Cytochrome c released from mitochondria into the cytoplasm plays a critical role in many forms of apoptosis by stimulating apoptosome formation and subsequent caspase activation. However, the mechanisms regulating cytochrome c apoptotic activity are not understood. Here we demonstrate that cytochrome c is nitrosylated on its heme iron during apoptosis. Nitrosylated cytochrome c is found predominantly in the cytoplasm in control cells. In contrast, when cytochrome c release from mitochondria is inhibited by overexpression of the anti-apoptotic proteins B cell lymphoma/leukemia (Bcl)-2 or Bcl-XL, nitrosylated cytochrome c is found in the mitochondria. These data suggest that during apoptosis, cytochrome c is nitrosylated in mitochondria and then rapidly released into the cytoplasm in the absence of Bcl-2 or Bcl-XL overexpression. In vitro nitrosylation of cytochrome c increases caspase-3 activation in cell lysates. Moreover, the inhibition of intracellular cytochrome c nitrosylation is associated with a decrease in apoptosis, suggesting that cytochrome c nitrosylation is a proapoptotic modification. We conclude that nitrosylation of the heme iron of cytochrome c may be a novel mechanism of apoptosis regulation.

Apoptosis is a cell death pathway that removes excess, damaged, autoactive, or infected cells from organisms. Apoptosis may be triggered by extrinsic stimuli or by cell surface receptors such as Fas. Fas is a member of the tumor necrosis receptor superfamily that mediates apoptosis when cross-linked by Fas ligand or by Fas agonist antibody (1–3). Fas cross-linking results in the formation of the death-inducing signaling complex composed of Fas, the adapter protein FADD/MORT1, and caspase-8 (4–8). Caspase-8, a member of the caspase family of cysteine proteases, is activated upon association with the death-inducing signaling complex (9 and subsequently activates downstream caspase members including caspases-3, -6, and -7 (10–13). These "effector caspases" cleave cellular proteins such as poly(ADP) ribose polymerase, leading to apoptotic cell death. In addition to directly activating downstream caspases, activated caspase-8 cleaves Bid, a member of the B cell lymphoma/leukemia (Bcl)-2 family of proteins (14–16). Truncated Bid trans-locates to the mitochondria where it induces the release of proapoptotic molecules from the mitochondria intermembrane space including cytochrome c and a sub-population of caspase zymogens.

Once released into the cytoplasm, cytochrome c plays a critical role in apoptotic pathways by binding to and inducing oligomerization of the protein Apaf-1. The redox activity of cytochrome c is not required for this apoptotic activity, but certain structural elements of the protein are necessary because apocytochrome c, denatured cytochrome c, and enzyme-digested cytochrome c have no proapoptotic activity (17, 18). After cytochrome c induces oligomerization of Apaf-1, the oligomer binds procaspase-9, leading to its autoactivation (19–22). The cytochrome c/Apaf-1/caspase-9 complex is called the apoptosome. Activated caspase-9 in the apoptosome activates downstream caspases such as caspase-3, leading to further propagation of the apoptotic cascade.

The Bcl-2 family of proteins prevent aberrant apoptosis by regulating the release of cytochrome c and other proapoptotic proteins from the mitochondrial intermembrane space (15, 18, 23–25). Anti-apoptotic family members such as Bcl-2 and Bcl-XL inhibit, whereas proapoptotic members such as Bax and Bid stimulate the release of cytochrome c. The mechanisms by which Bcl-2 family members regulate the release of mitochondrial proteins are controversial but may involve the formation of supramolecular openings in the outer mitochondrial membrane (26).

Nitric oxide (NO) is a free radical gas that provides another level of apoptosis regulation. The effects of NO on apoptosis are complex and may be either stimulatory or inhibitory (27). One of the mechanisms by which NO regulates biological processes including apoptosis is nitrosylation of proteins (28). Nitrosylation is a posttranslational modification involving the attachment of a NO group to a cysteine or transition metal. Although the function of many proteins can be modified by nitrosylation of critical cysteine residues and/or transition metals in *vitro*, only a limited number of proteins have been found to be endogenously nitrosylated in *vivo*. We have previously shown that endogenous nitrosylation of the catalytic site cysteine of a subset of caspase members serves as an on/off switch regulating caspase activity during apoptosis (29, 30). The subset of caspases regulated by S-nitrosylation resides predominantly in the mitochondria (30). This observation raised the possibility that mitochondria are a key site of protein nitrosylation in cells. To test this hypothesis, in the current studies we examined whether cytochrome c, another mitochondrial protein that plays a key role in apoptosis, is regulated by nitrosylation.

Cytochrome c has no free cysteines, and therefore, its heme iron is the most probable nitrosylation target. Cytochrome c can be heme nitrosylated in *vitro* (31–33) but has never been shown to be nitrosylated intracellularly. Indeed, guanylate cyclase and cytochrome oxidase are the only proteins whose function clearly has been demonstrated to be regulated by endogenous heme nitrosylation (34–38). The hemes of guanylate cyclase and cytochrome oxidase are 5-coordinate and bind NO...
much more rapidly than the 6-coordinate heme of cytochrome c (39). Therefore, it is unclear whether cytochrome c nitrosylation occurs intracellularly. However, cytochrome c undergoes a conformational change during apoptosis that may result in a 5-coordinate heme (40). This conformational alteration may increase the reactivity of cytochrome c with NO. To further explore these possibilities, in the current studies we determined whether 1) cytochrome c is heme-nitrosylated intracellularly during apoptosis, and 2) if so, whether nitrosylation regulates cytochrome c function during apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Immunoprecipitation—Cell lines were grown at 37 °C, 5% CO₂ in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Logarithmically, growing cells were stimulated with Fas agonist antibody (clone CH-11, 60–240 ng/ml, Upstate Biotechnology). In some experiments, cells were pretreated with NωNω-
monomethyl-l-arginine (l-NMA) (5 mM versus 1 mM l-arginine in the medium) prior to Fas stimulation. At various time points before and after Fas stimulation, mitochondrial and cytoplasmic fractions were prepared as described previously (30), except in some experiments cells were homogenized in buffer containing 0.25% sucrose, 5 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, pH 8, 2 mM MgCl₂, and protease inhibitors. Immunoprecipitation of mitochondrial and cytoplasmic fractions was performed as described previously (29, 30) using 10 μg of an anti-cytochrome c monoclonal antibody (BD Transduction Laboratories) or equal concentrations of an isotype-matched IgG1 control antibody (Sigma). The immunoprecipitated proteins were separated on a 12% SDS-PAGE gel and visualized using a silver staining kit as per the manufacturer’s instructions (Sigma) or analyzed by cytochrome c immunoblot using an anti-cytochrome c monoclonal antibody (BD Transduction Laboratories), a peroxidase-conjugated secondary antibody (Amersham Biosciences) and a chemiluminescence detection system per the manufacturer’s instructions (Amersham Biosciences). Cytochrome c or bovine serum albumin standards were used to quantitate the amount of cytochrome c in each immunoprecipitate.

Nitrosylation Measurements—Nitrosylation of proteins was determined by chemical reduction/chemiluminescence as described previously (41). Immunoprecipitated proteins were eluted from protein G-Sepharose beads (Amersham Biosciences) in 200 μl of 100 mM glycine, pH 3. The eluted proteins (50 μl) were injected into a 5-mL anaerobic solution containing 100 μM CuCl₂, 100 μM cysteine, and 0.01% antifoam (pH 3, 50 °C) purged continuously with argon in a Sievers 280 nitric oxide analyzer. Analysis of cytochrome c nitrosylation was performed as described in protocols. In some experiments, 45 mM potassium iodide and 10 mM I₂ in glacial acetic acid were used for chemical reduction (42). NO evolution was measured by chemiluminescence. Data were interpreted as raw photoelectric output (integrated using Sievers software) and as absolute NO evolved using NO standards generated by S-nitrosoglutathione or in vitro nitrosylated cytochrome c.

In Vitro Nitrosylation of Cytochrome c—Ferri-cytochrome c (horse heart, Sigma) (100–200 μM) was in vitro nitrosylated with DETA-NONOate (10 mM) in 0.1 M sodium phosphate buffer, pH 5, for 1 h in the dark at room temperature. Excess DETA-NONOate was removed by two sequential acetone precipitations.

UV-visible Spectroscopy—Spectrophotometric measurements were carried out using a Beckman Coulter DU 640B Spectrophotometer.

Caspase-3 Activation Assays—To assess caspase-3 activation by cytochrome c, cytosolic extracts prepared as described previously (43) were incubated for the indicated times at 30 °C with 1 μM dATP (Amersham Biosciences) alone or in combination with 10 μM control or in vitro nitrosylated cytochrome c. After the incubation, caspase-3 activation in the extracts was determined by immunoblot analysis using a caspase-3-specific mouse monoclonal antibody or a rabbit polyclonal that detects caspase-3 active fragments (BD Transduction Laboratories) as described previously (29).

Analysis by Acridine Orange Staining—Cells were pelleted, and 10 μl of cell slurry was mixed with 10 μl of acridine orange (50 μg/ml) diluted in phosphate-buffered saline. The percentage of cells with apoptotic morphology (nuclear and cytoplasmic condensation and nuclear fragmentation) was then analyzed on a wet mount slide using a Nikon Eclipse TE2000 fluorescent microscope. At least 200 cells in three separate fields were counted for each measurement.

RESULTS

Cytochrome c nitrosylation was analyzed during Fas-induced apoptosis of human mononuclear cell lines. Cytochrome c was purified by immunoprecipitation from mitochondrial and cytoplasmic fractions of the human monocytic cell line U-937 or the human T cell line CEM at 0, 1, and 2 h after Fas-stimulation with agonist antibody. Cytochrome c was efficiently immunoprecipitated with its specific antibody but not with equal concentrations of isotype-matched control antibody (Fig. 1a). A silver stain analysis revealed that immunoprecipitated cytochrome c was full-length and that as-
associated proteins did not significantly contaminate the immunoprecipitates (Fig. 1a).

The extent of nitrosylation of immunoprecipitated cytochrome c was determined by chemical reduction/chemiluminescence as described previously (30, 41). In this method, NO is displaced from S-NO and from a subpopulation of metal-NO bonds (including the Fe-NO bond of cytochrome c) in a saturated copper/cysteine solution and is detected by chemiluminescence. Measurements obtained using the copper/cysteine method were confirmed using 45 mM potassium iodide and 10 mM L-glutamic acid for chemical reduction (data not shown) (42). An analysis of cytochrome c immunoprecipitates indicated that little if any mitochondrial cytochrome c was nitrosylated either before or after Fas stimulation (Fig. 1b). In contrast, cytochrome c released into the cytoplasm was nitrosylated (Figs. 1b and 3c). The stoichiometry of NO to cytochrome c was 0.6 in immunoprecipitates obtained from the cytoplasm 1 h after Fas stimulation, indicating that a significant portion of cytoplasmic cytochrome c is nitrosylated at this time point. Cytoplasmic cytochrome c nitrosylation decreased 2 h after Fas stimulation, suggesting that the protein may be initially nitrosylated and then denitrosylated (Fig. 1b). Similar results were obtained using staurosporine as an apoptotic stimulus (data not shown). Cytochrome c nitrosylation was eliminated by pretreating immunoprecipitates with UV light, which cleaves NO from metal-NO and S-NO bonds, for 10–20 min, indicating that the NO signal was derived from protein nitrosylation rather than from contaminating nitrite (Fig. 1c). In addition, pretreatment of the immunoprecipitates with K$_2$Fe(CN)$_6$ (0.2 mM for 30 min) (Fig. 3c) but not with HgCl$_2$, which selectively displaces NO from S-NO bonds (data not shown), decreased the NO signal, suggesting that cytochrome c is heme-nitrosylated. Finally, pretreatment of cells with the nitric-oxide synthase (NOS) inhibitor l-NMA decreased cytochrome c nitrosylation, indicating that cytochrome c is endogenously nitrosylated (Fig. 3c).

To confirm that cytochrome c is nitrosylated on its heme iron during apoptosis, we analyzed the UV-visible absorption spectra of nitrosylated cytochrome c immunoprecipitates. In vitro nitrosylation of ferricytochrome c results in an iron-nitrosyl peak at 562 nm and a Soret shift from 405 to 411 (Fig. 2a) (31–33). Nitrosylated but not non-nitrosylated immunoprecipitated cytochrome c had a red-shifted Soret peak at 411 nm, consistent with heme nitrosylation (Fig. 2b). The high concentration of protein (~10 μg) required to visualize the 562 nm peak precluded the analysis of this peak in our immunoprecipitated samples. The red-shifted Soret peak of immunoprecipitated cytoplasmic cytochrome c was not the result of cytochrome c reduction (Fig. 2c), because the shift was stable at pH 3, whereas reduced cytochrome c is oxidized at this pH, resulting in a Soret peak shift back to 405 nm (Fig. 2d).

It is possible that cytochrome c is directly nitrosylated in the cytoplasm or is nitrosylated in the mitochondria and then rapidly released into the cytoplasm. To distinguish between these possibilities, we determined whether nitrosylated cytochrome c is found in mitochondria when its release into the cytoplasm is inhibited by overexpression of the anti-apoptotic proteins, Bcl-2 or Bcl-X$_L$ (23). Nitrosylation of immunoprecipitated cytochrome c was analyzed in Fas-stimulated U-937 cells stably transfected with Bcl-2, Bcl-X$_L$, or control expression vectors (44). In contrast to control cells, nitrosylated cytochrome c was found predominantly in the mitochondria 1 h after Fas stimulation in cells overexpressing Bcl-2 or Bcl-X$_L$ (Fig. 1d). These data suggest that cytochrome c is nitrosylated within mitochondria and then, in the absence of Bcl-2 and Bcl-X$_L$ overexpression, released into the cytoplasm.

Once released into the cytoplasm, cytochrome c forms a complex with Apaf-1 and caspase-9 called the apoptosome that cleaves and activates downstream caspases such as caspase-3. To determine whether nitrosylation of cytochrome c alters its ability to stimulate apoptosome formation and subsequent caspase-3 activation, we analyzed caspase-3 activation in cytosolic extracts stimulated with nitrosylated or control cytochrome c. The addition of in vitro nitrosylated cytochrome c as compared with equal concentrations of control cytochrome c to cytosolic extracts increased caspase-3 activation (Fig. 3a). The levels of the p20 active fragment were increased at 60 min, and the levels of both the p20 and p17 active fragments were increased after 120 min in lysates stimulated with nitrosylated cytochrome c as compared with control cytochrome c (Fig. 3a). These data suggest that nitrosylation is a posttranslational modification that enhances the proapoptotic function of cytochrome c, leading to increased caspase-3 activation.

To further establish a causal relationship between cytochrome c nitrosylation and apoptosis, we determined whether inhibition of intracellular cytochrome c nitrosylation decreased Fas-induced apoptosis. Pretreatment of CEM cells for 1 h with the NOS inhibitor L-NMA abrogated Fas-induced nitrosylation of cytosolic cytochrome c (Fig. 3, b and c) and was associated with a decrease in Fas-induced apoptosis (Fig. 3d). Thus, heme nitrosylation of cytochrome c may stimulate Fas-induced apoptosis.

**DISCUSSION**

In summary, our results suggest that nitrosylation of cytochrome c is a novel mechanism of apoptosis regulation in cells and a very early event in apoptotic signaling. The data indicate that apoptosis may be regulated in mammalian cells not only at the level of cytochrome c release from mitochondria but also by direct modification of cytochrome c. Because the heme edge of cytochrome c is involved in its association with Apaf-1 (45, 46), it is possible that heme nitrosylation of cytochrome c facilitates apoptosome formation and thereby stimulates caspase-3 activation. Ultimately, cytochrome c nitrosylation may provide a new therapeutic target for diseases associated with dysregulated apoptosis such as cancer, neurodegeneration, stroke, and autoimmunity.

The data provide one of the rare examples to date of endogenous heme nitrosylation regulating protein function. Guanylate cyclase and cytochrome oxidase are the other two proteins whose function has been clearly demonstrated to be regulated by endogenous heme nitrosylation (34–38). NO inhibits cytochrome oxidase activity by binding to the iron/copper binuclear center (36–38). NO activates guanylate cyclase by binding to the heme iron and inducing a conformational change in the enzyme (47). Nitrosylation may also be an allosteric regulator of cytochrome c function. However, our finding that cytochrome c is endogenously nitrosylated during apoptosis is unexpected because cytochrome c has a 6-coordinate heme that is significantly less reactive with NO than the 5-coordinate hemes of guanylate cyclase and cytochrome oxidase (39). One possible explanation for our results is that cytochrome c undergoes a subtle conformational change during apoptosis that increases the reactivity of the heme iron with NO. A number of investigators have demonstrated that cytochrome c undergoes a conformational change when bound to anionic phospholipid vesicles that model interactions of cytochrome c with the phospholipid-rich mitochondrial membranes (48–53). The conformational change involves an opening of the heme crevice, resulting in part from the loss of the iron-methionine 80 ligation. A similar conformational change in cytochrome c has been detected in cells early during apoptosis (40). The data raise the possibility that alterations in mitochondrial membrane lipids...
**FIG. 2. Cytochrome c is endogenously nitrosylated on its heme iron.**

**a.** Spectral characteristics of *in vitro* nitrosylated cytochrome c. The UV-visible absorption spectra of control (light blue line) and *in vitro* nitrosylated (dark blue dotted line) horse heart ferricytochrome c are shown (500 nM cytochrome c in 100 mM glycine, pH 3). The inset shows the 450–650-nm visible spectra of control and nitrosylated cytochrome c (100 μM).

**b.** Spectral characteristics of endogenously nitrosylated cytochrome c. The UV-visible absorption spectra of endogenously nitrosylated cytochrome c immunoprecipitated from the cytoplasm (dark blue dotted line) and non-nitrosylated cytochrome c immunoprecipitated from mitochondria (light

(Additional text continues...)
**Fig. 3. Cytochrome c nitrosylation enhances caspase-3 activation and apoptosis.** A, in vitro nitrosylation of cytochrome c increases caspase-3 activation. dATP (ATP) alone or in combination with control (cytochrome c, Cyt c) or in vitro nitrosylated cytochrome c (NO-Cyt c) was added to cytoplasmic extracts of CEM cells. Caspase-3 activation was determined after 60 and 120 min by assessing the levels of caspase-3zymogen (C3) and cleaved active fragments (p20 and p17) on caspase-3 immunoblots. The blot is representative of five independent experiments. B, silver stain of cytochrome c and control immunoprecipitates obtained from cells grown in the presence or absence of l-NMA. Cytochrome c immunoprecipitates obtained from the cytoplasmic fraction of CEM cells untreated (−) or pretreated (+) with the NOS inhibitor l-NMA for 1 h prior to stimulation with Fas agonist antibody for 0, 30, or 60 min were analyzed on a silver-stained gel. Bovine serum albumin (BSA) standards were used to quantitate the amount of cytochrome c in each immunoprecipitate. Immunoglobulin heavy chain (HC), light chain (LC), and Cyt c are shown. C, NOS inhibition decreases cytochrome c nitrosylation. The NO chemiluminescence signal from IgG1 control (Ig) or Cyt c immunoprecipitates obtained from the cytoplasmic fraction of CEM cells treated for 0, 30, or 60 min with Fas agonist antibody is shown. The background signal in control immunoprecipitates is because of nitrite contamination of the buffers. Only the cytochrome c immunoprecipitate obtained 1 h after Fas stimulation has a NO signal above background levels seen in control IgG immunoprecipitates. This increase in NO signal is eliminated by pretreating cells with l-NMA for 1 h (Cyt c +N) or by treating the immunoprecipitates with K$_5$Fe(CN)$_6$ (200 μM for 30 min) (Cyt c +Fe(CN)) d, l-NMA-induced inhibition of cytochrome c nitrosylation is associated with a decrease in Fas-induced apoptosis. CEM cells were grown in the presence or absence of l-NMA for 1 h and then were stimulated with Fas agonist antibody. After 1–5 h of Fas stimulation, the extent of apoptosis in untreated cells (0) or cells treated with Fas (F), l-NMA and Fas (F+N), or l-NMA alone (N) was determined by acridine orange staining as described previously (62). *p < 0.05 versus F+N, paired t test, n = 8.

**Fig. 4. Coordinated protein nitrosylation/denitrosylation regulates Fas signaling.** A, in resting cells, caspase zymogens (Casp) in the mitochondria (Mito) are inhibited by S-nitrosylation of their catalytic site cysteine (29, 30). B, after Fas stimulation, S-nitrosylated mitochondrial caspase zymogens are released into the cytoplasm (Cyto) and are denitrosylated. Concurrently, mitochondrial cytochrome c (Cyt c) is nitrosylated on its heme iron and released into the cytoplasm. C, nitrosylation of cytochrome c, in combination with denitrosylation of caspases, increases caspase activation by the apoptosome, a complex containing Apaf1, caspase-9, and cytochrome c. Increased caspase activation promotes apoptotic cell death.

During apoptosis may induce a conformational change in cytochrome c, resulting in a 5-coordinate heme. Such a conformational alteration may facilitate stable heme nitrosylation of cytochrome c during apoptosis. Nitrosylation is unlikely to be the result of more drastic conformational changes such as enzymatic degradation or denaturation, because immunoprecipitated nitrosylated cytochrome c runs the same size as full-length cytochrome c on gels and the antibody used for immunoprecipitation recognizes only native non-denatured cytochrome c.

We found that the NOS inhibitor l-NMA inhibited both cytochrome c nitrosylation and Fas-induced apoptosis in CEM cells. However, we and others (29, 54) have previously found that l-NMA stimulates Fas-induced apoptosis in Jurkat cells. This cell line variability may be due in part to the multiple proapoptotic and anti-apoptotic targets of NO in cells. The net effect of NO on apoptosis may depend on whether the proapoptotic effects of NO such as cytochrome c nitrosylation outweigh the anti-apoptotic effects such as caspase nitrosylation in a particular cell. In addition, the intracellular targets of NO may vary depending on the type and timing of an apoptotic stimulus, the source of NO, and the redox chemistry within a cell (55). Thus, a complex set of factors will determine the net effect of NO on apoptosis in a given cell type.

Our current finding that cytochrome c and previous finding that caspase zymogens (30) are nitrosylated in mitochondria raise the possibility that mitochondria provide a unique redox environment that facilitates protein nitrosylation (30). Caspase zymogens and cytochrome c are located in the mitochondrial intermembrane space where the relatively acidic pH (56) may promote both the formation and stability of metal-NO and S-NO bonds. In addition, mitochondria have a high concentration of lipid membranes that increase the rate of formation of metal-NO and S-NO bonds. Metabolic alteration may vary depending on the type and timing of an apoptotic stimulus, the source of NO, and the redox chemistry within a cell (57, 58).
Furthermore, complexes III and IV have been shown to reduce nitrite to NO raising the possibility that mitochondria can generate NO via NOS-independent mechanisms (59, 60). Thus, distinctive NO chemistry within mitochondria may allow this organelle to serve as a key site of intracellular protein nitrosylation. Whether nitrosylation targets cytochrome c for release from mitochondria remains to be investigated.

Finally, our results suggest that protein nitrosylation/denitrosylation may be a mechanism of signal transduction regulation comparable to phosphorylation/dephosphorylation as first postulated by Stamler (61). We have shown previously that caspase-3 is inhibited by S-nitrosylation in resting cells and then is activated by denitrosylation during apoptosis (29, 30). We now demonstrate that concurrent with caspase-3 denitrosylation, cytochrome c is nitrosylated, further enhancing caspase-3 activation. The combined data suggest that cytochrome c nitrosylation and caspase-3 denitrosylation act in concert to stimulate apoptosis (Fig. 4). The findings raise the possibility that coordinated nitrosylation/denitrosylation of proteins is a general mechanism of signal transduction regulation in cells.

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