Mitochondrial Regulation of Caspase Activation by Cytochrome Oxidase and Tetramethylphénylendiamine via Cytosolic Cytochrome c Redox State*

Received for publication, January 11, 2007, and in revised form, June 19, 2007. Published, JBC Papers in Press, August 9, 2007, DOI 10.1074/jbc.M700322200

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Cytochrome c release from mitochondria induces caspase activation in cytosols; however, it is unclear whether the redox state of cytosolic cytochrome c can regulate caspase activation. By using cytosol isolated from mammalian cells, we find that oxidation of cytochrome c by added cytochrome oxidase stimulates caspase activation, whereas reduction of cytochrome c by added tetramethylphénylendiamine (TMPD) or yeast lactate dehydrogenase/cytochrome c reductase blocks caspase activation. Scrape-loading of cells with this reductant inhibited caspase activation induced by staurosporine. Similarly, incubating intact cells with ascorbate plus TMPD to reduce intracellular cytochrome c strongly inhibited staurosporine-induced cell death, apoptosis, and caspase activation but not cytochrome c release, indicating that cytochrome c redox state can regulate caspase activation. In homogenates from healthy cells cytochrome c was rapidly reduced, whereas in homogenates from apoptotic cells added cytochrome c was rapidly oxidized by some endogenous process. This oxidation was prevented if mitochondria were removed from the homogenate or if cytochrome oxidase was inhibited by azide. This suggests that permeabilization of the outer mitochondrial membrane during apoptosis functions not just to release cytochrome c but also to maintain it oxidized via cytochrome oxidase, thus maximizing caspase activation. However, this activation can be blocked by adding TMPD, which may have some therapeutic potential.

Since the discovery by X. Wang (6) that cytochrome c release from mitochondria was central to apoptosis, there has been some question as to whether the two redox states of cytochrome c are equally effective in promoting caspase activation (7-10). But shortly after that discovery, there were two papers apparently showing that cytochrome c redox state had no effect on apoptosis (9, 11). Furthermore, Hampton et al. (9) showed that cytochrome c added to cytosol was rapidly reduced, and this reduction was enhanced by addition of dithiothreitol (DTT).2 DTT is routinely added when assaying caspase activation because caspases are inactivated by oxidation (12), and this complicates the analysis of whether the redox state of cytochrome c affects caspase activation, because agents that reduce caspases (such as DTT) also reduce cytochrome c, and agents that oxidize cytochrome c, such as H2O2 and ferricyanide, also oxidize caspases. The fact that cytosol readily reduces cytochrome c led investigators (9) to conclude that even if the redox state of cytochrome c affected caspase activation, it would not be relevant in the cell because cytochrome c in the cytosol would always be fully reduced.

We have reexamined whether the redox state of cytochrome c affects caspase activation, and we find that the reduced form of cytochrome c is less capable of causing caspase activation than the oxidized form. Furthermore, we find that mitochondria from apoptotic cells (but not healthy cells) are capable of fully oxidizing cytosolic cytochrome c via cytochrome oxidase. Agents, such as TMPD, that keep cytosolic cytochrome c reduced are capable of blocking apoptosis. These findings may have important conceptual and therapeutic consequences.

EXPERIMENTAL PROCEDURES

Materials—All reagents were of analytical grade. Human recombinant caspase-3 was purchased from Biomol. Isolated beef heart cytochrome c oxidase was a kind gift from Prof. Peter Rich. Unless otherwise stated, all chemicals were from Sigma.

Cell Cultures and Treatments—Murine macrophage J774 cells and human HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum and 50 μg/ml gentamycin at 37 °C in a humidified atmosphere containing 5% CO2. For induction of apoptosis, cells were plated in

2 The abbreviations used are: DTT, dithiothreitol; amc, 7-aminomethylcoumarin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; COX, cytochrome c oxidase; DMEM, Dulbecco’s modified Eagle’s medium; LDH, yeast lactate dehydrogenase/cytochrome c reductase; PBS, phosphate-buffered saline; TMPD, N,N,N',N’-tetramethylphenylene-1,4-diamine.
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Measurement of Redox State of Cytochrome c in Cell Homogenates and Cytosolic Extracts—Spectroscopic analysis of the redox state of cytochrome c was carried out using a Lambda 35 spectrometer (PerkinElmer Life Sciences) measuring the absorbance spectra between wavelengths of 500 and 600 nm at the indicated time after addition of 10 μM cytochrome c to the homogenates or cytosolic extracts (100–160 μg of total protein in 1 ml of lysing buffer). Reduction level of cytochrome c was expressed as absorbance at 550 nm minus absorbance at 535 nm and was normalized to the total protein concentration of homogenate or cytosolic extract used. Total reduction of cytochrome c was achieved by adding 1 mM ascorbate.

Immunocytochemistry for Cytochrome c—HeLa cells were plated on the chamber slides at a density 20 × 10⁴ cells/ml and cultured for 24 h before the experiment. After the experimental treatment with staurosporine/TMPD/ascorbate, cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were washed in PBS and permeabilized in PBS containing 0.1% saponin and 1% bovine serum albumin for 30 min prior to incubation with primary monoclonal antibodies against native cytochrome c (1:500 dilution; Pharmingen) for 1 h at room temperature. After washing three times with PBS, the slides were incubated with Cy3-conjugated affinity-purified goat antiserum (1:300 dilution in blocking solution; Jackson ImmunoResearch) for 1 h at room temperature and overlaid with mounting medium (Vectashield, Vector Laboratories), and staining was visualized using the confocal microscope (Olympus).

Statistical Analysis—Data are presented as means ± S.E. of at least three separate experiments. Statistical analyses were performed using Student’s t test. Differences were regarded to be statistically significant at p < 0.05.

RESULTS

Reduction of Cytochrome c Inhibits Caspase Activation in Vitro—We tested the ability of added cytochrome c to activate caspases in a cell-free system consisting of cytosol from J774 macrophages and a fluorogenic caspase-3 substrate DEVD-amc. As can be seen in Fig. 1A, addition of cytochrome c to cytosol (in the presence of dATP) greatly stimulated caspase activity in a concentration-dependent manner.

The redox state of cytochrome c was determined by measuring its absorption spectra in the range of 500–600 nm wavelengths. Pure cytochrome c, prior to addition to cytosol, was in the oxidized form, but it was rapidly reduced when added to the cytosolic extracts, coming to a steady state of about 80% reduction after 15 min of incubation. To maintain the added cytochrome c in a fully oxidized or reduced state, we treated the stock solutions of cytochrome c with various chemicals or enzymes and then added the mixtures to the cytosols. In the presence of purified cytochrome oxidase (COX), cytochrome c was immediately oxidized and remained in the fully oxidized state during a 30-min incubation with the cytosolic extracts. This resulted in a stimulation of caspase activation in cytosolic extracts; the increase in fluorescence because of DEVDase activity (compared with the control without cytochrome c) was seen already after 15 min of incubation in the presence of COX, whereas no caspase activation in the presence of cyto-

25-cm² cell culture flasks at a density 5 × 10⁶ cells/ml and incubated with 2 μM staurosporine in the presence/absence of 25 μM TMPD plus 0.2 mM ascorbate for 4 h.

For loading of cells with cytochrome c, cells were plated in 25-cm² cell culture flasks at a density 5–10 × 10⁶ cells/ml and incubated overnight. The next day cells were gently washed with DMEM, and 0.5 ml of DMEM containing 15 μM cytochrome c (or 15 μM cytochrome c plus 1.9 μM yeast LDH plus 1 mM lactate) was added to the flasks. Cells were scraped from the surface of the flasks using a plastic scraper, and the suspension of cells was passed 50 times through yellow Gilson pipette tip. After that, the volume of cell suspension was adjusted to 5 ml by adding DMEM with 5% fetal calf serum, and cells were incubated for 2 h.

Measurement of Caspase Activity—J774 or HeLa cells were washed twice with PBS, resuspended in 4 volumes of lysing buffer containing 250 mM sucrose, 10 mM KCl, 20 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, protease inhibitor mixture (1:100 dilution of stock solution (v/v); Sigma) and homogenized in a Dounce homogenizer (50 strokes with a glass pestle). The resulting homogenate was immediately used in further experiments, or cytosolic extracts were prepared by centrifugation at 14,000 rpm for 30 min in the Eppendorf microcentrifuge. To measure caspase activity, the supernatants (50–100 μg of total protein) were incubated with 100 μM DEVD-aminomethylcoumarin (amc) for 60 min at 37 °C. For evaluation of cytochrome c-induced caspase activation in vitro, cytosolic extracts were supplemented with 170–800 nM cytochrome c plus 1 mM dATP and incubated 15–60 min with 100 μM DEVD-amc. The release of 7-amc was measured using a fluorimeter (excitation at 380 nm and emission at 460 nm; PerkinElmer Life Sciences). The specificity of the reaction was tested in the presence of 80 μM N-acetyl-Asp-Glu-Val-Asp aldehyde (DEVD-CHO), an inhibitor of caspase-3; the rate of fluorescent substrate cleavage in cytosolic extracts was inhibitable with DEVD-CHO by more than 80%. When redox active agents (10 mM DTT, 1 mM ascorbate, 120 mM cytochrome c oxidase, 1.9 mM yeast lactate dehydrogenase plus 1 mM lactate, 25–200 μM TMPD) were used, cytochrome c was preincubated with them for 5 min and then added to the cytosols.

To measure the activity of human recombinant caspase-3, the enzyme (40 units) was added to 1 ml of buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol. The reaction was started by adding 100 μM DEVD-amc, and the increase of fluorescence was followed for 5–10 min. When TMPD was tested, it was added to the incubation mixture 5 min before the substrate DEVD-amc.

Assessment of Cell Death—After the treatment, cells were incubated with 1 μg/ml propidium iodide and 2 μg/ml Hoechst 33342 at room temperature for 10 min in the dark, followed by examination with a fluorescent microscope (Zeiss Axiovert S100). Propidium iodide-positive cells were considered as necrotic. Cells that were propidium iodide-negative but showed chromatin condensation and nuclear fragmentation were classified as apoptotic. Results are expressed as the percentage of the total cell number. In each experiment 6–10 independent fields (~1000 nuclei in total) were counted per each condition.
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To test whether cytochrome c reduction can affect caspase activation, we added lactate plus yeast lactate dehydrogenase (LDH), which directly reduces cytochrome c. In the presence of lactate and LDH, the rate of cytochrome c reduction in cytosols was increased from 2.9 ± 0.2 nmol/min (control, without LDH) to 9.3 ± 1.8 nmol/min in the presence of LDH, and after a 5-min incubation 45 and 78% of cytochrome c was in the reduced form in untreated control and LDH-treated cytosols, respectively. DEVD-cleavage activity was decreased by 37% in the presence of LDH plus lactate compared with cytosols incubated only with cytochrome c (Fig. 1C). This suggests that the reduced form of cytochrome c is less capable of activating caspases in vitro. To test whether COX could overcome the inhibition of caspase activation induced by LDH, we added COX 30 min after LDH, lactate, and cytochrome c. This resulted in oxidation of the cytochrome c and activation of the caspases; after 90 min of incubation with COX under these conditions, caspase activity was 0.102 ± 0.019 compared with 0.039 ± 0.009 nmol/min/mg (p < 0.05) in extracts incubated for the same time with LDH (before the addition of COX the activity of caspases was 0.043 ± 0.017 nmol/min/mg). This indicates that the inhibition because of LDH was reversible by COX.

We then looked for a small molecule compound that might be capable of reducing cytochrome c in cells. TMPD easily crosses membranes and rapidly reduces cytochrome c directly (13). Addition of TMPD to the cytosols containing cytochrome c immediately and fully reduced cytochrome c and completely blocked caspase activation in the cytosols supplemented with cytochrome c (Fig. 1C). TMPD reduced caspase activation (measured after 60 min of incubation with cytochrome c) by 33% when added to the extracts 15 min after cytochrome c, but it had only slight (15%) effect if added 30 min after cytochrome c when the caspases were already completely activated (data not shown). This indicates that TMPD inhibits the activation of caspases by cytochrome c but does not inhibit the caspases themselves after they have been activated. Altogether, these data indicate that the redox state of cytochrome c is important in the regulation of caspase activation in a cell-free system, and that the reduced form of cytochrome c has less capacity to activate caspases.

Previous studies (14) have shown that pinocytic loading of cytochrome c into the cells induced caspase activation and apoptosis. In agreement with this, we found that when macrophage cells were scraped-loaded with oxidized cytochrome c, this resulted in about 2-fold increase in caspase activity measured 2 h after the treatment (Fig. 1D). However, if the cells were scraped-loaded with cytochrome c plus LDH, there was no significant caspase activation in the cells, again suggesting that...
Reduced caspase activation, presumably because it is transported into cells. Ascorbate alone had no significant effect on staurosporine-treated cells to the control (untreated) level.

Ascorbate plus TMPD could prevent caspase activation by blocking cytochrome c release from mitochondria. To investigate this, cells were fixed, and cytochrome c localization within the cells was revealed by immunocytochemistry using a monoclonal antibody against cytochrome c. In untreated control cells, staining of cytochrome c was punctate indicating that cytochrome c was located in mitochondria (Fig. 3B) and only 0.7 ± 0.7% of cells had signs of cytochrome c immunoreactivity in cytosol. In contrast, diffuse labeling for cytochrome c was observed in staurosporine-treated cells (55.4 ± 3.8% of total number of cells) indicating a cytosolic location of cytochrome c (Fig. 3B). Cells treated with staurosporine plus TMPD and ascorbate also showed diffuse, whole cell staining of cytochrome c, 52.1 ± 6.7% of cells (Fig. 3B), i.e. there was no blockage of cytochrome c release by TMPD. This indicates that the suppression of caspase activation by TMPD was not related to the prevention of cytochrome c release from mitochondria, but rather TMPD affected some later steps in the apoptotic program between cytochrome c release and caspase activity, i.e. caspase activation itself.

The Redox State of Cytochrome c Is Regulated in Cell Homogenates—Because the redox state of cytosolic cytochrome c affected caspase activation, we were interested in what the redox state of cytochrome c would be in the cytosols of healthy and apoptotic cells, and what activities might be responsible for reducing or oxidizing cytochrome c in the cytosol. We compared the redox state of cytochrome c added to cytosols and homogenates of healthy and apoptotic cells, in part because homogenates contain mitochondria, whereas cytosol does not. It has been reported previously that cytosols have a high capacity to reduce cytochrome c (9). We examined the spectral changes in the range of 500–600 nm of cytochrome c

FIGURE 2. The effect of TMPD on caspase activation in staurosporine-treated J774 macrophage cells. Cells were incubated for 4 h with 2 μM staurosporine in the presence/presence of 25–200 μM TMPD, 0.2 mM ascorbate (where indicated); then cytosolic extracts were prepared, and caspase activity was measured using DEVD-amc as substrate. *, statistically significant difference from control; #, statistically significant difference from staurosporine-treated cells.
Cytochrome c Redox State Regulates Apoptosis

The most potent enzyme that oxidizes cytochrome c is mitochondrial cytochrome oxidase. Addition of purified cytochrome oxidase to cytosolic extracts rapidly oxidized added cytochrome c, and increased subsequent caspase activation (see above). Exogenous cytochrome c is not normally oxidized by cytochrome oxidase in intact mitochondria; however, when the mitochondrial outer membrane becomes permeable to cytochrome c as occurred in staurosporine-treated cells (Fig. 3B), cytochrome c may reach COX and be oxidized by it. To test whether COX was responsible for rapid oxidation of cytochrome c in homogenates of staurosporine-treated cells, we inhibited COX with 2 mM NaN₃ and then measured reduction of added cytochrome c. As can be seen from Fig. 5, NaN₃ substantially increased the rate of cytochrome c reduction in homogenates of staurosporine-treated cells; after 1 min of incubation the reduction level of cytochrome c was 2.7-fold and after 10 min was 9.1-fold higher in the presence of NaN₃ than in its absence. This suggests that cytochrome oxidase is involved in the oxidation of cytochrome c in staurosporine-treated cells when the mitochondrial outer membrane is permeabilized. NaN₃ also increased the rate of cytochrome c reduction in homogenates of control cells, but the increase was less pronounced, 1.4–1.8-fold after 1 and 10 min of incubation, respectively (Fig. 5). This may be due to the fact that during homogenization the mitochondrial outer membranes are partially damaged and become permeable to cytochrome c allowing access to cytochrome oxidase.

FIGURE 3. The effect of TMPD on caspase activation (A), release of cytochrome c from mitochondria (B), and cell death (C) in HeLa cells. HeLa cells were incubated with 2 μM staurosporine for 4 h in the absence/presence of 25 μM TMPD and 0.2 mM ascorbate (Asc). Caspase activity was measured following cleavage of DEVD-amc in cytosolic extracts of cells (A). For cytochrome c immunocytochemistry, cells after incubation were fixed and stained using anti-cytochrome c primary and Cy3-conjugated secondary antibody (B). Cell death (apoptotic or necrotic) is expressed as percentage of the total number of cells (C), * statistically significant difference from control; # statistically significant difference from staurosporine-treated cells.

DISCUSSION

Ever since the discovery by X. Wang that cytochrome c release from mitochondria was central to apoptosis (6), there have been persistent rumors and even some evidence that the redox state of cytochrome c makes a difference (7–10). But shortly after that discovery, there were two papers apparently showing that cytochrome c redox state had no effect on apoptosis. (i) The iron ion of cytochrome c could be replaced by a redox-inactive metal with no effect, and (ii) reduced and oxidized cytochrome c added to cytosolic extracts was equally effective (9, 11). However, the efficacy of redox-inactive cytochrome c shows only that cytochrome c does not have to change redox state to activate the apoptosome. The equal efficacy of reduced or oxidized cytochrome c when added to cytosol ignores the fact that cytosol has strong capacities to both reduce and oxidize cytochrome c, so that reduced or oxidized cyto-

The cytosolic extracts of cells (control; #, statistically significant difference from staurosporine-treated cells. necrotic) is expressed as percentage of the total number of cells (stained using anti-cytochrome c). As expected, reduction of cytochrome c was observed only within the 1st min after addition, and then cytochrome c was oxidized and stayed essentially fully oxidized for the rest of the incubation (Fig. 4, A and C). Similarly, only slightly slower reduction of cytochrome c was observed in cell homogenates, prepared from control or staurosporine plus TMPD plus ascorbate-treated cells (Fig. 4, B and C). Surprisingly, in staurosporine-treated cell homogenates a slight reduction of cytochrome c was observed only within the 1st min after addition, and then cytochrome c was oxidized and stayed essentially fully oxidized for the rest of the incubation (Fig. 4, B and C). In staurosporine plus TMPD and ascorbate-treated cell homogenates, cytochrome c was initially (during the first 2 min of incubation) rapidly reduced, then it was gradually oxidized, and after 10 min of incubation only 46% of added cytochrome c was in the reduced form. These data suggest that homogenates of staurosporine-treated cells contain some component that is capable of rapidly oxidizing added cytochrome c, but this component is missing in cytosolic extracts.

The cytosolic extracts of healthy or apoptotic cells. Initially, cytochrome c was in the oxidized form (~98%). When added to the cytosols from control or staurosporine-treated cells, it was gradually reduced (Fig. 4A) so that after 10 min of incubation with cytosols 35–40% of added cytochrome c was in the reduced form in both control and staurosporine-treated cell cytosols (Fig. 4C). As expected, reduction of cytochrome c was much faster in cytosols from staurosporine plus TMPD and ascorbate-treated cells; after 10 min of incubation about 80% of added cytochrome c was reduced (Fig. 4, A and C). Similarly, only slightly slower reduction of cytochrome c was observed in cell homogenates, prepared from control or staurosporine plus TMPD plus ascorbate-treated cells (Fig. 4, B and C).
Cytochrome c may be brought to the same steady-state redox state within a few minutes of addition. Hampton et al. (9) maintained the cytochrome c reduced or oxidized with ferrocyanide or ferricyanide, respectively, and found no gross difference in caspase activity between these two states. However, the ferrocyanide and ferricyanide directly inhibited activation, so they are not ideal agents for testing whether reduced and oxidized cytochrome c are equally capable of activating the apoptosome (9).

There have been reports since then that reduced cytochrome c is ineffective at inducing caspase activation if the cytochrome c is held reduced by reducing agents (7, 8, 10). Suto et al. (7) recently reported that oxidized cytochrome c rapidly activated caspase-3 and caspase-9 in a cytosolic fraction, whereas cytochrome c reduced by DTT, cysteine, or glutathione was completely ineffective at activating the caspases. Hancock et al. (8) list a variety of other evidence supporting the hypothesis that oxidized cytochrome c induces apoptosis, but reduced cytochrome c is incapable of doing so.

The fact that cytosol readily reduces cytochrome c led Hampton et al. (9) to conclude that even if the redox state of cytochrome c affected caspase activation, it would not be relevant in the cell because cytochrome c in the cytosol would always be fully reduced. However, we show here that cell homogenates, as opposed to cytosol, have the capacity to oxidize cytochrome c, and this capacity is increased in apoptotic cells to the extent that added cytochrome c is almost fully oxidized. Our findings that this oxidizing capacity is removed by centrifugation of the homogenates (to leave the cytosolic fraction), is inhibited by azide, and is replicated by adding purified cytochrome oxidase suggest that this activity is because of mitochondrial cytochrome oxidase itself. The finding that this oxid-
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dizing activity is greatly increased in homogenates from apoptotic cells (induced by staurosporine) is consistent with the mitochondria in these cells being permeable to cytochrome c (Fig. 3B). This suggests the following novel function for mitochondrial permeabilization during apoptosis: to oxidize cytosolic cytochrome c and thereby maximize caspase activation. It also suggests the possibility that caspase activation might be regulated by modulators of cytochrome oxidase activity, such as hypoxia, nitric oxide, or uncoupling. The reduced state of cytosolic cytochrome c might also be regulated by mitochondrial oxidants as superoxide reduces and hydrogen peroxide oxidizes cytochrome c, and mitochondrial oxidants are known to be involved in apoptosis in some conditions (17).

TMPD blocked cytochrome c-induced caspase activation in cytosolic extracts via reducing cytochrome c. Further TMPD blocked staurosporine-induced caspase activation and apoptosis in intact cells, without blocking cytochrome c release or without directly inhibiting caspase activity, indicating that it acted at the level of caspase activation. Thus TMPD may have some utility in blocking apoptosis in disease.

The kinetics of caspase activation shown in Fig. 1 has an apparent lag phase of 15 min after cytochrome c addition to cytosols. Hampton et al. (9) found a very similar lag. This apparent lag may be interpreted either (i) as the time required to activate the apoptosome or (ii) as the exponential increase in caspase-3 product expected for a progressive increase in caspase activity. According to the second interpretation, the first derivative (slope) of the curve gives the caspase-3 activity, and thereby maximizes caspase activation. Thus TMPD may have some utility in blocking apoptosis in disease.

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