



Review

Regulation of apoptosis by the redox state of cytochrome *c*Guy C. Brown^{a,*}, Vilmante Borutaite^b^a Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK^b Institute for Biomedical Research, Kaunas University of Medicine, LT 50009 Kaunas, Lithuania

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ABSTRACT

Cytochrome *c*, released from mitochondria into the cytosol, triggers formation of the apoptosome resulting in activation of caspases. This paper reviews the evidence for and against the redox state of cytochrome *c* regulating apoptosis, and possible mechanisms of this. Three research groups have found that the oxidized form of cytochrome *c* (Fe³⁺) can induce caspase activation via the apoptosome, while the reduced form (Fe²⁺) cannot. It is unclear whether this is due to the oxidized and reduced forms of cytochrome *c* having: (i) different affinities for Apaf-1, (ii) different abilities to activate Apaf-1 once bound, or (iii) different affinities for other components of the cell. Experiments replacing the Fe of cytochrome *c* with redox-inactive metals indicate that cytochrome *c* does not have to change redox states to activate caspases. In healthy cells, cytosolic cytochrome *c* is rapidly reduced by various enzymes and/or reductants, which may function to block apoptosis. However, in apoptotic cells, cytosolic cytochrome *c* is rapidly oxidized by mitochondrial cytochrome oxidase, to which it has access due to permeabilization of the outer membrane. Regulation of the redox state of cytochrome *c* potentially enables regulation of the intrinsic pathway of apoptosis at a relatively late stage.

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1. Introduction

The intrinsic pathway of apoptosis is mediated by various stimuli that cause the release of cytochrome *c* from mitochondria into the cytoplasm, triggering caspase activation [1,2]. Though the release of cytochrome *c* from mitochondria is irreversible, recent evidence suggests that the execution phase of apoptosis is highly regulated even after cytochrome *c* release [3,4]. There are several possible levels of such regulation and the redox state of cytosolic cytochrome *c* may be one of them [5]. This paper reviews the evidence for and against the redox state of cytochrome *c* regulating apoptosis, and possible mechanisms by which such regulation might be brought about.

2. The apoptosome

Once released from mitochondria, cytochrome *c* participates in assembling a multimeric, caspase-9-activating complex – the apoptosome. Studies on reconstituted apoptosomes using purified proteins indicate that Apaf-1, cytochrome *c*, pro-caspase-9 and dATP/ATP are the necessary and sufficient components of the complex, though some additional proteins may be involved (such as XIAP, Hsp70 and Aven

[6,7]. Apaf-1 is a cytosolic protein existing as an inactive, monomeric, closed conformation until the apoptotic signal – appearance of cytochrome *c* in cytosol – is received. Cytochrome *c* binds to the WD40 domains of Apaf-1, stabilizing an open conformation of Apaf-1 that hydrolyses dATP or ATP bound to the nucleotide-binding domain. This is followed by nucleotide exchange, which triggers oligomerisation into a heptamer of 7 Apaf-1 molecules, which is then capable of recruiting and activating pro-caspase-9 via the CARD domains of Apaf-1 and pro-caspase-9 [8]. It has been proposed that cytochrome *c* interacts only transiently with Apaf-1 triggering assembly of the apoptosome as some laboratories have been unable to find any cytochrome *c* in immunoprecipitated apoptosomes [3,9]. However, Zou et al. [10] did find significant amounts of cytochrome *c* in the mature apoptosome, although less than the amounts of Apaf-1 and caspase-9.

3. Early evidence on cytochrome *c* redox state and apoptosis

Cytochrome *c* exists in interconvertible reduced (haem Fe²⁺) or oxidized (haem Fe³⁺) forms. The structures of these two forms (see Fig. 1) are similar [11,12] but there are significant differences [13] leading to different physical properties of compressibility, stability, solvent accessibility, radius of gyration and maximum linear dimension [14]. The reduced form of cytochrome *c* also binds less to anions, and binds less tightly to negatively charged membranes [15].

Because the reduced and oxidized forms of cytochrome *c* have different physical and biochemical properties, one may ask whether they are equally capable of activating the apoptosome. However, shortly after the discovery of the role of cytochrome *c* in apoptosis

Abbreviations: Apaf-1, apoptosis activating factor 1; CARD, caspase recruitment domain; DTT, dithiothreitol; Hsp, heat shock protein; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; XIAP, X-chromosome-linked inhibitor of apoptosis

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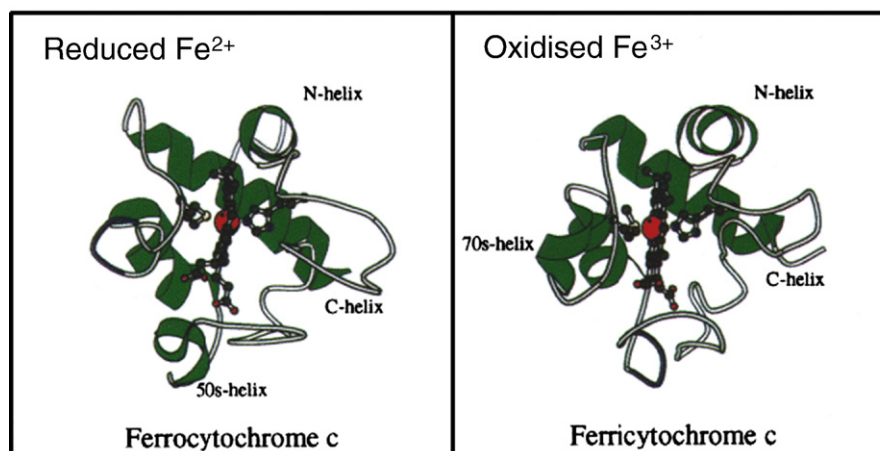


Fig. 1. Schematic representations of structures of oxidized (right) and reduced (left) horse heart cytochrome *c* (drawn with the program Molscript). The peptide backbone is represented as a white line except where it is alpha-helical when it is represented as green ribbon. The haem is seen edge on in the centre with iron atom in red. Reprinted with permission from [13]. Copyright (2008) American Chemical Society.

there were two reports apparently showing that the redox state of cytochrome *c* was not important for caspase activation through the apoptosome [16,17].

Kluck et al. [17] found that the haem Fe of cytochrome *c* could be replaced by a redox-inactive Cu or Zn with little effect on its pro-apoptotic function (50% reduced activity). However, the efficacy of redox-inactive cytochrome *c* shows only that cytochrome *c* does not have to change redox states to be effective. Kluck et al. [17] also found that oxidation of the cytochrome *c* in the cell-cytosolic extracts with 6 mM ferricyanide inhibited caspase activation. But they went on to show that this inhibition occurred directly on the caspases (and possibly Apaf-1) rather than via the redox state of cytochrome *c*. Indeed it has been shown that the caspases are inactivated by oxidation, probably at the active-site cysteine residue [18–20]. Thus although Kluck et al. [17] showed that cytochrome *c* does not have to change redox states to be effective, they were unable to test whether the reduced and oxidized forms were equally effective.

Interestingly Kluck et al. [17] found that there was a threshold of cytochrome *c* concentration to cause caspase activation (no activation by 20 nM cytochrome *c*); while at higher concentrations there was lag time before caspase activation (of 3 h at 40 nM cytochrome *c*, but just 15 min at 80 nM cytochrome *c*). In the conditions they used the cytochrome *c* was largely reduced (at least initially). We have found that this lag phase is decreased by keeping the cytochrome *c* oxidized (by adding cytochrome *c* oxidase) [21]. This may indicate that the oxidized and reduced forms of cytochrome *c* have different kinetics of activation of the apoptosome, or that the redox state of cytochrome *c* is changing during the incubation.

Hampton et al. [16] showed that cytochrome *c* added to cytosolic extracts was rapidly reduced, and this reduction was enhanced by addition of dithiothreitol (DTT). DTT is routinely used in assays of caspase activation to prevent their inactivation by oxidants. This complicates the analysis of the role of cytochrome *c* redox state in caspase activation, because agents that maintain caspases in the reduced state (such as DTT or reduced glutathione) also reduce cytochrome *c*, and agents that oxidize cytochrome *c*, (H_2O_2 or ferricyanide) also oxidize (and inhibit) the caspases. The finding that cytosol rapidly reduces cytochrome *c* lead Hampton et al. [16] to conclude that even if the redox state of cytochrome *c* affected caspase activation it would be irrelevant in the cells because cytochrome *c* in the cytosol would always be strongly reduced. However, we have found that while homogenates of healthy cells reduce added cytochrome *c*, those of apoptotic cells oxidize cytochrome *c* probably due to the cytochrome *c* oxidase of the cytochrome *c*-permeable apoptotic mitochondria [21].

Although Kluck et al. [17] and Hampton et al. [16] concluded that cytochrome *c* did not need to change redox states to activate caspases, they did find that activation was dependent on subtle structural features of cytochrome *c*. Kluck et al. [17] showed that yeast cytochrome *c* was unable to activate the caspases, although it has a very similar structure to the mammalian and fish cytochrome *c* that does activate. Hampton et al. [16] showed that biotinylated cytochrome *c* or cytochrome without the haem were incapable of activating the caspases. And they showed that (physiologically) high ionic strength inhibited cytochrome *c*-induced caspase activation, indicating that the interaction of cytochrome *c* with the apoptosome was largely ionic. Since the redox state of cytochrome *c* changes its net charge as well as a variety of subtle structural features [14] that affect its binding to proteins and membranes, the redox state might at least in principle affect its binding to the apoptosome.

4. Later evidence that cytochrome *c* redox state does affect apoptosis

Pan et al. [22] found that addition of oxidized cytochrome *c* to cell extracts induced apoptotic activity (measured by nuclear fragmentation), whereas addition of reduced cytochrome *c* had no effect. Furthermore, addition of cytochrome *c* reductase completely blocked the ability of the added oxidized cytochrome *c* to induce apoptotic activity. This activity was also inhibited by ascorbate, glutathione, cysteine and *N*-acetyl-cysteine, which are all capable of reducing cytochrome *c*. This work indicated that reduced cytochrome *c* was incapable of inducing apoptosis (at least at the concentrations, time and ionic strength used).

Suto et al. [23] found that addition of oxidized cytochrome *c* to a cytosolic extract resulted in processing and activation of both caspase 9 and caspase 3, whereas addition of reduced cytochrome *c* had no effect on either processing or activation of either caspase. They also found that addition of glutathione or cysteine to reduce the cytochrome *c* inhibited the ability of added oxidized cytochrome *c* to activate the caspases.

Similarly, we [21] found that cytochrome *c* added to cytosolic extracts was partially reduced, but if the cytochrome *c* was further reduced by adding cytochrome *c* reductase, ascorbate, DTT or TMPD then caspase activation was partially inhibited, whereas when the cytochrome *c* was oxidized by adding cytochrome *c* oxidase the rate of caspase activation was stimulated. Loading cytochrome *c* reductase into cells or incubating cells with ascorbate plus TMPD to reduce intracellular cytochrome *c* strongly inhibited staurosporine-induced apoptosis and caspase activation but not cytochrome *c* release,

indicating that reduction of cytosolic cytochrome *c* blocks caspase activation [21]. Furthermore, we found that mitochondria from apoptotic cells (but not healthy cells) are capable of fully oxidizing cytosolic cytochrome *c* via cytochrome oxidase. In contrast to previous work, cell homogenates (containing mitochondria) rather than cytosols were used, and this approach revealed an important difference between healthy and apoptotic cells: rapid oxidation of added cytochrome *c* in homogenates of cells undergoing apoptosis and rapid reduction of cytochrome *c* in homogenates of normal, non-apoptotic cells. It was also found that cytochrome *c* oxidation was carried out by mitochondrial cytochrome oxidase as the oxidation was prevented when cytochrome oxidase was inhibited by azide or if mitochondria were removed from the homogenate. Thus permeabilization of the outer mitochondrial membrane during apoptosis may have a function not only in the release of cytochrome *c* but also in facilitating its oxidation via cytochrome oxidase, thus maximizing activation of caspases.

The experiments of Pan et al. [22], Suto et al. [23], and Borutaite and Brown [18] are all consistent in that they show that oxidized cytochrome *c* induces caspase activation in cytosols, whereas reduced cytochrome *c* has little or no capacity to activate the caspases. But to what extent are these results consistent with the earlier results of Kluck et al. [17] and Hampton et al. [16], and subsequent work with the reconstituted apoptosome where cytochrome *c* is likely to be reduced due to the presence of DTT? It is difficult to know whether these results are consistent without comparing the kinetics of apoptosome activation with known concentrations and redox states of cytochrome *c*, and at similar ionic strengths, and over similar time scales. These kinetics are known to be complex (Kluck et al. [17]), which might in part reflect changes in the redox state of cytochrome *c*. The reduced and oxidized forms of cytochrome *c* may also compete for binding and/or activation of the apoptosome, so that the amount of activation may depend on the time and concentrations involved. For example, if the reduced form of cytochrome *c* binds to Apaf-1 with lower affinity than the oxidized form, this block on activation might be overcome simply by increasing the concentration of cytochrome *c* added to (potentially unphysiologically) high concentrations (or by reducing the ionic strength). Only careful experiments measuring the kinetics of the reconstituted apoptosome will resolve these issues.

5. Redox state of cytosolic cytochrome *c*

During apoptotic cytochrome *c* release, the mitochondrial membrane potential is known to depolarize, followed by repolarization (if caspases are inhibited) over a time course of about 1 h [24,25]. During the depolarization the cytosolic cytochrome *c* is presumably oxidized, and thus capable of activating the apoptosome. It is not clear what mediates the repolarization, but may involve upregulation of some cytochrome *c* reducing activity, either in the mitochondria or cytosol. One possibility is the high NADH-cytochrome *c* oxidoreductase activity of the outer mitochondrial membrane, which is limited by NADH supply from glycolysis. In preliminary experiments we found that this activity can strongly reduce cytosolic cytochrome *c* when mitochondria are added together with NADH (unpublished data).

iNOS and nNOS are found in the cytoplasm and can reduce cytochrome *c* at a rate over a hundred-fold greater than the rate at which they produce NO [26,27]. nNOS-expressing neurons are selectively protected from cell death induced by a variety of insults [28,29] and iNOS-induction is known to protect a variety of tissues. One mechanism of this protection might be reduction of cytosolic cytochrome *c* by NOS. However, once the mitochondria are permeabilized to cytochrome *c*, cytochrome oxidase is able to potentially oxidize cytosolic cytochrome *c*. We and others have shown that NO and hypoxia synergistically inhibit cytochrome oxidase [30–33]. Thus NO and hypoxia potentially block apoptosis by inhibiting cytochrome oxidase, resulting in reduction of cytosolic cytochrome *c* (the reduction being partly due to complex III of the permeabilized mitochondria).

Other cytosolic enzymes known to reduce cytochrome *c* include P450s, P450 reductases, *b*₅, *b*₅ reductases and neuroglobin. Neuroglobin is a recently found protein of the globin family [34]. Its expression level is high in neurons and retina – cells that are also relatively resistant to apoptosis. The biological function of neuroglobin is not entirely clear but it has been shown that upregulation of this protein protects neurons from hypoxic and ischemic damage [35]. Recently it was found that neuroglobin can rapidly reduce cytochrome *c* and it was proposed that the protective effect of neuroglobin against apoptosis in neurons might be mediated by this means [36].

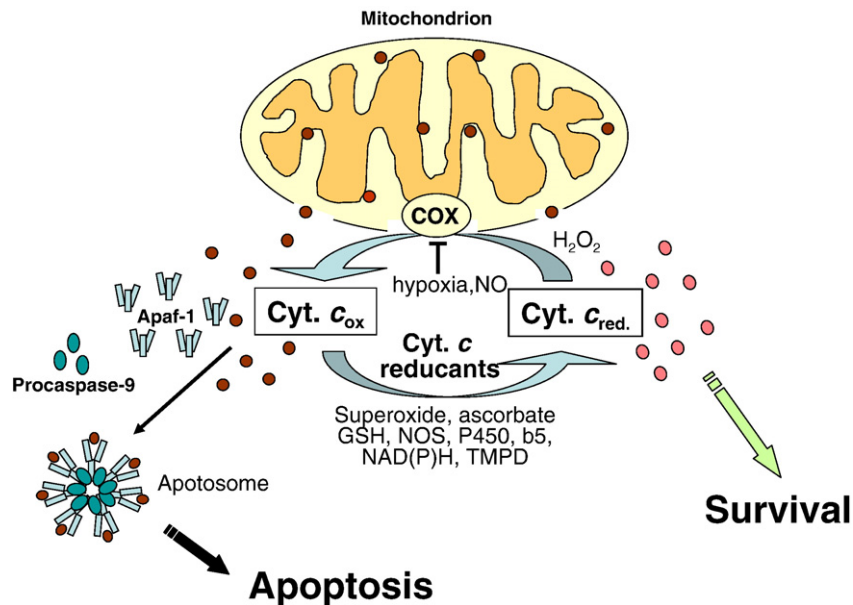


Fig. 2. Regulation of apoptosis by the redox state of cytosolic cytochrome *c*. Cytochrome *c* is oxidized by mitochondrial cytochrome oxidase (COX) and in this oxidized form (Cyt. *c*_{ox}) it binds to Apaf-1 forming the apoptosome which activates pro-caspase-9 leading to apoptosis. Cytosolic cytochrome *c* can be reduced (Cyt. *c*_{red}) by various reductants which include superoxide, ascorbate, reduced glutathione (GSH), some chemicals such as tetramethylphenylenediamine (TMPD) and reducing enzymes (cytochromes *b*₅, P450, NOS, neuroglobin, cytosolic NAD(P)H oxidases). This reduced cytochrome *c* cannot activate the apoptosome, and therefore does not promote apoptosis.

Cytosolic cytochrome *c* can also be reduced by ascorbate, glutathione, cysteine and superoxide, and can be oxidized by H₂O₂ [5]. But it is unclear whether these rates are fast enough in cells to compete with the enzyme catalysed rates. Cytochrome *c* release during apoptosis can cause increased mitochondrial superoxide production, which dismutates to hydrogen peroxide (via superoxide dismutase) and this can oxidize cellular glutathione (via glutathione peroxidase) [37]. Since the superoxide and glutathione can reduce cytosolic cytochrome *c*, while the hydrogen peroxide can oxidize, it is unclear what effect this stimulated mitochondrial oxidant production will have. Apoptosis can be regulated by many different redox molecules acting at different sites in the cell [5,38,39]. It is possible that once cytochrome *c* is released into the cytoplasm it comes into equilibrium with the 'cytosolic redox state' i.e. the system of oxidants and antioxidants that are broadly in equilibrium with reduced/oxidized glutathione. However, it seems more likely that reduction and oxidation of cytochrome *c* in the cytoplasm is dominated by specific enzyme catalysed reactions, such as that of cytochrome oxidase.

6. Mechanisms by which the redox state of cytochrome *c* may regulate apoptosis

As pointed out above, experimental evidence indicates that the redox activity of cytochrome *c* is not necessary for apoptosome formation or activation of caspases (i.e. it does not have to CHANGE redox states). However, there remain at least three possible mechanisms by which the redox state of cytochrome *c* might influence activation of the apoptosome:

- (a) The reduced and oxidized forms of cytochrome *c* might have different binding affinities for Apaf-1. To be consistent with the results above, the reduced form of cytochrome *c* would need to have a lower affinity or on rate for binding to Apaf-1. Binding of cytochrome *c* to Apaf-1 involves many residues on different areas of the surface, particularly lysine residues around the haem edge [13,40,41], and the relative positions of these residues does change with redox state [13], but as the binding is mainly electrostatic it is hard to know whether this will have much affect on the affinity.
- (b) The reduced and oxidized forms of cytochrome *c* might have different abilities to activate Apaf-1 (after binding). Binding of cytochrome *c* to Apaf-1 causes hydrolysis of bound dATP to dADP, followed by nucleotide exchange with free dATP, which then allows activation of the apoptosome, whereas if the nucleotide exchange does not occur the apoptosome becomes irreversibly inactivated. The reduced form of cytochrome *c* might bind to monomeric Apaf-1, but be incapable of causing dATP hydrolysis or exchange, thus blocking activation, and potentially leading to inactivation.
- (c) Reduced cytochrome *c* might be less capable of activating the apoptosome because it preferentially binds to other cell components (proteins, membranes, DNA) thus leaving less free to bind to the apoptosome. Cytochrome *c* has a net positive charge and is known to bind to DNA, to negatively charged membranes, and to a variety of cytosolic proteins (such as cytochrome *b₅* and the IP₃ receptor). The redox state of cytochrome *c* might affect the affinity of binding to these components. Cytochrome *c* also binds to ATP and this is known to block its activation of Apaf-1 [42], so if the reduced form of cytochrome *c* bound more ATP this might explain its inability to activate.

The redox state of cytochrome *c* might also regulate apoptosis upstream or downstream of caspase activation. According to the work of Kagan et al. [43,44], the oxidized form of cytochrome *c* can peroxidize mitochondrial cardiolipin, which apparently favours cyto-

chrome *c* release from the mitochondria. Since the reduced form of cytochrome *c* is unlikely to be capable of oxidizing cardiolipin, this might be one way in which the redox state of cytochrome *c* regulates apoptosis, although such regulation would be prior to cytochrome *c* release. Kagan's group have also provided evidence that once released from mitochondria the oxidized form of cytochrome *c* can peroxidize phosphatidylserine on the inside of the plasma membrane [45,46]. This oxidized form of phosphatidylserine is apparently preferentially exported to the outer leaflet of the plasma membrane, where it provides an 'eat-me' signal to phagocytes. Here again, since the reduced form of cytochrome *c* is unlikely to be capable of oxidizing phosphatidylserine, this might be one way in which the redox state of cytochrome *c* regulates apoptosis, although such regulation would be at the level of phosphatidylserine exposure.

7. Conclusions

Accumulating evidence suggests that the balance between reductive and oxidative pathways in cells determines the redox steady-state of cytochrome *c* (Fig. 2), and through this may regulate caspase activation by the apoptosome (as discussed in [32]). Apoptosis mediates programmed cell death, host defence and some pathology. Regulation of apoptosis is also important to the development of cancer and its treatment. Indeed there is evidence that activation of caspase-9 by cytochrome *c* is blocked in some cancer cells [47]. Therefore, understanding how apoptosis is regulated at various levels may have important clinical implications.

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