral function (reviewed in Ref. 1). Although it is well known that alkalosis is not protected by histones. Mutations in any of the genes coding for cytochrome oxidase, cytochrome bc1, NADH dehydrogenase, or ATPase complexes may lead to defective function of these enzymes. It has been shown that ROS generating agents can cause mitochondrial DNA damage (7). Furthermore, 4Fe-4S containing enzymes such as aconitases, are highly sensitive to redox-induced inactivation (8). Thus oxidative stress in the mitochondria can cause mitochondrial defects and increased oxidative stress to the organism.

Evidence has accumulated which supports a direct role for mitochondria in cell death. Mitochondrial respiration generates a proton gradient resulting from the mitochondrial transmembrane potential which is essential for mitochondrial function. It has been demonstrated that the mitochondrial permeability transition causes uncoupling of the respiratory chain with collapse of the mitochondrial membrane potential, calcium release, hypergeneration of superoxide radicals, and mitochondrial release of apoptotic proteins (9, 10). Mitochondrial release of cytochrome c has been shown to be a key step in staurosporin-induced apoptosis (11). In addition, activation of a

* This work was supported by National Institute of Health Grants HL03544, CA49797, and CA58835 (to D. K. St. C.), the Environmental Protection Agency (EPA 95466), the Kentucky Tobacco Research Board, and the American Heart Association. Part of this work has been presented at the Oxygen Society, Miami Beach, FL, abstract A-15, November 21–25, 1996. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: ROS, reactive oxygen species; BCECF-AM, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; DCF, 2,7-dichlorofluorescein diacetate; diRhO, dihydrorhodamine 123; F344, II, murine fibrosarcoma cell line; FIA, 4-hydroxy-2-nonenal, 1,5,5′,6′-tetrachloro-1,3,3′-tetramethylindacene; SOD, superoxide dismutase; pH4, extracellular pH; pHi, intracellular pH; [Ca2+]i, intracellular Ca2+; PBS, phosphate-buffered saline; HBBS, Hank’s balanced salt solution; TEMED, N,N,N′,N′-tetramethylmethylenediamine.

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member of the interleukin-1 converting enzyme family of cysteine proteases in tumor necrosis factor-induced apoptosis has been shown to be dependent on mitochondrial function (12). Furthermore, overexpression of bcl-2 prevented tumor necrosis factor-induced apoptosis and increased mitochondrial membrane potential, suggesting that mitochondria play an important role in apoptosis (13).

Aerobic organisms possess antioxidant defense systems that deal with ROS produced as a consequence of aerobic respiration. One important family of enzymes are the superoxide dismutases. These enzymes dismutate superoxide radicals into hydrogen peroxide and molecular oxygen, which can then be acted upon by other antioxidant enzymes such as glutathione peroxidase and catalase. In humans there are three forms of superoxide dismutase: cytosolic Cu/Zn superoxide dismutase (Mn-SOD), mitochondrial manganese superoxide dismutase (Mn-SOD), and extracellular superoxide dismutase (EC-SOD). Mn-SOD is a nuclear-encoded primary antioxidant enzyme that functions to remove superoxide radicals in mitochondria (14). The biological importance of Mn-SOD is demonstrated by the following: 1) inactivation of Mn-SOD and iron containing SOD genes in Escherichia coli increased mutation frequency when grown under aerobic conditions (15, 16). 2) Elimination of the Mn-SOD gene in Saccharomyces cerevisiae increased its sensitivity to oxygen (17). 3) Lack of Mn-SOD expression resulted in dilated cardiomyopathy and neonatal lethality in Mn-SOD knock-out mice (18). 4) Tumor necrosis factor selectively induced Mn-SOD, but not Cu/Zn-SOD, catalase, or glutathione peroxidase mRNA in various mouse tissues and cultured cells (19). 5) Transfection of Mn-SOD cDNA into cultured cells rendered the cells resistant to paraquat-, tumor necrosis factor-, and adriamycin-induced cytotoxicity, and radiation induced-neoplastic transformation (20–23). 6) Expression of human Mn-SOD genes in transgenic mice protected the mice from oxygen-induced pulmonary injury and adriamycin-induced cardiac toxicity (24, 25). Thus, the expression of Mn-SOD is essential for the survival of aerobic life and the development of cellular resistance to oxygen radical-mediated toxicity.

Because alkaline conditions reduce the concentration of protons which could subsequently affect mitochondrial membrane potential, we examined the possible role of mitochondrial antioxidant defenses in alkaline-induced apoptotic cell death. We report here that increased expression of mitochondrial superoxide dismutase protects cells against alkaline-induced mitochondrial damage, suppresses ROS generation and calcium release, as well as prevents nuclear condensation, DNA fragmentation, and cell death. Our results demonstrate that mitochondrial-mediated intracellular ROS generation is an important mechanism by which alkaline conditions cause cellular lethality and suggest that removal of superoxide radicals in the mitochondria is a critical step in the prevention of alkaline-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—A murine fibrosarcoma cell line (FSa-II) (26), which arose in a C3H/SeJ mouse was transfected with the pSV2-NEO plasmid or co-transfected with sense human Mn-SOD expression plasmid (pHAPR-1) plus the pSV2-NEO plasmid using Lipofectin as previously reported (27). Pure populations of cloned cells expressing low (SOD-L), high (SOD-H) level of Mn-SOD, or selectable marker alone (NEO) were used in all experiments. The cells were cultured in McCoy’s 5a medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta, Norcross, GA) and 1% antibiotics (5 mg of penicillin, 5 mg of streptomycin, and 10 mg of neomycin/ml, Life Technologies, Inc.) at 37 °C in humidified air containing 5% CO₂.

**Standard or Alkaline Conditions**—Standard conditions were obtained by culturing cells in humidified air containing 5% CO₂. Alkaline condition (pH 8.3) was obtained by culturing cells in humidified air containing 5% CO₂. Alkaline conditions were obtained by culturing cells in humidified air containing 0.2% CO₂, respectively. For experiments using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyfluorocarboxyfluorocarbocyanine ester (BCECF-AM), fura-2, 2,7-dichlorodihydrofluorescein diacetate (DCF), 5',5',6',6'-tetrachloro-1',1',3',3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), or dihydrodihomadine 123 (dRh10), a modified Hanks’ balanced salt solution (HBSS) containing 10.0 mM Hepes, 1.0 mM MgCl₂, 2.0 mM CaCl₂, 2.7 mM glucose, and 0.001% of Gentamicin (Sigma) adjusted to pH 7.3 ± 0.05 and 8.3 ± 0.05 was used.

**SOD Activity Gel Assay**—To determine the growth characteristics under various culture conditions, the cells were plated in 60-mm tissue culture dishes at 5 × 10⁴ cells per dish and cultured under normal culture (pH ≈ 7.3) conditions continuously or 24 h after plating, then the cells were transferred to alkaline conditions (pH 8.3) and cultured for 4 days. The cells were trypsinized and the number of cells were counted using a hemacytometer daily. The average cell number at 24 h after plating for each cell line was normalized to one, and the relative cell number was plotted as a function of days after plating.

**Cell Survival Assay**—Survival after 24 h of culture under alkaline conditions (pH 8.3) was analyzed by *in vitro* colony formation. A stock culture near confluence was trypsinized and the number of cells were counted by a hemacytometer. Five hundred cells for SOD-L and SOD-H, and 1000 cells for NEO cells were plated in plastic dishes. Twenty hours after plating, the cells were transferred to alkaline conditions (pH 8.3) for 24 h, then returned to standard conditions. After incubation at 37 °C with 5% CO₂ for 11 days, the cells were stained with crystal violet, and colonies containing more than 50 cells were counted using a dissecting microscope. All experiments were repeated at least three times. The surviving fraction was calculated by the following equation,

\[
\text{Surviving factor} = \frac{\text{Number of colonies formed}}{\text{Number of cells plated} \times \text{plating efficiency}} \quad \text{(Eq. 1)}
\]

**SOD Activity Gel Assay**—A non-denaturing gel assay for SOD activity was performed according to the methods previously described (29) with slight modifications. Cells were sonicated in 50 mM potassium phosphate buffer (pH 7.8). 250 μg of protein/lane was electrophoresed through a nondenaturing ribogelatin gel consisting of a 5% stacking gel (pH 6.8) and a 12% running gel (pH 8.8) at 4 °C. To visualize SOD activity, gels were first incubated in 2.43 mM nitro blue tetrazolium (Fisher Scientific, Pittsburgh, PA) in deionized water for 20 min and then in 0.028 mM riboflavin, 280 mM TEMED (Life Technologies, Inc., Grand Island, NY) in 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the dark. Gels were then washed in deionized water and illuminated under fluorescent light until clear zones of SOD activity were evident.

**DNA Gel Electrophoresis Assay**—DNA ladder formation assay was performed according to the method described by Darzynkiewicz et al. (29) with minor modifications. Briefly, normal pH or alkaline pH-treated cells were trypsinized and collected by centrifugation and fixed in 70% ethanol. This was done by transferring 1 ml of cell suspension in PBS (pH 7.3) into tubes containing 10 ml of 70% ethanol on ice. The cells were stored in the fixative at ~20 °C for up to 2 weeks. The cells were then spun at 1000 × g for 5 min and the ethanol was thoroughly removed. The cell pellets (1 × 10⁷ cells), in 1.5-ml Eppendorf tubes, were resuspended in 80 μl of phosphate-citrate buffer, consisting of 192 parts of 0.2 M Na₂HPO₄ and 8 parts of 0.1 M citric acid (pH 7.8), at room temperature, for 12 h. After centrifugation at 1000 × g for 5 min, the supernatant was transferred to new tubes and concentrated by vacuum in a vacuum concentrator (Jouan Co., Winchester, VA) for 20 min. A 5-μl aliquot of 0.25% Nonidet P-40 (Sigma) in distilled water was then added, followed by 10 μl of RNase A (Sigma, 25 mg/ml DNase free). Twenty-four h after incubation at 37 °C, 3 μl of a solution of proteinase K (1 mg/ml, Sigma) was added and the extract was incubated for an additional 2 h at 37 °C. The extracted DNA from 1 × 10⁷ cells was analyzed by electrophoresis through a 1.8% agarose gel and photographed under UV light after staining with ethidium bromide.

**Microscopic Assay of Nuclear Chromatin Condensation and Fragmentation**—Cells grown on glass coverslips were stained with the fluorescent dye Hoechst 33342 (Molecular Probes, Eugene, OR). Cells were fixed for 30 min in a solution containing 4% paraformaldehyde in PBS and then incubated in PBS with 1 μg/ml of the dye for 30 min. Cells were washed twice with PBS, then twice with water. Fluorescence was visualized using a 40 × oil immersion lens inverted microscope (Nikon, Tokyo, Japan); the dye was excited at 340 nm and emission was filtered with a 510-nm barrier filter. Photographs of microscope fields were taken using a 35-mm Nikon camera and Kodak film. To quantify the extent of apoptosis, the number of cells with fragmented DNA were counted in 4 random 40 × fields.

**Measurement of Extracellular pH and Intracellular pH**—Measure-
ment of extracellular pH (pH_{e}) was performed using a pH meter (Corn- ing pH meter 240; Corning, NY) with a general purpose combination electrode (476530, Corning). The pH conditions were standardized with buffer solutions at pH 7.00 ± 0.01 and pH 10.00 ± 0.02 at 25°C (Fisher). Intracellular pH (pH_i) was determined by using the pH-sensitive fluorescent dye, BCECF (30–32). Cells were incubated in the modified Hanks’ solution adjusted to pH 7.3 or 8.3 for 60 min, then loaded with acetoxyethyl ester derivative (BCECF-AM) (Molecular Probes, Eugene, OR) at a final concentration of 3 μg/ml for 30 min at 37°C. The cells were washed with high [K+] solution (142.3 mM KCl, 1.5 mM CaCl_2, 1.0 mM MgCl_2, 20.0 mM HEPES, 5.5 mM glucose, and 0.01 mM EDTA) and harvested with a rubber policeman. The intensity of 535 nm emission fluorescence from the excitation wavelengths 440 and 490 nm, respectively, were measured with a spectrophotometer (Shimadzu, Kyoto, Japan). The 440/490 ratio was used to obtain the pH value from a standard curve.

**Measurement of Intracellular Free Calcium Levels ([Ca^{2+}]_{i})**, **Cellular ROS, and Mitochondrial Membrane Potential**—Procedures for quantifying [Ca^{2+}]_{i}, in individual cells by fluorescence ratio imaging of the Ca^{2+} indicator dye fura-2 are detailed in our previous studies (33). Cells were incubated in the presence of 2 μg/ml acetoxyethyl ester form of the Ca^{2+} indicator dye fura-2 (Molecular Probes) for 30 min. Cells were washed with the modified HBSS adjusted to pH 7.3 or 8.3 and incubated with the modified HBSS at pH 37°C for various time periods. Cells were imaged with a Zeiss Axiovert inverted microscope using a fluoro x 40, N.A. 1.3 fluorescence objective and an Attocouler intensified CCD camera. Images were acquired using a Zeiss Axioflou system. The ratio of fluorescence emission using two different excitation wavelengths (340 and 380 nm) was used (following background subtraction and shade correction) to determine [Ca^{2+}]_{i}. The system was calibrated using solutions containing no Ca^{2+} (0 Ca^{2+} plus 1 mM EGTA) or a saturating level of Ca^{2+} (1 mM) according to the formula [Ca^{2+}]_{i} = K_{F0}/(F_{0} - F)/K_{F0} for our system K_{F0} was 0.85, R_{max} was 10.40, F_{0}/F_{max} was 13.99, and K_{F0} was 224.0. Values represent the average [Ca^{2+}]_{i} in the cell body. The ratio of the Ca^{2+} at pH 7.3 to pH 8.3 was calculated.

Relative levels of ROS were determined by confocal laser microscopy image analysis of cultured cells loaded with DCF as detailed previously (34). Cell culture medium was replaced with modified HBSS at pH 7.3 or 8.3, and incubated for 60 min at 37°C. Then cells were loaded with DCF by incubation for an additional 30 min in the presence of 50 μM dye. Images of DCF fluorescence were acquired using a confocal laser scanning microscope (Molecular Dynamics, Sararosta 2000) coupled to an inverted microscope (Nikon). The dye was excited at 488 nm and emission was filtered using a 535-nm barrier filter. The intensity of the laser beam and the sensitivity of the photodetector were held constant to allow quantitative comparisons of relative fluorescence intensity of the cells between groups. Cells were chosen for analysis on a random basis and scanned only once since the laser light could itself induce photobleaching, resulting in increased cell fluorescence. Values for average fluorescence intensity/cell were obtained using “ImageSpace” software supplied by the manufacturer (Molecular Dynamics).

Relative levels of mitochondrial ROS was determined by confocal laser microscope image analysis of cultured cells loaded with dhRho (Molecular Probes, Eugene, OR) using the same procedures described for DCF measurements. The final concentration of the dye used in the study was 10 μg/ml.

Relative levels of mitochondrial membrane potential was also determined using confocal laser microscope image analysis of cultured cells loaded with JC-1 (Molecular Probes) similar to that of DCF, except the excitation wave length was 514 nm. The final concentration of the dye used in the study was 1 μg/ml.

**Immunoperoxidase Staining for 4-Hydroxy-2-nonenal**—Flasks with monolayers were prepared for immunostaining with polyclonal antibody raised to 4-hydroxy-2-nonenal (HNE)-modified keyhole limpet hemocyanin. The antibody was tested for cross-reactivity toward malonaldehyde and sodium acetylcysteine derivatives of acrolein, trans-2-pentanal, and trans-2-nonenal, compounds structurally similar to HNE. Competitive Western blot experiments, recognizes cysteine, histidine, and lysine-HNE adducts (36). The epitope recognized by the antibody appears to be the hemiacetal form of the HNE-derived portion of protein-HNE adducts. Cells were fixed with 10% buffered formalin at room temperature for 10 min, rinsed with PBS, and post-fixed with absolute ethanol for 10 min. Immunostaining was performed as previously described (20). Briefly, fixed cells were rinsed with PBS, and endogenous peroxidase was blocked by incubation with 5% hydrogen peroxide for 5 min. Nonspecific antigenic sites were blocked with swine serum for 30 min. Excess serum was then removed and the anti-diaminobenzidine antibodies were incubated overnight at 4°C. The grids were stained with lead citrate and uranyl acetate, and observed in a Hitachi H-300 electron microscope (Hitachi Co., Hitachi, Japan). At least 20 cells (20–40) were photographed and described in a blinded fashion by a pathologist (T. D. O.). The photographs presented are representative of cells examined.

**Statistical Analysis**—Data were evaluated using the SAS system (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed for multiple comparisons of each dependent variable. ANOVA with complete block design was used for experiments performed at different times (37). p value < 0.05 was considered to be statistically significant. All data was presented as mean ± S.E.

**RESULTS**

**Intracellular pH and Mn-SOD Activity**—The intracellular pH of FSA-II, NEO, SOD-L, and SOD-H which were incubated for 90 min at pH 7.3 or 8.3 were 7.41–7.49 and 7.86–7.99, respectively (Table I). The results indicate that pH_i at pH 7.3 are 0.1 to 0.2 higher, while pH_i at pH 8.3 are 0.3 to 0.4 lower than the pH_i in all four cell lines studied. There were no differences in pH_i observed among the four cell lines, both at pH_i 7.3 and 8.3. To monitor if changes in pH affected the cell death, the Mn-SOD activity was determined. The Mn-SOD activity was significantly different among the four cell lines. The Mn-SOD activity in FSa-II, NEO, SOD-L, and SOD-H cells cultivated at pH 7.3 or 8.3 for 24 h. Each lane was loaded with 250 μg of protein and electrophoresed through a 10% polyacrylamide gel at 4°C. Lane 1, FSA-II at pH 7.3; lane 2, NEO at pH 7.3; lane 3, SOD-L at pH 7.3; lane 4, SOD-H at pH 7.3; lane 5, FSA-II at pH 8.3; lane 6, NEO at pH 8.3; lane 7, SOD-L at pH 8.3; lane 8, SOD-H at pH 8.3. The pHi in FSa-II, NEO, SOD-L, and SOD-H cells cultured at pH 7.3 or 8.3 for 90 min. The pHi in FSa-II, NEO, SOD-L, and SOD-H cells cultured at pH 7.3 or 8.3 for 90 min. The pHi in FSa-II, NEO, SOD-L, and SOD-H cells cultured at pH 7.3 or 8.3 for 90 min. The pHi in FSa-II, NEO, SOD-L, and SOD-H cells cultured at pH 7.3 or 8.3 for 90 min. The pHi in FSa-II, NEO, SOD-L, and SOD-H cells cultured at pH 7.3 or 8.3 for 90 min.
Mn-SOD activity in the transfected cells, SOD activity gel assays were performed. The results demonstrated that the activity of the Cu/Zn-SOD was unchanged for all cell lines at both pH 7.3 and 8.3 (Fig. 1). The activity of Mn-SOD in FSa-II and NEO cells was not detectable, suggesting the activity of Mn-SOD is very low for these two cell lines. The Mn-SOD activity in the SOD-L and SOD-H was clearly detectable and the human Mn-SOD activity in the SOD-H cells was greater than the SOD-L cells. These results confirmed that the human Mn-SOD in the transfected cells remains stably expressed and there was no major change in detectable Mn-SOD or Cu/Zn-SOD activity under alkaline conditions.

Effect of Alkaline pH on Growth Kinetics and Cell Survival—Fig. 2 shows growth kinetics of the four cell lines at pH 7.3 and 8.3. At pH 7.3 the growth curves were not different among the four cell lines. At pH 8.3, growth of FSa-II and NEO cells was suppressed, while SOD-L and SOD-H cells had only a slightly slower rate than that at pH 7.3 (Fig. 2).

Fig. 3 shows results of surviving fraction studies of the four cell lines at pH 8.3. At pH 8.3, the surviving fractions for SOD-L and SOD-H cells were significantly greater compared with the nontransfected cells (p < 0.03, NEO versus SOD-L; p < 0.005, FSa-II versus SOD-H; p < 0.002, NEO versus SOD-H; p < 0.002, Non-SOD versus SOD).

The Effect of Alkaline Conditions on Cell Death—To determine if reduced cell survival at alkaline pH was due to apoptotic cell death, we performed DNA fragmentation and nuclear condensation assays. Fig. 4A shows the results of the DNA fragmentation assay for the four cell lines cultured at pH 7.3 or 8.3 for 48 h. Only FSa-II and NEO cells exhibited DNA ladder formation at pH 8.3. No visible DNA fragmentation was found in the SOD-L and SOD-H cells at pH 8.3.

To further verify the presence of apoptosis, photomicrographs following Hoechst staining were taken. Fig. 4B shows chromatin condensation and nuclear fragmentation after staining with Hoechst 33342 for 24 h at pH 7.3 and 8.3. At pH 8.3, the FSa-II and NEO cells showed chromatin condensation and nuclear fragmentation features typical of apoptosis (Fig. 4B). The frequency of chromatin condensation and fragmentation was determined; 4% of nuclei in both FSa-II and NEO cells cultured at pH 8.3 showed apoptotic changes, while no apoptotic nuclei were detected in SOD-L and SOD-H cells. Note that cell number in the same surface area were greatly reduced for FSa-II and NEO at pH 8.3, while there was no substantial change in cell number for the SOD-L and SOD-H cells (Fig. 4B).

Alkaline pH Alters Mitochondrial Membrane Potential and Induces Oxidative Stress—Since alkaline pH alters the intracellular proton concentration, we compared the mitochondrial membrane potential of all four cell lines at pH 7.3 and 8.3 using a dye sensitive to mitochondrial membrane potential, JC-1. The intensity of JC-1 increased in FSa-II and NEO cells but not in SOD-L and SOD-H cells, indicating that the mitochondrial membrane potential was increased in FSa-II and NEO cells, but not in SOD-L and SOD-H cells following 90 min of incubation at pH 8.3 (Fig. 5).

To determine if changes in mitochondrial membrane potential were accompanied by changes in intracellular ROS, we measured the levels of intracellular ROS using the ROS-sensitive dye DCF. Fig. 6A shows the results of DCF staining for the four cell lines at pH 7.3 and 8.3. The staining was performed at 90 min of culturing at pH 7.3 or 8.3. There was a substantial increase in fluorescence intensity in FSa-II and NEO cells but not in SOD-L and SOD-H cells at pH 8.3 (Fig. 6A). For a
quantitative comparison, the average intensity of DCF fluorescence was determined in the four cell lines following incubation for 90 min at pH 8.3. The results confirmed the qualitative results shown in Fig. 6A, i.e. the FSa-II and NEO cells exhibited a large increase in ROS concentration, whereas little or no change was detected in the SOD-L and SOD-H cells (Fig. 6B).

To determine if the increased intracellular ROS observed with DCF fluorescence was accompanied by an increase mitochondrial ROS, a dye-sensitive to change in mitochondrial ROS, dhRho was used. Similar changes to those observed with DCF were documented with dhRho fluorescence following 90 min incubation at pH 8.3 (Fig. 6B), indicating that ROS was increased in the mitochondria of the FSa-II and NEO cells but not in the SOD-L and SOD-H cells.

Alkaline pH Induces Lipid Peroxidation—To determine if ROS generation is accompanied by increase lipid peroxidation product, the levels of HNE, a highly toxic aldehyde product of lipid peroxidation (38, 39) and a sensitive marker of oxidative damage and lipid peroxidation (40) was evaluated by immunohistochemical staining. As shown in Fig. 7A, in control cells (Fig. 7A), apoptotic bodies and dead cells showed strong staining for HNE. In SOD cells (Fig. 7C), apoptotic bodies and dead cells were rarely seen and HNE staining was not observed. Non-immune serum controls were uniformly negative (Fig. 7B and D).

Alkaline Conditions and Calcium—To determine whether alkaline-induced cell death was associated with changes in intracellular calcium, the amount of cellular free calcium at pH 7.3 and 8.3 was compared in all four cell lines. The intracellular calcium levels at pH 7.3 were 156.1 ± 1.8, 135.9 ± 3.0, 157.8 ± 3.2, and 159.8 ± 3.1 for FSa-II, NEO, SOD-L, and SOD-H, respectively. At pH 8.3 there were large increases in intracellular free calcium after 90 min in the FSa-II and NEO cell lines (Fig. 8). A modest increase in intracellular free calcium was also detected for the SOD-L and SOD-H cell lines (Fig. 8). The increased intracellular free calcium in the two control cell line (FSa-II and NEO) was significantly greater than that in the SOD-transfected cell lines (p < 0.001).

Ultrastructural Pathology—To identify subcellular organelles affected by alkaline pH, ultrastructural morphology of
the control and SOD transfected cells were examined using electron microscopy. Fig. 9 shows electron micrographs for NEO and SOD-L cell lines incubated for 6 h at pH 8.3. Many changes were found in the control cells, including nuclear condensation and mitochondrial damage (Fig. 9b). In the control cells, damaged mitochondria were often found in lysosomes (arrow), while mitochondria in the SOD cells (Fig. 9a) appeared normal.

**DISCUSSION**

Alkaline pH causes cell death although the mechanism leading to cell death is not presently understood. The present investigation was conducted to examine the link between alkaline conditions, mitochondrial antioxidant status, and cell death. This study is the first to demonstrate that oxidative stress is an early and critical event leading to cell death, and mitochondria are primary target organelles of injury in alkaline conditions. It has been demonstrated that alteration of mitochondrial function is an early feature of programmed cell death (41). Our results show that under alkaline conditions, the mitochondrial transmembrane potential increased, suggesting an alteration of mitochondrial function as an early event in alkaline-induced cell death. The intracellular pH in the cells overexpressing Mn-SOD was not different from that in the control cells, and there was no significant change in the mitochondrial membrane potential of these cells, suggesting that the increased production of superoxide radicals in the control cells was associated with increased mitochondrial membrane potential.

It is well established that mitochondrial respiration generates superoxide radicals (4, 5). Under normal physiological conditions, a small percentage of the oxygen consumed for respiration is converted into superoxide radicals (2). It has also been demonstrated that alkaline conditions are associated with an increase in oxygen consumption. Exposure of cultured French bean cells to an elicitor preparation from a fungal pathogen showed a rapid increase in oxygen uptake and a transient alkalinization of the apoplast (42). The oxygen uptake increased while the hydrogen ion concentration decreased in dogs and humans during hyperventilation (43–45). In anesthetized paralyzed patients, there was an increase in oxygen consumption with alkalosis (46). Thus, it is possible that under alkaline conditions, the generation of superoxide radicals from mitochondrial respiration is increased. Alkaline pH may act like a sink to draw protons away from the mitochondria, which normally maintains a proton gradient through respiration. Thus, under alkaline conditions, the mitochondria compensate
Mitochondria are believed to participate in the intracellular calcium network (49). The relationship between mitochondrial damage and increased intracellular free calcium has been extensively studied (for a review, see Ref. 33). It has been demonstrated that perturbation of calcium homeostasis is tightly associated with cell death (see Ref. 50 for a further review). Increased intracellular calcium can lead to increased ROS generation through activation of calcium-dependent proteases (51). On the other hand, increased intracellular ROS can cause disturbances in calcium homeostasis. Thus, it is not yet clear whether generation of ROS or increase intracellular calcium is the earlier event leading to cell death. Our results which indicate that removal of ROS prevents intracellular calcium increases suggest that ROS production precedes the increased calcium levels observed under alkaline conditions. However, our results do not exclude the possibility that ROS-independent pathways of cell death can occur under certain circumstances. In fact, it has been shown that cells cultured under a near anaerobic atmosphere, where ROS generation is reduced, inhibit apoptotic cell death induced by oxygen generating agents but not by antibodies to Fas/APO or interleukin 3 (52).

Several potential mediators responsible for mitochondria-mediated cell death have recently been identified. Activation of specific members of the interleukin-1β converting enzyme cysteine protease family (9, 10) and cytochrome c release from mitochondria (11) have been shown to be key participants of apoptotic cell death. More recently, it has been shown that mitochondrial function is necessary for the activation of the CPP-32 like protease by tumor necrosis factor but not staurosporin (12). Our results clearly indicate that mitochondrial damage is a critical event in alkaline-induced cell death. Thus, it is possible that damaged mitochondria release cytochrome c for the loss of protons by increasing respiration which in turn will also produce more superoxide radicals. This hypothesis is supported by our findings that a significant increase in ROS levels was observed in control cells but not Mn-SOD-transfected cells. The identification of HNE in high amounts in parental but not in Mn-SOD-transfected cells suggests oxidative stress as a result of altering pH in the parental cells. It is interesting to note that in control cells, the increase in total ROS levels correlated with increased mitochondrial ROS. Furthermore, in Mn-SOD-transfected cells, there was no measurable increase in the levels of both mitochondrial ROS and total cytosolic ROS, suggesting that removal of superoxide radicals in the mitochondria reduced cellular oxidative stress at a distance from the mitochondria. Since SOD is specific for the removal of superoxide radicals (47), these results also suggest that superoxide is the initial radical that leads to the increased ROS detected. Why would removal of superoxide radicals in the mitochondria affect the levels of total cytosolic ROS? It has been hypothesized that in addition to its important role in respiration, mitochondrial respiration may also play a role in maintaining the cellular redox status by eliminating cytosolic superoxide radicals (48). The cytoplasmic superoxide radical scavenging of mitochondria enhances the spontaneous dismutation of superoxide which diffuses into the mitochondrial intermembrane space. The mitochondrial intermembrane space has a localized proton-rich environment that can protonate superoxide radicals to form hydroperoxyl radicals, which then can diffuse into mitochondrial matrices and are dismutated by Mn-SOD. Thus, superoxide consumption in the mitochondria creates a gradient for superoxide radicals which favors diffusion from the cytosolic to the mitochondrial space (48).

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and/or activate these proteases in the alkaline-induced cell death process. Although the relative time course for each event to occur after alkaline treatment remains to be established, the finding that the mitochondria were taken up by lysosomes by 6 h after treatment indicate that mitochondrial damage occurred much earlier. Furthermore, the facts that the function of Mn-SOD to remove superoxide radicals in mitochondria and mitochondria damage and cell death are prevented by overexpression of Mn-SOD, places oxidative stress and mitochondrial damage as early steps leading to cell death under alkaline conditions.

The finding that expression of Mn-SOD prevents alkaline-induced cell death may also have practical implications. Increased Mn-SOD expression in response to oxidative stress has been demonstrated in cultured cells and in animals. We have previously shown that ionizing radiation induced Mn-SOD activity in mouse heart (53) and increased Mn-SOD activity protected transgenic mouse against adriamycin-induced cardiac toxicity (25). Production of ROS by paraquat, ionizing radiation, and adriamycin, have been well documented (54, 55). The finding that alkaline conditions is accompanied by increasing oxidative stress adds to the growing list of pathological conditions where oxidative stress plays an important role in determining the fate of a cell and suggests that alkaline-induced cell death may be preventable by increasing levels of mitochondrial antioxidants.

REFERENCES

Prevention of Mitochondrial Injury by Manganese Superoxide Dismutase Reveals a Primary Mechanism for Alkaline-induced Cell Death


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