Superoxide in Apoptosis

MITOCHONDRIAL GENERATION TRIGGERED BY CYTOCHROME c LOSS*

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Activation of apoptosis is associated with generation of reactive oxygen species. The present research shows that superoxide is produced by mitochondria isolated from apoptotic cells due to a switch from the normal 4-electron reduction of O2 to a 1-electron reduction when cytochrome c is released from mitochondria. Bcl-2, a protein that protects against apoptosis and blocks cytochrome c release, prevents superoxide production when it is overexpressed. The switch in electron transfer provides a mechanism for redox signaling that is concomitant with cytochrome c-dependent activation of caspases. The block of cytochrome c release provides a mechanism for the apparent antioxidant function of Bcl-2.

Reactive oxygen species (ROS) are important regulators of apoptosis. Pro-oxidants and redox cycling agents, such as H2O2 (1), diamide (2), etoposide (3), and semiquinones (4), can induce apoptosis. Other apoptotic stimuli, such as treatment with TNFa (5), lipopolysaccharide (6), ceramide (7), growth factor withdrawal (8), and human immunodeficiency virus infection (9), can stimulate the ROS production. Studies with TNFa and ceramide have shown that these agents activated ROS generation by mitochondria (7). The recent proposed model of p53-induced apoptosis also placed ROS as a central signaling event (10, 11). Antioxidants and thiol reductants, such as NAC (12), overexpression of thioreredoxin (13), and MnSOD (14), can block or delay apoptosis. On the other hand, ROS can provide protective mechanisms under some conditions, such as the activation of NF-kB by TNF-induced ROS production (15, 16). Thus ROS, and the resulting cellular redox change, can be part of the signal transduction pathway during apoptosis. Yet the mechanisms of ROS generation and its relationship with the well studied caspase activation have not been resolved.

A role of ROS during apoptosis was initially proposed based upon the observation that Bcl-2, a general inhibitor of apoptosis in mammalian cells, has an apparent antioxidant function (17, 18). Bcl-2 is largely localized to the mitochondrial outer membrane, and when overexpressed, it protects cells from lipid peroxidation and thiol oxidation induced by menadione and hydrogen peroxide (17, 18). Bcl-2 also protects p53 cells from apoptosis under conditions that ROS generation is limited (19, 20). Thus, its anti-apoptotic function is not limited to the antioxidant property. Nonetheless, this apparent antioxidant property of Bcl-2 has not been explained.

When human myeloid leukemia HL 60 and U-937 cells undergo apoptosis induced by staurosporine (21), etoposide (21), cytosine b-D-arabinofuranoside (22), and ionizing radiation (23), there is an early release of cyt c from mitochondria, and the released cyt c participates in the activation of caspase 3. Bcl-2 family proteins prevent cyt c from entering the cytosol either by blocking cyt c release (21, 22) or by binding directly with cyt c (23). Cyt c is localized in the mitochondrial intermembrane space and is part of the mitochondrial electron transport chain. A variety of mitochondrial poisons are well known to stimulate ROS generation by a mechanism that involves inhibition of electron transfer, accumulation of reducing equivalents in the middle portion of the electron transfer chain, and direct one-electron transfer to O2 to produce superoxide (for review, see Ref. 24). This switch appears to occur principally at the level of coenzyme Q (ubiquinone/ubiquinol) and thus could also occur as a consequence of cyt c loss.

In this study, we used staurosporine-treated HL 60 cells to study the mechanism of ROS generation during apoptosis. Results show that the cellular redox change occurred following mitochondrial release of cytochrome c and was in parallel to the caspase activation. The release of cytochrome c was associated with inhibited mitochondrial respiration and stimulated mitochondrial superoxide production, and overexpression of Bcl-2 inhibited all of these processes.

EXPERIMENTAL PROCEDURES

Cell Culture—HL 60 cells transfected with bcl-2 (bcl-2) or vector alone (neo) were cultured as described (21). Overexpression of Bcl-2 was monitored by Western blot. Cells were passaged every 2–3 days and seeded at 0.25 × 10⁶/ml, and apoptosis was induced by adding 1 μM staurosporine.

Measurement of Intracellular and Mitochondrial Redox Change—After treatment, cells were pelleted and resuspended in 5% perchloric acid/saturated borax, supplemented with 5 μM 2′,7′-dichlorofluorescein diacetate, and 15 μM dihydrorhodamine (DHR) for 20 min, washed with phosphate-buffered saline, and loaded onto a Becton-Dickinson FACStation. FL-1 fluorescence was also recorded with high pressure liquid chromatography as described (25), and the amount of acid-insoluble protein was measured by the Bradford method.

Measurement of Intracellular and Mitochondrial Redox Change—After treatment, cells were pelleted and resuspended in 5% perchloric acid/saturated borax, supplemented with 5 μM 2′,7′-dichlorofluorescein diacetate, and 15 μM dihydrorhodamine (DHR) for 20 min, washed with phosphate-buffered saline, and loaded onto a Becton-Dickinson FACStation. FL-1 fluorescence was recorded, and cell debris was electronically gated out based on the forward light scatter. 300 μM tert-butylhydroperoxide treatment was used as a positive control. The DHR fluorescence was also recorded with confocal microscopy. Cells double-stained with 60 nM DiOC6 and 15 μM DHR were washed and resuspended in 15 μl of phosphate-buffered saline and placed on a no. 1 thickness coverslip. The green and red fluorescence were simultaneously recorded with a Bio-Rad MRC 1024 laser scanning confocal microscopy system, with the excitation wavelength set at 488 nm. Images were acquired and processed with Laser-Sharp software, using identical background and gain settings.
Six to eight areas were randomly picked, and representative ones are presented in Fig. 2.

Measurement of Mitochondrial Membrane Potential (mtΔΨ)—After treatment, cells were loaded with either 2 μM rhodamine 123 (28) or 60 nM DiOC6 (29) for 10 min, and mtΔΨ was measured by FACSscan.

Measurement of Substrate-stimulated Oxygen Consumption—After treatment with staurosporine for the indicated periods of time, 2 × 10⁶ cells were washed with phosphate-buffered saline and resuspended in mitochondrial respiration buffer (30). O₂ consumption was measured in a 2.0-ml chamber with a Clark-type oxygen electrode (Yellow Springs) calibrated with air and sodium dithionite (30). Cells were permeabilized with 0.01 mg/ml digitonin for 1 min, and substrate-stimulated respiration was recorded by adding 2.5 mM succinate and 0.25 mM ADP. The substrate-stimulated O₂ consumption rate over the rate after digitonin permeabilization was calculated as the respiratory control ratio.

Measurement of Superoxide Generation in Isolated Mitochondria—Mitochondria were isolated from neo and bcl-2 cells with nagarse-digitonin treatment (31). Measurements were performed in a SLM/Amino DW-2000 spectrophotometer at 30 °C. 40 μM acetylated cytochrome c was included in the incubation buffer, and the absorbance change was monitored at 550 nm (32). Reaction was initiated by adding 3 mM succinate. KCN (0.5 mM) was used to inhibit normal electron flow and stimulate superoxide production (positive control), and bovine erythrocyte superoxide dismutase (SOD) (Sigma; 100 units) was added to verify that the reduction of acetylated cytochrome c was due to superoxide generation.

Western Blot Analysis of Mitochondrial cyt c Release—Mitochondrial and cytosolic fractions were prepared with digitonin-nagarse treatment. Each lane was loaded with 50 μg of protein, and Western blot was performed as described (21), using a gift antibody from Dr. R. Jemmerson (University of Minnesota).

RESULTS AND DISCUSSION

To determine whether an oxidant signal precedes or follows mitochondrial release of cyt c, we measured the redox state of the intracellular GSH pool as a function of time following treatment of neo cells with 1 μM staurosporine (Fig. 1). Under these conditions, cyt c release was detectable already at 1 h (Fig. 1B), but oxidation of cellular GSH/GSSG did not occur until after 2 h (Fig. 1A). By 6 h, the redox potential E₉₅ was oxidized by 72 mV (from −239 ± 6 to −167 ± 9 mV), a change similar to the 86 mV oxidation in apoptotic HT29 cells (data not shown). In cells overexpressing Bel-2, cyt c release was blocked (Fig. 1B), and the redox change was inhibited (Fig. 1A). The measured mitochondrial GSH/GSSG ratio changed with a similar time course (Fig. 1A, inset). Thus, cyt c loss preceded the generation of an oxidant signal and as previously reported, Bel-2 blocked cyt c release (21) and had an apparent antioxidant function (17, 18), as measured by E₉₅ of the GSH/GSSG pool and the mitochondrial GSH/GSSG ratio.

Antioxidants, such as NAC, can inhibit apoptosis in some systems (12). Although the staurosporine-induced caspase activation and subsequent DNA fragmentation could be completely inhibited by overexpression of Bel-2, they were not sensitive to NAC (Fig. 1C). Thus in staurosporine-induced apoptosis, the caspase activation seems to be redox-insensitive, and the effects of Bel-2 cannot be attributed to its apparent antioxidant function.

Because the redox signal was not observed until after cyt c release, we then examined whether the mitochondrial is a possible source of the redox signal. For this, we used DHR, a non-fluorescent compound that accumulates in the mitochondria and can be oxidized to the fluorescent rhodamine (33). FACSscan showed that DHR was oxidized in staurosporine-treated neo cells but not in cells overexpressing Bel-2 (Fig. 2A and B). Confocal microscopy confirmed that this fluorescence was mainly localized to the mitochondria, together with the mtΔΨ-driven dye DiOC₆ (Fig. 2C). Thus, the results suggest that ROS may be generated in the mitochondria upon cyt c release and that this is prevented by Bel-2 in association with its inhibition of cyt c release.

Babior and co-workers (30, 34) found that substrate-dependent mitochondrial O₂ consumption was inhibited with loss of cyt c in a Fas-activated model of apoptosis. Measurements in mitochondria of Neo cells treated with staurosporine also showed that substrate-stimulated O₂ consumption was inhibited (Fig. 3A). Thus, the loss of cyt c is associated with an interruption of normal electron flow that could divert electron transfer to the generation of superoxide.

Activation of the mitochondrial permeability transition (MPT) is a major controlling mechanism in some apoptotic systems (for review, see Ref. 35), and this could also contribute to cyt c release (data not shown) and superoxide production (36, 37). To determine whether the MPT had occurred in Neo cells treated with staurosporine, we used FACS analysis with rhodamine 123 or DiOC₆. Results showed that ΔΨ was largely retained under conditions where cyt c was released and the redox signal was generated (Fig. 3, B and C). At later times, the ΔΨ was lost (Ref. 21 and data not shown), indicating that the redox change preceded the MPT and was not a result of MPT.

To determine whether mitochondria that have lost cyt c during apoptotic signaling have increased substrate-dependent superoxide production, we measured stimulated superoxide production in mitochondria isolated from staurosporine-
Results showed that superoxide generation was enhanced in mitochondria isolated from staurosporine-treated neo cells. By 4 h, the rate was 5.28 ± 0.33 nmol/mg protein per min (Fig. 4A). This was dependent upon respiratory substrate and was inhibited by superoxide dismutase.

To determine whether superoxide production could be blocked by preventing cytochrome c release, superoxide production by mitochondria from staurosporine-treated bcl-2 cells was measured. Results showed that mitochondria from cells over-expressing Bcl-2 had no increase in superoxide production following staurosporine treatment (Fig. 4B). Addition of cyanide, a direct mitochondrial electron transport inhibitor, resulted in superoxide generation in mitochondria from Bcl-2-overexpressing cells at a rate similar to that from neo cells. Thus, the results show that the effect of Bcl-2 was not due to a direct electron-scavenging or superoxide-metabolizing activity of Bcl-2 itself but rather to the prevention of \( \text{O}_2^\cdot \) production.

These observations demonstrate that the antioxidant activity of Bcl-2 is indirect and clarify an important issue concerning Bcl-2 function. However, this finding also raises a fundamental question about the redox signaling in cells. The redox state of the GSH/GSSG pool is substantially oxidized (239 mV) compared with the NADPH pool (2394 mV) (38) that drives GSSG reduction. If the steady state \( \text{E}_0 \) of GSH/GSSG reflects a balance between the rate of GSH oxidation (GPx-GSH peroxidase) and GSSG reduction (GRx-GSSG reductase). The balance of electron flow through cytochrome oxidase to produce \( \text{H}_2\text{O} \) compared with transfer to \( \text{O}_2 \) to generate \( \text{O}_2^\cdot \) is controlled by the cytochrome c content in the intermembrane space. By controlling cyt c loss from mitochondria, Bcl-2 can alter the cytosolic GSH/GSSG redox state.

The present demonstration of a mechanism for ROS generation during apoptosis does not resolve whether ROS has a role in apoptosis signaling. Despite the fact that an oxidant signal occurs during apoptosis by diverse stimuli, so far the available pieces of evidence suggest that caspase activation is irrelevant to the redox state. In vitro data using cell-free systems indi-
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cated that caspase 3 activation was independent of the redox state of cyt c (39, 40). Similarly, when we added 5 mM NAC to the neo cells, we obtained no inhibition of caspase activation, as measured by DNA fragmentation (Fig. 1C) and phosphatidylserine translocation (data not shown). Thus when ROS is generated concomitantly with a direct activating mechanism of caspases, it might be only a side effect that is unrelated to the key signaling events leading to the caspase-executed cell death.

However, a range of other possible functions for ROS could exist beyond the activation of death proteases. Caspases are inhibited by oxidants (41, 42). The superoxide generated could function as a mechanism to inactivate caspases and prevent their destruction of cells that phagocytose apoptotic bodies (41). ROS may also counteract the lethal effects of caspases in non-apoptotic cells. Caspase 3 has recently been found to function in the processing of pro-interleukin 16, a non-apoptotic function (46). Alternatively, the switch of mitochondrial electron transport from its normal pathway through cyt c, which does not result in free radical production, to one in which electrons are transferred to O2 with production of superoxide, may provide a fail-safe mechanism that complements cyt c-dependent caspase activation for the execution of cell death. The dramatic 75-mV drop across the MPT pore has critical thiols, which upon oxidation can trigger its opening (48) and release the apoptosis-inducing factors. The MPT pore is known to be another regulatory mechanism of NF-kB (44, 45). In TNF-a-induced apoptosis, NF-kB has been shown to control the expression of several protective genes, such as c-IAP2 and MnSOD (13, 14). Despite its known to be another regulatory mechanism of NF-κB, which does not result in its activation for the execution of cell death. The dramatic 75-mV drop across the MPT pore has critical thiols, which upon oxidation can trigger its opening (48) and release the apoptosis-inducing factors. The MPT pore is known to be another regulatory mechanism of NF-kB (44, 45). In TNF-a-induced apoptosis, NF-kB has been shown to control the expression of several protective genes, such as c-IAP2 and MnSOD (13, 14). Despite its known to be another regulatory mechanism of NF-κB, which does not result in its activation for the execution of cell death. The dramatic 75-mV drop across the MPT pore has critical thiols, which upon oxidation can trigger its opening (48) and release the apoptosis-inducing factors. The MPT pore is known to be another regulatory mechanism of NF-kB (44, 45). In TNF-a-induced apoptosis, NF-kB has been shown to control the expression of several protective genes, such as c-IAP2 and MnSOD (13, 14). Despite its known to be another regulatory mechanism of NF-κB, which does not result in its activation for the execution of cell death. The dramatic 75-mV drop across the MPT pore has critical thiols, which upon oxidation can trigger its opening (48) and release the apoptosis-inducing factors. The MPT pore is known to be another regulatory mechanism of NF-kB (44, 45). In TNF-a-induced apoptosis, NF-kB has been shown to control the expression of several protective genes, such as c-IAP2 and MnSOD (13, 14). Despite its known to be another regulatory mechanism of NF-κB, which does not result in its activation for the execution of cell death. The dramatic 75-mV drop across the MPT pore has critical thiols, which upon oxidation can trigger its opening (48) and release the apoptosis-inducing factors. The MPT pore is known to be another regulatory mechanism of NF-kB (44, 45). In TNF-a-induced apoptosis, NF-kB has been shown to control the expression of several protective genes, such as c-IAP2 and MnSOD (13, 14). Despite its known to be another regulatory mechanism of NF-κB, which does not result in its activation for the execution of cell death.
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