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Minireview

Cytochrome *c* in the apoptotic and antioxidant cascades

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Abstract Recent progress in studies on apoptosis has revealed that cytochrome *c* is a pro-apoptotic factor. It is released from its places on the outer surface of the inner mitochondrial membrane at early steps of apoptosis and, combining with some cytosolic proteins, activates conversion of the latent apoptosis-promoting protease pro-caspase-9 to its active form. Cytochrome *c* release can be initiated by the pro-apoptotic protein Bax. This process is blocked by the anti-apoptotic proteins Bcl-2 and Bcl-x_L. The role of cytochrome *c* in apoptosis may be understood within the framework of the concept assuming that the evolutionary primary function of apoptosis was to purify tissues from ROS-overproducing cells. In this context, the pro-apoptosis activity of cytochrome *c* might represent one of the anti-oxidant functions inherent in this cytochrome. Among other cytochrome *c*-linked antioxidant mechanisms, the following systems can be indicated. (1) Cytochrome *c* released from the inner mitochondrial membrane to the intermembrane space can operate as an enzyme oxidizing $O_2^{-\bullet}$ back to O_2. The reduced cytochrome *c* is oxidized by cytochrome oxidase (or in yeasts and bacteria, by cytochrome *c* peroxidase). (2) The intermembrane cytochrome *c* can activate the electron transport chain in the outer mitochondrial membrane. This bypasses the initial and middle parts of the main respiratory chain, which produce, as a rule, the major portion of ROS in the cell. (3) The main respiratory chain losing its cytochrome *c* is inhibited in such a fashion that antimycin-like agents fail to stimulate ROS production.

Key words: Cytochrome *c*; Apoptosis; Bcl-2; Bax; Cytochrome b_5; Reactive oxygen species; Permeability transition pore; Mitochondrion

1. Introduction

In 1996 I considered possible reasons why mitochondria are involved in apoptosis [1]. It was assumed that the programmed cell death (apoptosis) was invented by biological evolution as a mechanism to purify the cell population from ROS-overproducing cells. Since mitochondria are usually the main source of ROS in the cell, it is not surprising that they are involved in apoptosis, being equipped with pro-apoptotic proteins. Apoptosis-inducing factor (AIF), an ICE-like protease, is one of them [2–4]. AIF is localized in the mitochondrial intermembrane space. It is released to the cytosol due to breakdown of the outer membrane of mitochondria when the permeability transition pore (PTP) is opened.

The PTP is known to be induced by ROS. This may represent a mechanism of induction of ROS-linked apoptosis. Other PTP inducers are also apoptogenic, while PTP inhibitors are anti-apoptotic, a fact indicating that different tissue functions requiring apoptosis may be realized via similar mechanisms [3,4].

In 1996–1997, one more pro-apoptotic protein was identified in mitochondria. This proved to be cytochrome *c*, a well known component of the mitochondrial respiratory chain. In this paper I shall summarize related observations, trying to answer the question why evolution selected cytochrome *c* for this role. In this connection, various possible antioxidant effects of cytochrome *c* will be considered.

2. Cytochrome *c* mediates apoptosis

Cytochrome *c* is a small (in mammals, 104 amino acid residues), very stable hemoprotein containing covalently bound heme *c* as a prosthetic group. Its sequence and the three-dimensional structure at atomic resolution have been known for many years. It was also shown that cytochrome *c* is bound to the outer surface of the inner mitochondrial membrane. As for the function of cytochrome *c*, biochemists believed that it is rather simple, namely to transport electrons from cytochrome c_1 to cytochrome oxidase. This transport per se is not coupled to energy transduction so the role of cytochrome *c* seems to be restricted to the function of a shuttle connecting two respiratory chain energy transducers, i.e. the bc_1 complex (complex III) and cytochrome oxidase (complex IV) (for review, see [5]).

The dogma of apparent simplicity of the cytochrome *c* function was shaken in 1996 when Wang and co-workers [6] discovered that this protein is somehow involved in apoptosis. As a model of apoptosis, treatment of cell-free extract from HeLa cells with dATP was used. It was found that the addition of dATP to the extract resulted in typical apoptotic changes, i.e. (i) cleavage of the caspase-3 precursor yielding active caspase-3 (an apoptosis-linked protease), (ii) nuclear DNA fragmentation and (iii) specific morphological changes in the cell nucleus. Fractionation of the extract revealed the existence of at least two factors necessary to the above effect. Cytochrome *c* proved to be one of them. In other experiments it was shown that treatment of HeLa cells with staurosporine, a potent pro-apoptotic agent, causes release of cytochrome *c* from mitochondria to cytosol. Similar relationships were revealed in human embryonic and monoblastic cell lines.

In 1997, the above observations were confirmed and extended by Wang's [7] and Newmeyer's [8,9] groups. It ap-

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Abbreviations: $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical H^+ potential difference; $\Delta\Psi$, transmembrane electric potential difference; AIF, apoptosis-inducing factor; Apaf-1 and Apaf-3, apoptosis-activating factors 1 and 3; ICE, interleukin-converting enzyme; PTP, permeability transition pore; ROS, reactive oxygen species; Z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethylketone

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peared that staurosporine-induced release of cytochrome *c* to cytosol of human acute myeloid leukemia cells is blocked by overexpression of the anti-apoptotic Bcl-2 protein which is present in the outer mitochondrial membrane [7]. Moreover, it was found that cytochrome *c* did not leave mitochondria treated with hypotonic (150 mM) sucrose provided that the Bcl-2 superproducing cell line was used [7].

Newmeyer's group has reported the release of cytochrome *c* from mitochondria of the CEM cell line treated with some apoptogens (ultraviolet treatment, etoposide, staurosporine, actinomycin D or H_2O_2) and its retention in the Bcl-2-overproducing line. Moreover, addition of the baculovirus-expressed Bcl-2 to *Xenopus* egg mitochondria slowed down the release of cytochrome *c* from these mitochondria incubated in the presence of cytosol. The caspase-3 activation by combined action of a cytosolic fraction and purified cytochrome *c* was confirmed [8]. Cytochrome *c* preparations from various vertebrate species, but not from *Saccharomyces cerevisiae*, were able to initiate all signs of apoptosis. Cu- and Zn-substituted cytochrome *c* had strong pro-apoptotic activity although they were inactive as electron carriers [9].

Quite recently, Wang and co-authors [10,11] identified the cytosolic partner of cytochrome *c* in the apoptotic cascade. This proved to be a protein showing significant sequence homology to Ced-4, a *Caenorhabditis elegans* factor activating pro-caspase-3. The protein, called apoptosis-activating factor 1 (Apaf-1), possesses a nucleotide binding site apparently responsible for binding of dATP, a cofactor of apoptosis. It was shown that addition of (i) Apaf-1, (ii) cytochrome *c* (previously Apaf-2) and (iii) dATP to pro-caspase-9 (previously Apaf-3) results in conversion of pro-caspase-9 to active caspase-9. Caspase-9 causes cleavage of pro-caspase-3 to caspase-3, the key enzyme involved in apoptosis (see below, Fig. 1).

In the above mentioned publications of Wang and Newmeyer, it was stressed that cytochrome *c* release from mitochondria is not accompanied by a mitochondrial $\Delta\Psi$ collapse [7,8]. On the other hand, Wang and coworkers [12] showed that apoptosis induced by the anticancer drug arabinosylcytosine (Ara-C) in human myeloid leukemia HL-60 cells, also related to cytochrome *c* release, causes the loss of $\Delta\Psi$. All these effects were inhibited by overexpression of Bcl-x_L, a very close relative of Bcl-2, possessing, like Bcl-2, anti-apoptotic activity.

According to Wang and Studzinski [13], in the same type of cells the apoptosis-inducing release of cytochrome *c* is blocked by 1,2,5-dihydroxyvitamin D_3. The effect was shown to be mediated by the anti-apoptotic protein Mcl-1 rather than by Bcl-2 or Bcl-x_L. The vitamin increased in mitochondria the levels of Mcl-1 as well as of the proliferation-associated protein Raf-1.

Kufe and his colleagues [14] reported that a human myeloid leukemia line overexpressing Bcl-x_L failed to (i) release cytochrome *c* from mitochondria to cytosol and (ii) undergo apoptosis in response to such apoptogenic treatment as ionizing radiation. Further analysis revealed the existence of a complex of Bcl-x_L and cytochrome *c* both in the irradiated and in non-treated cells. No such complex was found when pro-apoptotic Bcl-x_S, a Bcl-x_L derivative of a shorter length, was studied. A complex of Bcl-x_L and cytochrome *c* was also found when purified recombinant hexa-His-Bcl-x_L bound to a nitrocellulose filter was treated with bovine heart cytochrome *c* for 1 h. In this system, Bcl-x_S was found to prevent formation of the Bcl-x_L-cytochrome *c* complex. Experiments using an immunogold labeling method have localized Bcl-x_L to mitochondria in the Bcl-x_L-overproducing cells.

Independently, Manon and co-authors [15] analyzed yeast cells expressing human Bcl-x_L and Bax, another member of the Bcl-2 family which, like Bcl-x_S, shows pro-apoptotic activity. In the Bax-expressing cells, two effects were found, namely a decrease in the amount of cytochrome oxidase and a dramatic release of cytochrome *c* to the cytosol. On the other hand, the bc_1 complex and H^+-ATPase were unaffected. Coexpression of Bcl-x_L almost fully prevented the effects of Bax.

In 1996, the X-ray structure of Bcl-x_L was published by Muchmore et al. [16]. It proved to be strikingly similar to the pore-forming subunit of diphtheria toxin, which transports the ADP-ribosylating subunit of the toxin across the cell membrane (for discussion, see [17]). In 1997, it was reported that both anti- and pro-apoptotic proteins (Bcl-2, Bcl-x_L [18,19] and Bax [17,19], respectively) can form 20–300 pS single channels in model planar phospholipid membranes. Under some conditions, the channels become much larger (>1 nS) [17,19]. Bcl-2 and Bax showed mild cation and anion selectivities, respectively. The P_{K^+}/P_{Cl^-} values were 2.4 for Bcl-2 and 0.32 for Bax [19]. Incorporation of both proteins into the membrane took place at acidic pH only (just as in the case of diphtheria toxin) [19].

The X-ray and planar membrane data may be of crucial importance for understanding the whole Bcl-2 story. Let us assume that pro-apoptotic members of the Bcl-2 family such as Bcl-x_S or Bax allow cytochrome *c* to escape from the mitochondrial intermembrane space. This might be done by formation of a cytochrome *c*-specific transport pathway including (i) loose binding of cytochrome *c* with, e.g., Bax on the inner surface of the outer mitochondrial membrane, (ii) its translocation across the membrane via the diphtheria toxin-like part of Bax, and (iii) release of cytochrome *c* on the outer surface of the outer membrane. However, according to P. Nicotera (personal communication), not only cytochrome *c* but also adenylate kinase is released from the mitochondrial intermembrane space at an early step of apoptosis. Thus Bax apparently forms a non-specific large pore permeable for proteins. It is also possible that Bax breaks down the outer mitochondrial membrane, directly affecting it like digitonin or forming pores in the inner membrane, resulting in swelling of the mitochondrial matrix and, as a consequence, in disruption of the outer membrane.

As for the anti-apoptotic proteins (Bcl-2, Bcl-x_L), they fail to permeabilize the outer membrane for cytochrome *c* but can tightly bind this cytochrome. In other words, their membrane-affecting activity seems to be suppressed whereas the cytochrome *c* binding activity is increased compared with pro-apoptotic members of the same protein family.

Apparently both the pro- and anti-apoptotic proteins in question are competent in formation of oligomers (this effect might be essential for formation of a large channel). Since all of them are rather similar structurally, heterogenic oligomers (e.g. containing Bcl-2 and Bax) can also be formed. The oligomer-forming partners are inactive. This could explain the Bax-Bcl-2 crosstalk (e.g. [15]).

The real situation appears to be even more complex if we take into account that mechanisms of apoptosis may, to some degree, depend upon the type of the apoptosis-inducing stim-

ulus. Gottlieb and coworkers [20,21] reported that apoptosis caused by antibodies to Fas protein, in contrast to that caused by H_2O_2, staurosporine and some other stimuli used by Wang and Newmeyer, is not accompanied by cytochrome *c* release from mitochondria. Nevertheless, mitochondrial respiration is blocked at the cytochrome *c* level. The inhibition seems to be induced by the so-called post-mitochondrial fraction whose activity (i) was arrested by an ICE-protease inhibitor, carbobenzoxy-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), and (ii) required the outer mitochondrial membrane to be permeabilized by digitonin or the incubation medium to be acidified to pH 6.8. Addition of cytochrome *c* to the digitonin-treated mitochondria, inhibited by the 'post-mitochondrial fraction', restored the normal respiration rate.

Unfortunately, the nature of the respiration-inhibiting factor(s) remain obscure. The above-mentioned 'post-mitochondrial fraction' was prepared as follows. Homogenate obtained by nitrogen cavitation of Jurkat cells suspended in a solution of 100 mM sucrose, 5% Percoll and some other ingredients, was centrifuged at $2500\times g$ for 5 min. Centrifugation of the $2500\times g$ supernatant ($10\,000\times g$, 15 min) provided (i) a pellet called 'mitochondrial fraction' and (ii) the supernatant ('post-mitochondrial fraction'). The 'mitochondrial fraction' was found to contain about two thirds of the cytochrome oxidase pool of the homogenate whereas one third of it was localized in the 'post-mitochondrial fraction'. This means that the 'post-mitochondrial fraction' contained a rather large portion of the mitochondrial inner membranes, most probably as swollen ('light') mitochondria which did not precipitate at $10\,000\times g$ in the sucrose-Percoll medium used.

Further studies of the same group showed that the 'post-mitochondrial fraction' from CEM cells that overexpress Bcl-2 both prevents and reverses inhibition of mitochondrial respiration by the 'post-mitochondrial fraction' from Fas antibody-treated Jurkat cells [21]. This reversibility indicates that in the inhibited mitochondria, cytochrome *c* does not go far from the inner mitochondrial membrane. One possibility is that it is released from the outer surface of the inner membrane, crosses the intermembrane space and combines with Bax on the inner surface of the outer membrane.

It should be stressed that the effect described by Gottlieb's group represents a very early apoptotic event. A 5 min treatment of the Jurkat cells with anti-Fas antibodies proved to be sufficient to initiate some inhibitory activity of the 'post-mitochondrial fraction' [21].

Release of cytochrome *c* to cytosol might be the next step of the process. Formally speaking, this speculation is inconsistent with the facts that Z-VAD-fmk fails to prevent cytochrome *c* release to cytosol [8] whereas respiratory inhibition by the 'post-mitochondrial fraction' is abolished by this protease inhibitor [21]. However, this discrepancy may be due to the fact that different apoptogenic stimuli were used by Kluck et al. [8] and Adachi et al. [21]. The role of the Z-VAD-fmk-sensitive protease is unclear. This is hardly hydrolysis of the cytochrome *c* polypeptide chain since (i) the inhibition of respiration is reversible [21] and (ii) the spectrum of cytochrome *c* in the inhibited mitochondria is not changed [20]. It seems possible that the above protease is identical to AIF which is also sensitive to Z-VAD-fmk [3,4]. AIF has been shown to be released from mitochondria due to swelling caused by the PTP opening [2–4] so the fraction of light mitochondria could be the source of AIF. An effect of AIF added to mitochondria was already described by Kroemer's group. It was found that the added AIF stimulates the opening of the PTP [4].

3. Place of cytochrome *c* in the antioxidant system of the cell

As already mentioned in Section 1, apoptosis can be initiated by an increase in the concentration of ROS (for reviews, see [22,23]). Apparently, for aerobic organisms, ROS-linked apoptosis is the last line of antioxidant defence at the cellular level. This process was postulated to purify the cell population from those cells which proved to be dangerous for the tissue since they become, for some reason, ROS superproducers [22–24]. Since mitochondria generate, as a rule, the main portion of ROS in the cell [1,22–24], it is not surprising that AIF, for example, specialized in mediating apoptosis, is localized in mitochondria. As for cytochrome *c*, it originally specialized in a function other than apoptosis. Nevertheless, its role in this process may, in my opinion, be understood within the framework of the same antioxidant concept.

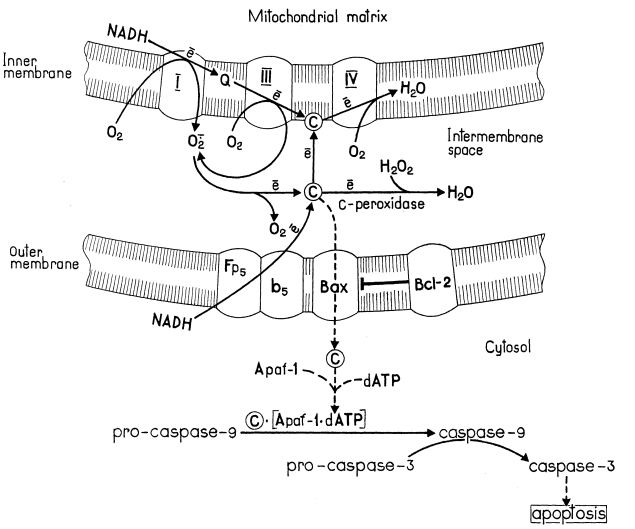

Fig. 1. Possible role of cytochrome *c* in the antioxidant defence system of mitochondria and in apoptosis. I, III and IV, corresponding respiratory chain complexes (NADH-CoQ reductase, $CoQH_2$-cytochrome *c* reductase and cytochrome *c* oxidase, respectively); *c* and b_5, cytochrome *c* and cytochrome b_5, respectively; Fp_5, NADH-cytochrome b_5 reductase; Bcl-2 and Bax, anti- and pro-apoptotic proteins of the Bcl-2 family, respectively; Apaf-1, apoptosis-activating factor 1. It is assumed that cytochrome *c*, desorbed from the inner mitochondrial membrane to the intermembrane space, oxidizes superoxide which is produced by complexes I and III. Moreover, it receives electrons from an external respiratory chain (Fp_5 and b_5) localized in the outer mitochondrial membrane, bypassing the $O_2^{-\bullet}$-producing complexes I and III. Cytochrome *c* reduced by $O_2^{-\bullet}$ or by b_5 is reoxidized by cytochrome oxidase or cytochrome *c* peroxidase. If these mechanisms fail to prevent large-scale $O_2^{-\bullet}$ production, Bax-mediated release of cytochrome *c* to the cytosol is assumed to be initiated. This release may be due to cytochrome *c* translocation by Bax or, more probably, to the Bax-mediated disruption of the outer mitochondrial membrane. Cytosolic cytochrome *c* activates the apoptotic cascade so the $O_2^{-\bullet}$-superproducing cell is discarded by apoptosis. To simplify the scheme, I did not show the ROS-induced PTP opening and mitochondrial swelling, the event also resulting in disruption of the outer membrane of mitochondria. AIF, another intermembrane pro-apoptotic protein, is also omitted. It is shown that Bcl-2 combines with Bax and inactivates it. Moreover, Bcl-2 may apparently attenuate apoptosis by (i) preventing opening of the PTP and (ii) binding of released cytochrome *c*.

The role of cytochrome *c* in the antioxidant system of mitochondria and in apoptosis is illustrated in Fig. 1. The following processes possibly involved in the cytochrome *c*-linked defence mechanism are indicated in the figure.

(1) Oxidation by cytochrome c of superoxide which is generated by complexes I and III of the respiratory chain. As a result cytochrome *c* is reduced and $O_2^{-\bullet}$ is converted back to O_2. The reduced cytochrome *c* is then oxidized by complex IV, resulting in the formation of H_2O from O_2.

In yeast, cytochrome *c* peroxidase localized in the intermembrane space [25] can substitute for cytochrome oxidase as oxidant of the $O_2^{-\bullet}$-reduced cytochrome *c*. This enzyme is also found in the periplasmic space of some aerobic bacteria (for references, see [26]). The cytochrome *c*-cytochrome *c* peroxidase system appears to be a scavenger simultaneously for both $O_2^{-\bullet}$ and H_2O_2:

$$2\mathrm{Cyt.c}^{3+} + 2O_2^{-\bullet} \rightarrow 2\mathrm{Cyt.c}^{2+} + 2O_2 \quad (1)$$

$$2\mathrm{Cyt.c}^{2+} + H_2O_2 + 2H^+ \rightarrow 2\mathrm{Cyt.c}^{3+} + 2H_2O \quad (2)$$

In an anaerobically grown, respiratory chain-deficient mutant of yeast, lacking cytochrome *c*, cytochrome *c* peroxidase and other respiratory enzymes, aeration results in immediate and specific induction of synthesis of cytochrome *c* and cytochrome *c* peroxidase. Later catalase is also induced [27–29].

The ability of soluble cytochrome *c* to oxidize $O_2^{-\bullet}$ to O_2 is widely used by researchers as a method of $O_2^{-\bullet}$ measurement. Apparently, to oxidize $O_2^{-\bullet}$ at a high rate in mitochondria, cytochrome *c* should be released to the intermembrane solution. As for cytochrome *c* bound to complexes III or IV, it is hardly competent in fast oxidation of $O_2^{-\bullet}$ just as of ascorbate, another hydrophilic reductant of soluble cytochrome *c*. In line with this suggestion, we recently found that addition of cytochrome *c* to mitochondria in the resting state (state 4) strongly suppresses the formation of H_2O_2 (R.A. Simonyan, V.P. Skulachev and A.A. Starkov, in preparation).

(2) The cytochrome c-mediated oxidation of external NADH by complex IV via NADH-cytochrome b_5 reductase and cytochrome b_5. This energy non-coupled pathway localized in the outer mitochondrial membrane (for review, see [1]) bypasses the initial and middle segments of the respiratory chain which produce $O_2^{-\bullet}$ in state 4 where ADP is exhausted [30]. Just these segments are known to be targets for antimycin A, rotenone and a large number of hydrophobic xenobiotics. Inhibition of the respiratory chain by these agents strongly increases $O_2^{-\bullet}$ production by mitochondria [22,30–32]. Activation of the external electron transport pathway overcomes the inhibition and, hence, should decrease the $O_2^{-\bullet}$ formation.

Release of cytochrome *c* to the intermembrane space of liver mitochondria caused by cold exposure of rats was described in our group by Mokhova and co-authors [33,34]. This observation exemplifies the case when mitochondria in vivo regulate affinity of cytochrome *c* to the inner membrane respiratory chain complexes and its concentration in the intermembrane space.

The cytochrome b_5-mediated electron transport pathway is inherent in liver and, to a lesser degree, in kidney and some other tissues. It is absent from muscles which possess another non-coupled external NADH oxidation system of unknown mechanism (for review, see [5]).

(3) The cytochrome c desorption to the intermembrane space can decrease the superoxide production activated by inhibition of the respiratory chain at the antimycin A-sensitive site. This effect was first described by Turrens et al. [35] and was recently reproduced in this group (Simonyan and Starkov, unpublished). It can be explained by inhibition of oxidation of the complex III FeS cluster (and, hence, inhibition of the $CoQH_2$ oxidation to $CoQH^\bullet$) due to removal of cytochrome *c* from the inner membrane. This effect resembles the myxothiasol inhibition of $O_2^{-\bullet}$ generation in antimycin A-treated mitochondria [22,32].

(4) The Bax- or/and PTP-mediated release of cytochrome c to the cytosol, in cooperation with some cytosolic factors, induces apoptosis. It is not clear yet whether the effect of Bax could be triggered by an increase of the ROS concentration in the cell. However, it has already been proved that ROS induce opening of the PTP (for reviews, see [22,23,36]) and Bcl-2 inhibits this effect [4].

Cytosolic cytochrome *c* can, in principle, be reduced by $O_2^{-\bullet}$ in the cytosol and then can be oxidized by cytochrome oxidase provided that the outer mitochondrial membrane is broken or cytochrome *c*-permeable. However, this process is apparently of low rate because of great dilution of cytochrome *c* in the cytosol.

Desorption of cytochrome *c* from the inner mitochondrial membrane is of crucial importance for the scheme shown in Fig. 1. This process was found in our group to occur under some physiological conditions related to stimulation of oxidative metabolism. In liver, this takes place in response to cold exposure of rats (see above). Interestingly, this process is greatly stimulated by thyroid hormones [34]. According to our hypothesis [22–24,37], thyroid hormones stimulate the antioxidant system of the cell to compensate for an increase in ROS production due to up-regulation of respiratory metabolism which is induced by the same hormones. The following potential antioxidant effects of thyroid hormones can be mentioned:

1. Increase in non-ohmicity of the inner mitochondrial membrane (effect of mild uncoupling occurring at high $\Delta\bar{\mu}_{H^+}$) [38,39] which should prevent a strong increase in the rate of ROS production by mitochondria in the resting state [30]. At least partially this effect may be due to induction by thyroid hormones of synthesis of (i) the uncoupler protein-2 [40] and (ii) the mitochondrial ATP/ADP antiporter [41], an anion carrier involved in uncoupling by fatty acids [5,42–44].
2. The PTP opening in a cyclosporin A-insensitive fashion [45].
3. Induction of synthesis of mitochondrial α-glycerophosphate dehydrogenase [46,47] and cytosolic malic enzyme [46,48], events stimulating the flux of reducing equivalents from mitochondria to cytosol and a bypass of initial steps of the coupled respiratory chain (complex I) which is especially active in generation of ROS.
4. Induction of synthesis of cytochrome *c* [49]. This effect is strong in liver and less strong in kidney, which correlates with the amount of cytochrome b_5 in these tissues.

Stimulation of release of the membrane-bound cytochrome *c* to the intermembrane space [34] might represent one more antioxidant effect of thyroid hormones.

In conclusion, the pro-apoptotic effect of cytochrome *c* may

be regarded as one of the antioxidant functions inherent in this protein. This can explain why cytochrome *c* proved to be involved in apoptosis. An alternative possibility is that cytochrome *c* was randomly selected by evolution as a mitochondrial pro-apoptotic factor.

4. Addendum

Quite recently Vander Heiden et al. [50] reported in *Cell* that not only staurosporine but also anti-Fas antibodies cause release of all the cytochrome *c* pool from mitochondria to cytosol. This observation contradicts the above-mentioned publication of Gottlieb's group. The reason for this discrepancy is unclear.

In the same paper [50], it was reported that Bcl-2 completely prevents the cell death induced by oligomycin and antimycin A treatments. Inhibition of respiration by these two agents was not affected by Bcl-x_L although Bcl-x_L prevented release of cytochrome *c*. This means that oligomycin and antimycin A kill the cell indirectly, due to induction of apoptosis rather than directly, i.e. because of inhibition of H^+-ATPase and complex III, respectively. The well-known ability of oligomycin and antimycin A to increase the ROS production by mitochondria may give rise to apoptosis in these experiments.

According to Vander Heiden et al. [50], apoptosis allows antibodies to subunit IV of cytochrome oxidase to reach their target which is unavailable in control cells. This fact points to disruption of the outer mitochondrial membrane in apoptotic cells.

Unfortunately the authors of this interesting publication tried to measure mitochondrial $\Delta\Psi$, using rhodamine-123 in all the experiments shown in the figures. This probe, in contrast to another probe JC-1, fails to monitor mitochondrial $\Delta\Psi$ in intact cells [51]. As a results, the authors came to some confusing conclusions (like the assumption that "Bcl-2 ... regulates the accumulation of hydrogen ions in the intermembrane space" (by forming "an ion channel in the outer membrane"), etc.

These conclusions were used by Reed in the same issue of *Cell* [52] as a basis for the hypothesis that the pro-apoptotic Bax activates K^+ influx into mitochondria via the K^+ uniporter whereas the anti-apoptotic Bcl-x_L prevents K^+ influx by activating the K^+/H^+ antiporter. However, there is no evidence that the above-mentioned porters are really involved. Even more, cooperation of the K^+ uniporter and the K^+/H^+ antiporter must result in complete uncoupling and collapse of $\Delta\Psi$, which hardly occurs in the Bcl-x_L-overproducing cells.

In his article, Reed also mentioned that "experiments using irreversible pan-caspase inhibitory peptides have provided evidence that apoptosis induced by growth factor deprivation, anticancer drugs, and Bax overexpression is potentially inhibited when caspases are inactivated but cell death is not stopped. Under these circumstances, cytochrome *c* is still released, $\Delta\Psi$ drops, ROS are generated and the cell dies by a caspase-independent mechanism that resembles necrosis. Thus release of cytochrome *c* from mitochondria has two ways of killing cells: (a) by activating caspases via Apaf-1, and (b) by interrupting electron transport chain, thus preventing oxidative phosphorylation, promoting free-radical production, and eventually depriving the cell of ATP" [52]. This means that we should distinguish two cytochrome *c*-related phenomena:

1. Immediate effects of release of cytochrome *c* from the inner mitochondrial membrane, an event decreasing, as we suggest, the level of ROS; if, nevertheless, the ROS level increases, it causes apoptosis provided that caspase activation is possible.
2. Remote consequences of the respiratory chain inhibition due to cytochrome *c* release.

In case (2) an increase in ROS formation may occur due to inhibition of respiration and exhaustion of energy sources supporting maintenance of high levels of antioxidants in the cell.

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