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Toxicology 208 (2005) 259–271
TOXICOLOGY
www.elsevier.com/locate/toxicol

An epigrammatic (abridged) recounting of the myriad tales of astonishing deeds and dire consequences pertaining to nitric oxide and reactive oxygen species in mitochondria with an ancillary missive concerning the origins of apoptosis

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Abstract

Mitochondria play a central role in the life and death of cells. These organelles serve as the major energy-producing powerhouse, whereby the generation of ATP is associated with the utilization of molecular oxygen. A significant fraction (2–3%) of molecular oxygen consumed by mitochondria may be reduced in a one-electron fashion to yield a series of reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical. ROS are capable of damaging components of the electron transport apparatus and can, in turn, disrupt mitochondrial functioning, limiting cellular ATP levels and ultimately resulting in cell death. ROS-induced disruption of electron transport can perpetuate production of deleterious ROS and propagate mitochondrial damage. Consequently, mitochondria are highly enriched with water-soluble and lipid-soluble antioxidants (glutathione, ascorbate, Vitamin E, and coenzyme Q) and antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, catalase, thioredoxins, and peroxiredoxin. Another important antioxidant acting as a very effective scavenger of reactive lipid (peroxyl, alkoxyl) radicals is nitric oxide (NO) generated by mitochondrial nitric oxide synthase. However, NO can also be very disruptive to mitochondria function, a process facilitated by its high reactivity with superoxide. This interaction results in the formation of peroxynitrite, an oxidant capable of causing oxidative/nitrosative stress, further aggravating mitochondrial dysfunction, causing ATP depletion and damage to cells. Thus, in the most general sense, the effects of NO in mitochondria may be either protective or deleterious depending on specific conditions of local redox environment (redox potential, ratio of oxidized to reduced glutathione, transition metals, and the presence of other oxygen- and nitrogen-centered radicals).

Keywords: Apoptosis; Phosphatidylserine; Nitric oxide

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0300-483X/$ – see front matter © 2004 Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2004.11.027

1. Mitochondria in the apoptotic process: life in the belly of the beast

It is becoming increasingly apparent that mitochondria play a key role in mediating apoptosis (for reviews see Kuwana and Newmeyer, 2003; Saelens et al., 2004). Apoptosis in response to cellular stress occurs through a process, which results in the release of pro-apoptotic proteins from the mitochondrial intermembrane space (Kuwana et al., 2002). These pro-apoptotic proteins initiate cell death by a cascade of events that are either caspase-dependent or caspase-independent and include cytochrome *c*, apoptosis-inducing factor (AIF), endonuclease G, Smac/Diablo (second mitochondrial-derived activator of caspase/direct IAP-binding protein with low PI) and the serine protease HtrA2/Omi (high-temperature requirement protein A2). Both reactive oxygen species (ROS) and nitric oxide (NO) are known to be critical mediators of the apoptotic process and their actions in mitochondria have been studied extensively (Moncada and Erusalimsky, 2002; Ueda et al., 2002; Cadenas, 2004; Juhaszova et al., 2004). Whether ROS or NO facilitate or prevent apoptosis is highly dependent on the intracellular redox balance in cells and, in particular, within mitochondria. The overall redox state is determined by the presence of both water- and lipid-soluble antioxidants including oxidized and reduced glutathione, ascorbate, Vitamin E, and coenzyme Q, and antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, catalase, thioredoxins, and peroxiredoxins (Inoue et al., 2003; Fernandez-Checa, 2003; Landar and Darley-Usmar, 2003; Banmeyer et al., 2004). The toxicity of both ROS and NO is also dependent on the presence and accessibility of transition metals as well as other oxygen- and nitrogen-centered radicals (Radi et al., 2002; Fillebeen and Pantopoulos, 2002; Boyd and Cadenas, 2002; Quinlan et al., 2002). However, due to its free radical nature, NO can exert dual actions in cells acting as an antioxidant by scavenging peroxyl and alkoxyl lipid radicals (Ebadi and Sharma, 2003; Kagan et al., 2003), or by functioning as a pro-oxidant by reacting with superoxide anion to form peroxynitrite (Ebadi and Sharma, 2003; Dedon and Tannenbaum, 2004). This long-lived and highly reactive oxidant has been found to readily damage mitochondria (Levonen et al., 2001). The well-established correlation of beneficial or deleterious effects with local levels of NO make it tempting to speculate that the concentration of NO determines its overall effects. In this regard, relatively low physiological levels of NO are frequently associated with its regulatory and protective (antioxidant) properties, whereas excessive formation of ROS and NO cause dysregulation of mitochondrial function. Recent discoveries elucidating the biochemical mechanisms that mediate the effects of NO in mitochondria more clearly explain the physiological or potential pathophysiological actions of this reactive intermediate.

2. A loyal servant

It is now known that NO can play a pivotal role in regulating normal mitochondrial functioning. This is based on the findings that NO readily binds to and modulates the function of cytochrome *c* oxidase, also known as complex IV in the electron transport chain. Cytochrome *c* oxidase was first identified as a potential target for intracellular NO more than 30 years ago (Gibson and Greenwood, 1965). This enzyme is a critical regulator of mitochondrial respiration, controlling its activity by catalyzing the reduction of oxygen into water in the final stage of electron transport. It is well established that NO reversibly inhibits cytochrome *c* oxidase by competing with oxygen for the binuclear-binding site (Rubbo et al., 2000; Boyd and Cadenas, 2002; Ebadi and Sharma, 2003; Palacios-Callender et al., 2004). However, until recently the dependency of the NO–cytochrome *c* oxidase interaction on oxygen tension obscured this fundamental physiological mechanism. In an elegant series of experiments, it was demonstrated that, at low oxygen tensions, the production of NO via endothelial nitric oxide synthase in respiring endothelial cells in response to physiological mediators results in a decrease in the rate of oxygen consumption, an effect that is reversed by an inhibitor of nitric oxide synthase (Fig. 1; Borutaite et al., 2001; Ramachandran et al., 2002). These experiments clearly established that, in oxygen poor environments, similar to those found in the distal vasculature, due to the extremely high affinity of NO for cytochrome *c* oxidase, even the nanomolar concentrations of NO generated in tissues under basal conditions are likely to be important in the regulation of oxygen consumption. Although it has long been held that the control of oxygen consumption by mitochondria is largely (∼50%)

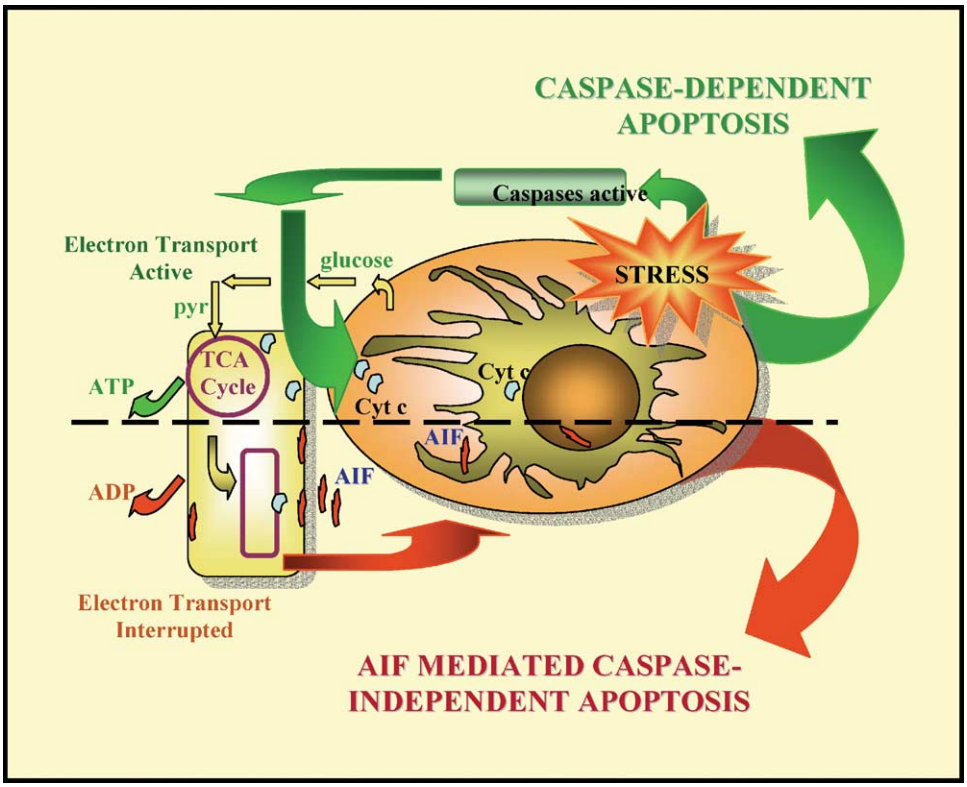

Fig. 1. Two distinct routes to apoptosis. Life-threatening cellular stress may initiate a cascade of events mediated by the activity of the caspase proteases that ultimately converge on the mitochondria causing the release of cytochrome *c* and irreversibly committing the cell to death (upper pathway, green arrows). Alternatively, mitochondrial lesions leading to the interruption of electron transport may also initiate an apoptotic cascade. In this pathway, the release of apoptosis-inducing factor (AIF) and related mediators from the mitochondria activates cellular responses also culminating in the release of cytochrome *c*, DNA fragmentation and cell death (lower pathway, red arrows). The mitochondria in the left is enlarged in this schematic to portray the organelle's dependency on cytosolic substrates. Insufficiencies in glucose-derived pyruvate or molecular oxygen may also inhibit oxidative respiration and result in caspase-independent apoptosis.

exerted by ATP-turnover reactions, through the interaction between oxygen and NO, cytochrome *c* oxidase also exerts considerable control over the rate of respiration through pO_2-dependent changes in its K_m for NO (Moncada and Erusalimsky, 2002). In this regard, while cytochrome *c* oxidase has a relatively low affinity for NO at high concentrations of oxygen, in an oxygen poor environment, the affinity for NO rises dramatically. In addition, interaction with NO, and reduction of the electron transport chain, increase the K_m of the enzyme for oxygen (Palacios-Callender et al., 2004). When NO is bound by cytochrome *c* oxidase, the terminal oxidase of the electron transport chain, electron transport is obstructed and respiration and oxygen consumption slow dramatically. The exquisite sensitivity of the oxidase to oxygen tension is an important regulatory mechanism for electron transport. Thus, lying proximal to the ATP-turnover reactions, cytochrome *c* oxidase acts to regulate respiration in response to the availability of oxygen (Moncada and Erusalimsky, 2002; Thomas et al., 2003; Nisoli et al., 2004).

Interestingly, in the presence of high concentrations of NO, this regulatory mechanism has the potential to fuel pathology. When electron transfer is interrupted at cytochrome *c* oxidase, significant amounts of superoxide are produced (Wenzel et al., 2003; Carreras et al., 2004; Duchen, 2004; Tyurina et al., 2004a). In the presence of NO, superoxide is rapidly converted to peroxynitrite. Initially, peroxynitrite formation effectively removes NO allowing cytochrome *c* oxidase to bind

oxygen and resume respiratory activity. However, when excessive quantities of NO are generated during inflammation or neuronal hyper-stimulation, significant quantities of potentially tissue injuring peroxynitrite may be formed (Fig. 1; Souza et al., 2001; Dedon and Tannenbaum, 2004; Keynes and Garthwaite, 2004).

3. The garnerer of graves

High levels of NO can obstruct mitochondrial respiration at several other sites in the electron-transport chain (Ramachandran et al., 2002). For example, NO has been found to effectively inhibit complex I. Three potentially important pathways may be involved in this inhibition: (i) nitrosylation of protein cysteines; (ii) formation of iron–nitrosyl complexes with Fe–S centers; and (iii) damage of a sensitive site by peroxynitrite (Riobo et al., 2001). Coenzyme Q, a mediator active in electron transport between complexes I, II and complex III, is also sensitive to NO (Poderoso et al., 1999). This entails direct oxidation of fully reduced ubiquinol by NO to an ubisemiquinone radical. This latter intermediate can propagate oxidative stress by acting as an electron donor for molecular oxygen to produce superoxide. In addition, when levels of NO are elevated, blockage of mitochondrial complex III is also likely to occur. In this instance, NO inhibits respiration through nitrosylation of cytochrome *b* heme. Furthermore, as indicated above, NO can directly bind to the binuclear center of complex IV (cytochrome *c* oxidase) precluding binding of oxygen and causing inhibition of respiration (Cleeter et al., 1994). Inhibition of respiratory complexes is associated with their reduction and resultant production of superoxide, which can dismutate to yield hydrogen peroxide or react with NO forming peroxynitrite. Both of these potent oxidants can serve as a source of oxidizing equivalents to feed the peroxidase reactions that mediate apoptosis (see further below).

4. Pathways to death

Within the past decade the work of numerous investigators has led to the identification of many events and signals leading to apoptosis (Kuwana and Newmeyer, 2003; Saelens et al., 2004). A schematic outlining these events is presented in Fig. 2. In this model, cellular injury results in activation of the pro-apoptotic Bcl2 family of signaling molecules, the production of ROS and/or alterations in calcium homeostasis. Each of these pathways triggers mitochondrial release of pro-apoptotic molecules that initiate events leading to death (Duchen, 2004; Tsujimoto, 2003). One of the best-characterized pathways involves the release of cytochrome *c* and activation of the caspase family of proteases (for reviews, see Ceccatelli et al., 2004; Salvesen and Abrams, 2004). Upon release into the cytoplasm, cytochrome *c* activates Apaf-1, which initiates the caspase cascade. Another key event in this pathway is caspase-3-mediated cleavage of the protein inhibitor of caspase-activated DNase (ICAD). The resultant entry of caspase-activated DNase (CAD) into the nucleus results in oligonucleosomal DNA fragmentation (Nimmanapalli and Bhalla, 2003; Annunziato et al., 2003; Thorburn, 2004). As indicated in the Introduction, many additional pro-apoptotic factors are released by mitochondria in response to apoptotic signals including Smac/Diablo and HtrA2/Omi. It is intriguing that HtrA2/Omi also possesses a serine protease activity and contributes to another path leading to apoptosis, which is generally referred to as caspase-independent cell death (Vaux and Silke, 2003).

5. A clandestine assassin within

Another body of emerging evidence suggests that the mitochondrion itself is also able to initiate apoptosis. This caspase-independent pathway is mediated by the release of a distinct subset of mitochondrial factors such as AIF and endonuclease G (Cregan et al., 2004; Abraham and Shaham, 2004). In healthy cells, AIF is localized in the mitochondrial intermembrane space where, based on the presence of an FAD-binding domain in its N-terminal and apparent redox activity, it is thought to function as an oxidoreductase. However, similar to the bifunctional activity of cytochrome *c*, when AIF is released from mitochondria it translocates to the nucleus where it triggers chromatin condensation and nuclear fragmentation. Moreover, once released into the cytosol, AIF induces the production of ROS and initiates the expression of phosphatidylserine in the plasma membrane (Punj and Chakrabarty, 2003; Cregan et al., 2004).

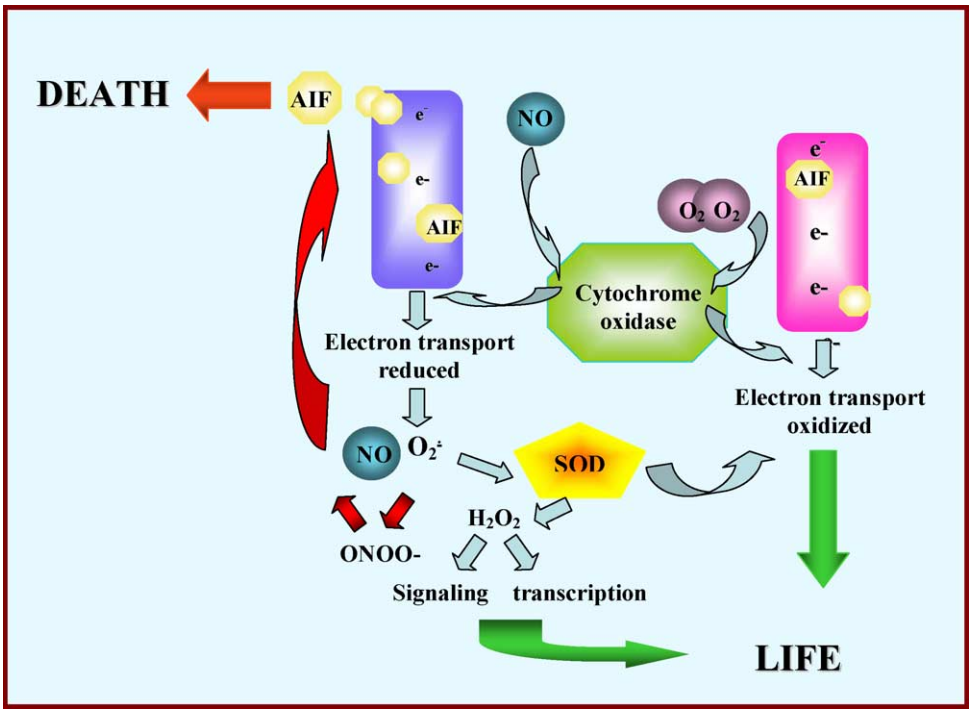

Fig. 2. Cytochrome *c* oxidase: guardian of life and death. Association of NO with cytochrome oxidase results in a reduction of the electron-transport chain, slowing oxidative metabolism and supporting the generation of superoxide anions. In the low levels of oxygen encountered in the distal vasculature, the reduced character of the electron transport apparatus facilitates association of cytochrome oxidase with oxygen, and oxidative respiration is resumed. Under these conditions, the enzyme superoxide dismutase converts superoxide anions into hydrogen peroxide removing the potentially toxic radical. This diffusible reactive intermediate can activate signaling pathways modulating the proteome and ultimately leading to cellular protection. In the presence of high levels of NO, as well as when oxygen is limiting, the formation of peroxynitrite from the reaction between superoxide anions and NO is favored. The peroxynitrite formed may interact with mitochondrial lipids and proteins resulting in macromolecular damage and lipid oxidation. Perturbations to the mitochondrial membranes lead to the release of AIF and other factors into the cytosol initiating caspase-independent routes to cell death.

While the nature and timing of the events leading to apoptosis are relatively well characterized, the specific mechanisms mediating these biochemical reactions are not well established (Zimmermann et al., 2001; Danial and Korsmeyer, 2004). It has recently been shown that through interactions with the members of Bcl-2 family members, the mitochondria-specific phospholipid cardiolipin is oxidized and this may play a central role in the regulation of mitochondrial transition pore formation (Newmeyer and Ferguson-Miller, 2003). This transition pore mediates the extensive mitochondrial swelling, depolarization of the inner mitochondrial membrane, and the uncoupling of oxidative phosphorylation that are hallmarks of apoptosis. These alterations results in the loss of ATP synthesis and ultimately, fragmenting of the mitochondria (Zamzami and Kroemer, 2001). The transition pore is thought to be controlled by a complex of molecules referred to as the permeability transition pore complex. Although the complete molecular composition of this pore is still unclear, some of the proteins of the core have been identified and include the mitochondrial voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophyllin D. The formation of this complex mediates opening of an ion channel in the inner mitochondrial membrane. This subsequently deflates the mitochondrial proton gradient and allows water to enter the matrix swelling the organelle and leading to the release of apoptosis-inducing proteins (Jacotot et al., 1999; Sharpe et al., 2004).

While complicated two-stage interactions of cytochrome *c* with cardiolipin have been shown to be crit-

ical to mitochondrial transition pore formation (Iverson and Orrenius, 2004), the catalytic mechanisms involved have not yet been fully described. However, one finding of particular interest is the determination that cytochrome *c* acts as a self-catalyst of its own release via stimulating cardiolipin oxidation. In these studies, interactions of cytochrome *c* with anionic phospholipids, particularly cardiolipin and phosphatidylserine, markedly altered the accessibility of cytochrome *c* heme to small molecules due to weakening of the bond between the heme and a methionine residue (Met_{80}; Tuominen et al., 2001, 2002). This results in activation of cytochrome *c* leading to the initiation of the enzyme's peroxidase activity (Zucchi et al., 2003). Hydrogen peroxide treatment of cardiolipin-containing liposomes preincubated with cytochrome *c*, or isolated liver mitochondria, results in selective oxidation of cardiolipin by the peroxidase activity of cytochrome *c*. Oxidation occurs through the formation of an oxoferryl species with a very high oxidizing potential (Kagan et al., 2003). Thus, a fraction of cytochrome *c* tightly bound to cardiolipin in the mitochondria is effectively a ready-to-act peroxidase. The triggering of this cytochrome-peroxidase depends on the availability of oxidizing equivalents such as hydrogen peroxide or peroxynitrite. This suggests that the pro-apoptotic function of cytochrome *c* may be initiated in mitochondria well before its release into the cytosol. Therefore, we have hypothesized that a redox catalytic step is required for the subsequent formation of the mitochondrial permeability pore, release of cytochrome *c*, apoptosome formation, and caspase activation (Jiang et al., 2003; Tyurina et al., 2004a,b; Kagan and Quinn, 2004). Interestingly, as a redox-active compound, NO may play an important role in regulating this catalytic step. In this regard, our previous work has characterized NO as a potent reductant of oxoferryl species of hemoproteins, and is capable of preventing peroxidation of phospholipids in cell membranes (Gorbunov et al., 1997; Yalowich et al., 1999). Potentially, acting as a reductant, NO may regulate cardiolipin oxidation in normally functioning mitochondria, preventing the pro-apoptotic effects of inadvertently generated hydrogen peroxide. However, this antioxidant function of NO is apparently not effective in the presence of pro-apoptotic signals to mitochondria since the presence of NO does not prevent disruption of mitochondrial electron transport and the formation of superoxide provoked by these mediators. Under these conditions, the reaction of NO with superoxide is favored over the competing reaction of NO with activated oxoferryl-species of cytochrome *c*. In this manner, NO-dependent control of cytochrome *c* peroxidase activity is diminished. Moreover, the subsequent accumulation of reactive intermediates including hydrogen peroxide and peroxynitrite creates conditions favoring activation of the peroxidase function of cytochrome *c*. Similar to many other circumstances in which NO exhibits its two-face Janus-like behavior, the role of NO as an anti-apoptotic regulator of cytochrome *c* peroxidase activity in normal mitochondria switches to that of an enhancer of pro-apoptotic cascades upon the cell's commitment to death (Jiang et al., 2003; Tyurina et al., 2004b; Kagan and Quinn, 2004).

6. The sword of Solomon

Living cells continually integrate stress-inducing signals from the extra cellular environment. Adaptation to stress and cellular survival is mediated by a wide range of survival signaling pathways that are controlled by kinases, ceramides, and lipid mediators (Sabri et al., 2003; Dickinson et al., 2003; Wada and Penninger, 2004). In a similar manner, regulated cell death via apoptosis is also mediated by distinct pathways which, as discussed above, comprise two broad groups, the death receptor conduit and the mitochondria-initiated pathways. Investigations focusing on mechanisms of programmed cell death have been provided new findings, which distinguish between the pathways. These studies demonstrated that that the divergent paths leading to apoptosis are intimately connected. Caspase-8-mediated cleavage of Bid, a member of the Bcl-2 protein family, has been identified as a common event linking the death-inducing signaling complex and AIF/mitochondrial pathways (Li et al., 1998; Roucou et al., 2002; Wei et al., 2004). Following cleavage, the truncated Bid (tBid) translocates to the mitochondria where it induces the release of cytochrome *c* irreversibly committing the cell to apoptosis. The mechanisms by which tBid rapidly and selectively localizes in the mitochondria are not known. However, a post-translational *N*-myristoylation of tBid following its cleavage by caspase-8 has been identified as an activating switch, targeting tBid to the outer mitochondrial

membrane. Additional studies indicate that cardiolipin also links the two death pathways through the mitochondrial targeting of tBid by binding to a specific domain in tBid. Interestingly, cardiolipin, a lipid enriched in the mitochondria, is found at high concentrations throughout the inner membrane, and in particular at the contact sites between the inner and outer membrane of this organelle. Immunogold tomography has revealed that these contact sites are the precise locations that are the preferential targets for association with tBid (Kim et al., 2004). In further support of these findings, it has recently been determined that in the presence of Bcl-2 or Bcl-xL, cleavage and translocation of tBid occur, but the release of cytochrome *c* is blocked (Kim et al., 2004). It is tempting to speculate that association of specific Bcl_2/Bcl-xL family members and cardiolipin regulates the association of cytochrome *c* with this lipid. In this scheme, cardiolipin–BCL_2 protein interaction is the mechanism underlying interruption of the apoptotic cascade by these proteins. However, this mechanism has yet to be validated. It is noteworthy, however, that modified molecular species of cardiolipin such as monolyso-cardiolipin have particularly high affinity for Bid (Esposti et al., 2003).

7. The slippery connection

Through its electrostatic/hydrophobic interactions with negatively charged cardiolipin, cytochrome *c* is activated to a peroxidase, leading to oxidation of cardiolipin and release of cytochrome *c* out of mitochondria into the cytosol (Kagan et al., 2003). As indicated above, cardiolipin is a unique lipid that is seldom found in the cytosol; in fact cardiolipin is rarely localized outside of mitochondria. However, we have recently determined that the activation of cytochrome *c* to a peroxidase can also occur in the cytosolic environment. Under these conditions, the peroxidase function of cytochrome *c* is activated through interactions with other anionic phospholipids including phosphatidylserine. In a manner similar to that of cardiolipin, phosphatidylserine triggers the peroxidase form of cytochrome *c* which, in turn, selectively catalyzes its own oxidation (Kagan et al., 2003). More importantly, this redox activity of phosphatidylserine-bound cytochrome *c* appears to be required for externalization of phosphatidylserine on the plasma membrane during intrinsic apoptosis. Because externalized phosphatidylserine acts as a universal recognition signal essential for effective tethering, engulfment, and phagocytosis of apoptotic cells by macrophages (Fadok, 2003), redox-dependent catalysis of phosphatidylserine oxidation is likely to be an important component of the clearance of apoptotic cells. Potentially, antioxidant protection of phosphatidylserine against peroxidation during apoptosis disrupts completion of the phosphatidylserine-segment of the apoptotic program causing deficiencies in phosphatidylserine-dependent signaling. As a result of the decreased externalization of phosphatidylserine and the resultant deficiency of oxidized phosphatidylserine on the surface of apoptotic cells, recognition of the apoptotic cell by phagocytes is impaired (Fadeel and Kagan, 2003; Tyurina et al., 2004b). Furthermore, by acting as an effective scavenger of oxidizing lipid radicals, NO can inhibit phosphatidylserine oxidation during apoptosis (Fabisiak et al., 2000). These findings imply that NO can block phosphatidylserine signaling and clearance of apoptotic cells by macrophages. Surprisingly, despite inhibition of phosphatidylserine oxidation, NO does not significantly affect egression of phosphatidylserine to the cell surface. This is predominantly due to direct inhibition by NO, presumably by *S*-nitrosylation, of aminophospholipid translocase, the major enzymatic pump internalizing phosphatidylserine and maintaining its transmembrane gradient in normal non-apoptotic cells (Daleke, 2003). This represents another example of the essential multifunctional actions of NO in cells. The significance of this process becomes evident if one takes into account that clearance of apoptotic cells through the phosphatidylserine-signaling pathway is one of the major regulators of the inflammatory response. In fact, phosphatidylserine-dependent signaling switches off production of pro-inflammatory cytokines and stimulates the generation and release of anti-inflammatory cytokines by macrophages (Savill and Fadok, 2000; Henson et al., 2001). Given the fact that during inflammation, activated macrophages release significant amounts of NO, NO-mediated interruption of phosphatidylserine signaling may lead to deregulation of cytokine balance and to propagation of the inflammatory response.

While the effects of NO resulting from its direct interaction with heme iron, for example, during the activation of guanylate cyclase, are well understood,

its more subtle activities regulating other signal transduction pathways are less well defined (Richter et al., 1999; Brown and Borutaite, 2001). These functions are often exquisitely dependent on conditions within the local environment including redox state, pH, and oxygen tension, obscuring the activities of NO and making it difficult to identify the reactive species involved (Adler et al., 1999; Gow and Ischiropoulos, 2001). In the mitochondria, this is complicated by the absence of a gene or mRNA sequence encoding a mitochondrial nitric oxide synthase, and the fact that no modification of any cellular nitric oxide synthase isozyme targeting it has been identified. Although NO production by isolated mitochondria has been characterized and the role of NO within mitochondria is well established and is critical for respiratory function, there are still significant questions regarding the source of this radical gas (Giulivi, 2003; Kanai and Peterson, 2004; Shiva and Darley-Usmar, 2003; Valdez et al., 2004). Potentially, the elucidation of the fine regulatory mechanisms by which NO controls such functions as the peroxidase activity of cytochrome *c* may lead to an understanding of the somewhat baffling requirement for nitric oxide synthase activity and low levels of NO in mitochondria.

8. Epilogue

8.1. Clues from the ancients

Despite the recent deciphering of the subtle mechanisms mediating some of the actions of NO, the concept that NO can participate in both pro- and anti-apoptotic activities obscures the analysis of the physiological roles of this molecule in mitochondria. Fortunately, within the past few years the sequencing of numerous genomes from each of the three domains of life (Archaea, Bacteria, and Eukarya) has provided sufficient data to gain insight into early cellular evolution (for reviews, see Ouzounis and Kyrpides, 1996; Forterre, 2001; Koonin and Aravind, 2002; Koonin, 2003). Results stemming from investigations on the emergence of eukaryotes indicate that pro-eukaryote unicellular organisms developed the machinery for apoptosis as an altruistic mechanism to control growth (Welburn and Maudlin, 1997; Lee and Kimelman, 2002; Madeo et al., 2002). The ability of the host to induce apoptosis in the symbiont was likely a requirement for protecting the host, and conversely, regulation of host apoptosis is essential for protecting the symbiont. These mechanisms are still employed by eukaryotic cells during deleterious parasitic infection (James and Green, 2004; Morris et al., 2004). In this regard, analysis of protein domains of the nitric oxide synthases, as well as the proteins of apoptosis suggests that the origins of these proteins directed their apoptotic roles. These phylogenetic analyses clearly indicate that the two paths leading to apoptosis evolved prior to the appearance of eukaryotes (Punj and Chakrabarty, 2003; Cande et al., 2002; Fig. 3). In addition, emerging studies strongly suggest that the signaling complex/caspase pathway and the mitochondrial/AIF path arose in different prokaryote precursor cells (Leist and Jaattela, 2001; Cande et al., 2002; Koonin and Aravind, 2002; Punj and Chakrabarty, 2003). These findings support a hypothesis in which, during primordial development, each of the proto-eukaryote's precursor cells, whose fusion ultimately resulted in its formation, possessed primitive machinery required for a distinct route to cell death.

8.2. The plot thickens . . .

Current studies point to a eubacterial sources for the generation of the caspase proteases (Nunez et al., 1998; Leist and Jaattela, 2001; Koonin and Aravind, 2002). In addition it has been suggested that the caspase-like proteases were strongly selected in eubacteria where they were employed as a mechanism for development, differentiation, and defense (Cecconi, 1999; Debrabanta et al., 2003). Moreover, investigations into the genesis of the nitric oxide synthases suggest that this protein also emerged from eubacteria (Cox et al., 2001; Adak et al., 2002; Zemojtel et al., 2003; Fig. 3). In contrast, the proto-mitochondrial cell, putatively derived from Archea, expressed several proteins that mediate apoptosis by the caspase-independent route, most notably AIF (Cande et al., 2002; Frank et al., 2003; Punj and Chakrabarty, 2003). Interestingly, examination of the AIF crystal structure together with structure–function analysis has demonstrated that following its release into the cytosol, this mitochondrial protein translocates to the nucleus and binds DNA. This oxidoreductase subsequently initiates large-scale (50 kb) DNA fragmentation (Chose et al., 2002; Fumarola and Guidotti, 2004).

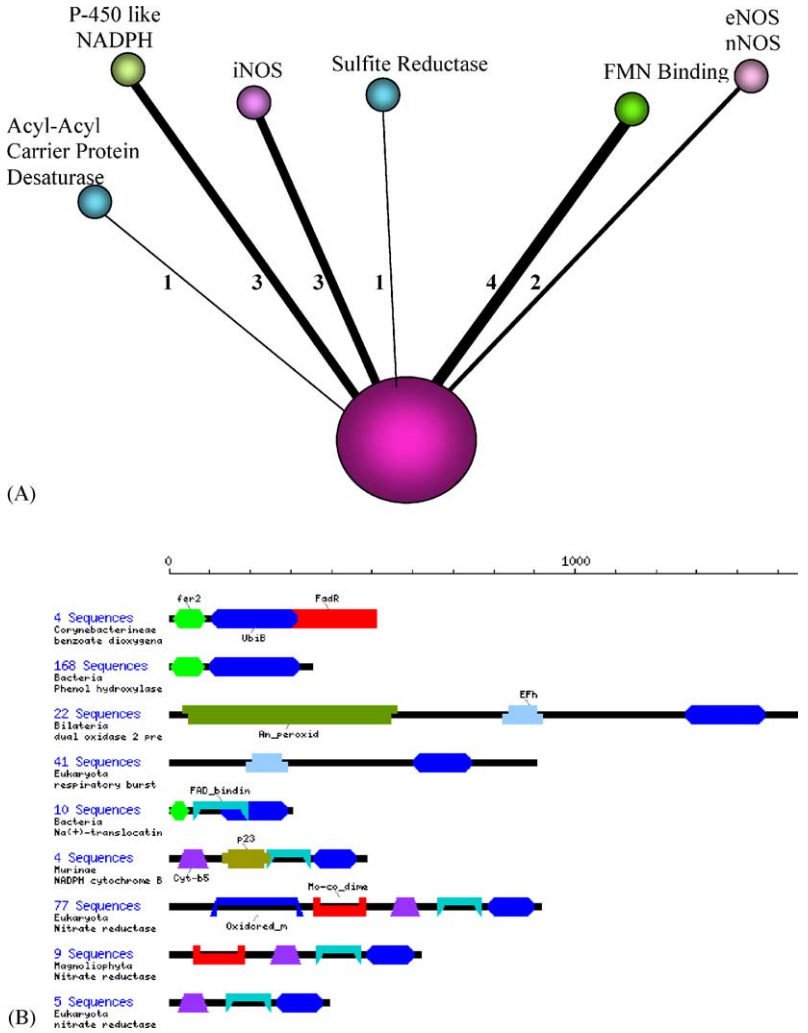

Fig. 3. (A) Cluster 277, a new cluster of homologous proteins related to nitric oxide synthase. SWISSPROT and TrEMBL databases were used to analyze the amino acid sequence from the most ancient nitric oxide synthase sequenced (dNOS) for hierarchical classification using *Protomap* (Copyright© 2000 Golan Yona and the ProtoMap authors), 161 proteins exhibited significant homology and were added to the cluster database. Vortex: all proteins of cluster, small spheres: functional groups. The number of homologous motifs connecting the functional related groups is next to each line. (B) Domain relationships of proteins homologous to dNOS. The amino acid sequence from the most ancient nitric oxide synthase sequenced was searched for homologous domains using NCBI Conserved Protein Domain Database (Marchler-Bauer et al., 2003). Resulting proteins were limited to those with Flavodoxin, Oxidoreductase NAD-binding, and FAD-binding domains. The architecture of the conserved domains is exhibited. Taken together these data imply a eubacterial origin for the nitric oxide synthases.

8.3. . . . and all is revealed

It is widely held that eukaryotic cells evolved following the establishment of a symbiotic relationship between a eubacterial host and a resident proto-mitochondrial symbiont (for reviews, see Spring, 2003; Emelyanov, 2003; Andersson et al., 2003). In the resulting eukaryote, the host cell contributed primitive caspase proteases and nitric oxide synthases, and the proto-mitochondrial symbiont supplied AIF and protein machinery for caspase-independent cell death. These proteins were crucial to the successful generation of a

eukaryotic cell as they facilitated the development of a true symbiosis. In this scheme, the eubacteria used nitric oxide to regulate the respiratory activity of the proto-mitochondria, and caspase activity to control its growth. Conversely, the proto-mitochondria countered aggressive activity by the eubacterial host using the proteins of the AIF pathway. The symbiotic fusion provided the basic units required for mitochondrial respiration and a system for programmed cell death. Ultimately, the proteins of advanced eukaryotes and multicellular organism evolved from these primitive precursors, which were combined and adapted to enhance survival. When viewed in this light it is hardly surprising that a single organelle, the mitochondrion, ultimately possesses the ability to regulate life and death.

Acknowledgments

Supported by NIH Grants CA100994 and ES006897 to JDL, CA093798 to DEH, and HL064145 and HL070755 to VEK. DEH and JDL are also support by the NIEHS Center Grant ES005022.

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