Mitochondrial regulation of caspase activation by cytochrome oxidase and tetramethylphenylenediamine (TMPD) via cytosolic cytochrome c redox state
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Running title: Cytochrome c redox state regulates apoptosis
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Abstract
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. 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 TMPD (tetramethylphenylenediamine) or yeast lactate dehydrogenase/cytochrome c reductase blocks caspase activation. Scrape-loading of cells with this reductase inhibited caspase activation induced by staurosporin. Similarly, incubating intact cells with ascorbate plus TMPD to reduce intracellular cytochrome c strongly inhibited staurosporin-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 were 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.

Introduction
The intrinsic pathway of apoptosis is initiated by various stimuli that cause the release of cytochrome c from mitochondria into the cytoplasm (1). Once released, cytochrome c binds to the cytosolic adaptor protein Apaf-1 causing the formation of the so-called ‘apoptosome’ leading to activation of caspase-9, which then cleaves downstream caspases such as caspase-3 (2). The release of cytochrome c from mitochondria has been thought to be an irreversible step for the apoptotic process (3). However, recent evidence suggests that the execution phase of apoptosis is highly regulated even after cytochrome c is released (4;5). And one of possible levels of such regulation could be the redox state of cytochrome c.

Since the discovery by Xiadong Wang that cytochrome c release from mitochondria was central to apoptosis (6), 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). 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, lead 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 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 finding 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 kind gift from Dr 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 25 cm² cell culture flasks at a density 5 x 10⁶ cells/ml and incubated with 2 µM staurosporin in the presence/absence of 25 µM TMPD plus 0.2 mM ascorbate for 4 hours.

For loading of cells with cytochrome c, cells were plated in 25 cm² cell culture flasks at a density 5-10 x 10⁶ cells/ml and incubated overnight. 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 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 vol. of lyzing 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 cocktail (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 14000 rpm for 30 min in the Eppendorf microcentrifuge. To measure caspase activity, the supernatants (50-100 µg total protein) were incubated with 100 µM DEVD-amc for 60 min at 37°C. For evaluation of cytochrome c-induced caspase activation in vitro, cytosolic extracts were supplemented with 170 nM – 800 nM cytochrome c plus 1 mM dATP and incubated 15-60 min with 100 µM DEVD-amc. The release of 7-amino-methyl-coumarin (amc) was measured using a Perkin-Elmer fluorimeter (excitation at 380 nm, emission at 460 nm). The specificity of the reaction was tested in the presence of 80 µM DEVD-CHO, an inhibitor of caspase-3: the rate of fluorescent substrate cleavage in cytosolic extracts was inhabitable with DEVD-CHO by more than 80%. When redox active agents (10 mM DTT, 1 mM ascorbate, 120 nM cytochrome c oxidase, 1.9 µM 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 U) 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 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.

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 Perkin Elmer Lambda 35 spectrometer measuring the absorbance spectra between wavelengths of 500 nm and 600 nm at indicated time after addition of 10 µM cytochrome c to the homogenates or cytosolic extracts (100-160 µg total protein in 1 ml 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 chamberslides at a density 20 x 10⁴ cells/ml and cultured for 24 h before the experiment. After the experimental treatment with staurosporin/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 3 times with PBS the slides were incubated with Cy3-conjugated affinity pure goat anti-mouse secondary antibodies (1:300 dilution in blocking solution; Jackson ImmunoResearch Laboratories) for 1 h at room temperature, coverslipped with mounting medium (Vectashield, Vector Laboratories) and staining visualized using the confocal microscope (Olympus).

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 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 due to DEVDase activity (compared to the control without cytochrome...
c) was seen already after 15 min incubation in the presence of COX, whereas there was no caspase activation in presence of cytochrome c but absence of COX at this time point (Fig 1B). After a 30 min incubation, the DEVD-cleaving activity in extracts treated with cytochrome c together with COX was 88% higher than in extracts incubated only with cytochrome c (Fig. 1C). This suggests that cytochrome c oxidation can stimulate the rate of caspase activation in vitro.

In order 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 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 to 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. In order 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 incubation with COX under these conditions caspase activity was 0.102 ± 0.019 nmol/min/mg compared to 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 due to LDH was reversible by COX.

We then looked for a small molecule compound that might be capable of reducing cytochrome c in cells. N,N,N',N'-tetramethylphenylene-1,4-diame (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 incubation with cytochrome c) by 33% when added to the extracts 15 min after cytochrome c, but 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 caspses 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 regulation of caspase activation in 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 reduced by LDH, there was no significant caspase activation in the cells, again, suggesting that reduced cytochrome c is not able to cause caspase activation. Presence of exogenous cytochrome c in the incubation medium of intact cells or just scraping and pipetting of the cells without cytochrome c did not induce caspase activation (Fig. 1D).

**TMPD prevents staurosporin-induced caspase activation in cells.** Staurosporin is known to cause mitochondria-mediated apoptosis in cells (15;16). To test whether TMPD can block cytochrome c-mediated caspase activation in cells we treated J774 macrophage cells with staurosporin in the presence/absence of TMPD for 4 h and then measured the activity of caspases in cytosolic extracts. Incubation of cells with staurosporin resulted in a 3-fold increase in DEVD-cleaving activity (Fig.
TMPD suppressed this activity in a concentration dependent manner: 25 \( \mu \text{M} \) TMPD inhibited caspase activation by 46% and 200 \( \mu \text{M} \) TMPD completely blocked caspase activation. TMPD can be rapidly oxidized by cells via cellular cytochrome c and cytochrome oxidase (13), therefore it may not last long enough to fully prevent caspase activation in cells. To overcome this, cells were supplemented with ascorbate, which can rapidly reduce TMPD and maintain TMPD in the reduced form. As can be seen in Fig 2A, in the presence of 0.2 mM ascorbate, even 25 \( \mu \text{M} \) TMPD lowered DEVD-cleaving activity of staurosporin-treated cells to the control, untreated, level. Ascorbate alone had no significant effect on staurosporin-induced caspase activation, presumably because it is transported into cells relatively slowly.

Because oxidizing agents, such as H\(_2\)O\(_2\), can inactivate caspases directly (12) it was important to determine what effect TMPD (although a reducing agent) had on pre-activated caspases. However, TMPD had no significant effect on caspase activity when added directly to the cytosols prepared from staurosporin-treated cells where caspases were already active: DEVD-cleaving activity was 0.62 ± 0.16 nmol/min/mg protein in staurosporin-treated cell extracts, and when the extracts were supplemented with 25 \( \mu \text{M} \) TMPD or 0.2 M ascorbate the activity was 0.48 ± 0.14 and 0.50 ± 0.10 nmol/min/mg, respectively. This indicates that TMPD does not inhibit caspase activity directly.

Similarly, TMPD (up to 200 \( \mu \text{M} \) concentration) alone or together with ascorbate had no significant effect on the activity of isolated active recombinant caspase-3: the rate of substrate cleavage expressed as increase in relative fluorescence per min was 5.14 ± 0.49 in the absence of TMPD, and 4.38 ± 1.01, 3.74 ± 0.10, 4.73 ± 0.29 in the presence of 25 \( \mu \text{M}, 200 \mu \text{M} \) TMPD and 25 \( \mu \text{M} \) TMPD plus 0.5 mM ascorbate, respectively. These findings confirm that TMPD does not significantly inhibit caspases directly.

Similar results were obtained in experiments on HeLa cells (Fig. 3). Incubation of HeLa cells with 2 \( \mu \text{M} \) staurosporin for 4 h caused a 4-fold increase in caspase activity which was reduced almost to control level in the presence of 25 \( \mu \text{M} \) TMPD plus 0.2 mM ascorbate (Fig. 3A). Ascorbate alone had no effect on staurosporin-induced caspase activation in HeLa cells (Fig. 3A). TMPD plus ascorbate also substantially reduced staurosporin-induced cell death measured at 24 h: treatment with staurosporin caused apoptosis in about 40% and necrosis in 50% of cells, and these numbers were reduced in the presence of TMPD plus ascorbate to 6% apoptosis and 25% necrosis (Fig. 3C). TMPD itself was not toxic to the cells (Fig. 3C).

TMPD might in principle inhibit apoptosis upstream of cytochrome c release, rather than at the level of caspase activation. We therefore tested whether TMPD could prevent caspase activation by blocking cytochrome c release from mitochondria. To investigate this, cells were fixed and cytochrome c location 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 cytosols. In contrast, diffuse labeling for cytochrome c was observed in staurosporin-treated cells (55.4 ± 3.8% of total number of cells) indicating a cytosolic location of cytochrome c (Fig. 3B). Cells treated with staurosporin 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 programme between cytochrome c release and caspase activation, 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 previously reported 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 added to cytosolic extracts of healthy or apoptotic cells. Initially cytochrome c was in the oxidized form (~98%). When added to the cytosols from control or staurosporin-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 staurosporin-treated cell cytosols (Fig. 4C). As expected, reduction of cytochrome c was much faster in cytosols from staurosporin-plus TMPD and ascorbate-treated cells: after 10 min incubation about 80% of added cytochrome c was reduced (Fig. 4A,C). Similar, only slightly slower reduction of cytochrome c was observed in cell homogenates, prepared from control or staurosporin-treated cells (Fig. 4B,C). Surprisingly, in staurosporin-treated cell homogenates a slight reduction of cytochrome c was observed only within the first minute after addition, and then cytochrome c was oxidized and stayed essentially fully oxidized for the rest of the incubation (Fig. 4B,C). In staurosporin-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 incubation only 46% of added cytochrome c was in the reduced form. These data suggest that homogenates of staurosporin-treated cells contain some component that is capable of rapidly oxidizing added cytochrome c, but this component is missing in cytosolic extracts.

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 occur in staurosporin-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 staurosporin-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 staurosporin-treated cells: after 1 min incubation the reduction level of cytochrome c was 2.7-folds and after 10 min – 9.1-folds higher in the presence of NaN₃ than in its absence. This suggests that cytochrome oxidase is involved in oxidation of cytochrome c in homogenates of staurosporin-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-folds after 1 min and 10 min 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.

Discussion
Ever since the discovery by X. Wang that cytochrome c release from mitochondria was central to apoptosis (6), there have been persistent rumours 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 Fe 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 were 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 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 (7) recently reported that oxidized cytochrome c rapidly activated caspases 3 and 9 in a cytosolic fraction, whereas cytochrome c reduced by DTT, cysteine or glutathione was completely ineffective at activating the caspases. Hancock (8) lists 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 lead 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), and is inhibited by azide, and is replicated by adding purified cytochrome oxidase, suggests that this activity is due to mitochondrial cytochrome oxidase itself. The finding that this oxidizing activity is greatly increased in homogenates from apoptotic cells (induced by staurosporin) is consistent with the mitochondria in these cells being permeable to cytochrome c (Fig 3B). This suggests a 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 redox 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 (e.g. (17)).

TMPD blocked cytochrome c-induced caspase activation in cytosolic extracts via reducing cytochrome c. Further TMPD blocked staurosporin-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, while the second derivative gives the rate of caspase activation. If we follow this interpretation, Fig 1B indicates that oxidized
cytochrome c greatly increases the rate of caspase activation. While if we follow the first interpretation, the figure indicates that oxidized cytochrome c activates the apoptosome much more quickly.

We did not investigate the mechanism by which the redox state of cytochrome c regulates caspase activation. One possibility is that the reduced form of cytochrome c has a lower affinity for (or lower ability to activate) Apaf-1 than the oxidized form. Alternatively, the reduced form of cytochrome c may have a higher affinity for some competing binding site in the cell. The reduced state of cytochrome c differs from the oxidized state in charge and a variety of subtle structural changes (18). Physiological ionic strength greatly increases the quantity of cytochrome c required to activate caspases in cytosolic extracts, suggesting that the binding to Apaf-1 is largely ionic (9;19).

Whatever the mechanism, the findings that the cytosolic redox state of cytochrome c regulates caspase activation, and this in turn is regulated by mitochondria during apoptosis, have important conceptual and possibly therapeutic consequences.

REFERENCES

This research was supported by a project grant from the British Heart Foundation (BHF).

The abbreviations used are: amc - 7-amino-methyl-coumarin; Apaf-1 – apoptosis activating factor-1; CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate; COX – cytochrome c oxidase; DMEM – Dulbecco’s Modified Eagle Medium; DTT - dithiothreitol; LDH - yeast lactate dehydrogenase/cytochrome c reductase; NTA – nitrilotriacetic acid; PBS – phosphate buffered saline; TMPD - N,N,N',N'-tetramethylphenylene-1,4-diamine.

Figure legends

**Fig. 1.** The effect of added cytochrome c on caspase activation in cytosolic fraction of J774 macrophage cells. A – time-course of caspase activation in cell free system after addition of various concentrations of cytochrome c. B – time-course of activation of caspases in the presence of 170 mM cytochrome c and 120 nM COX. C – effects of oxidized and reduced cytochrome c on caspase activation in cell-free system. Cytochrome c was preincubated with 120 nM COX, 1.9 µM yeast LDH plus 1 mM lactate or 25 µM TMPD for 5 min (where indicated), then 100 µg cytosolic extract was added and after 30 min incubation caspase-3-like, DEVD-cleaving activity was measured following increase in fluorescence due to cleavage of DEVD-amc. D – scraping-loading of cells with cytochrome c induces caspase activation within the cells. Cells were loaded with 15 µM cytochrome c plus/minus 1.9 µM yeast LDH as described in Experimental Procedures. After 2 h incubation caspase activity was measured in cytosolic extracts of the cells. * - statistically significant difference from control, # - statistically significant effect of COX, LDH or TMPD compared to cytosols treated with cytochrome c (+cyt.c).

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

**Fig. 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 staurosporin for 4 h in the absence/presence of 25 µM TMPD and 0.2 mM ascorbate. 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 staurosporin-treated cells.

**Fig. 4.** Reduction of cytochrome c in cytosolic extracts and whole cell homogenates of HeLa cells. HeLa cells were incubated with 2 µM staurosporin and 25 µM TMPD plus 0.2 mM ascorbate (where indicated) for 4 h as previously. Control cells were incubated for the same time but without staurosporin or TMPD/ascorbate. Cytosolic extracts (A) or homogenates (B) were incubated with added 10 µM cytochrome c. Cytochrome c reduction level was assayed following spectral changes in the interval of wavelengths 500-600 nm and measuring the height of characteristic peak at 550 nm. Fully reduced state of cytochrome c was achieved by adding 1 mM ascorbate and that level was taken as 100% reduction (C).

**Fig. 5.** The effect of NaN3 on the redox state of cytochrome c in HeLa cell homogenates. Cells were incubated with staurosporin for 4 h, cell homogenates were prepared and incubated with 10 µM cytochrome c in the presence/absence of 2 mM NaN3.
Fig. 1A
Fig. 1B
Fig. 1C

DEVDase, nmol/min/mg

Control + Cyt.c + Cyt.c + COX + LDH + Cyt.c + TMPD

* +170 nM Cyt.c 

#
Fig. 1D
Fig. 2
Fig. 3A
Fig. 3B

Control  +Staurosporin  +Staurosporin +TMPD +Ascorbate
Fig. 3C

Cell death, %

- Apoptosis
- Necrosis

Control +Stauro +TMPD +Asc

+Stauro

+Stauro +TMPD +Asc

TMPD
Cytosols

![Graph showing Cytosol reduction, ΔA_{500-535}/mg protein over time for different treatments: Control, +Stauro, +Stauro +TMPD +Asc, with error bars indicating variability.]
Homogenates

![Graph showing Cyt.c reduction, $\Delta A_{550-535}$/mg protein over time for different treatments.](Fig. 4B)

- Control
- +Stauro
- +Stauro +TMPD +Asc

Fig. 4B
Fig. 4C
Fig. 5
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J. Biol. Chem. published online August 9, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M700322200

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