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News and Commentary

'What nourishes me, destroys me'†: towards a new mitochondrial biology

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† The title is a quotation from the only known portrait of Christopher Marlowe. The words 'Quod me nutrit me destruit' appear mysteriously in the corner of the painting.

It is a privilege to witness a revolution in thinking in Biology, but that is surely what we have seen in the past few years in our understanding of the roles of mitochondria in cell biology. Ten years ago, it all seemed simple: the mitochondrion was 'the powerhouse of the cell', the cliche of every biology textbook. Chemiosmotic principles were solved, the mechanism of ATP synthesis was understood at the molecular level.[1] What more could there be? And yet now we know otherwise – mitochondria are intimately involved in the delicate processes that sustain the balance between cell life and cell death. And of course, as the problem becomes the focus of attention in ever more laboratories, so new and surprising observations emerge. For some years, the proposal that mitochondria might play a central role in apoptotic cell death was thought improbable, despite the observation that Bcl-2, that exemplar anti-apoptotic protein, was localised to the mitochondrial outer membrane.[2] Since the cluster of publications emerged in 1994–1995 showing that cytochrome *c* (cyt *c*), that most innocuous of pink proteins, is a key initiator of apoptotic cell death (for an unusual review, see http://www.ergito.com/gtexpts/wang.htm), the mitochondrion has moved centre stage and has become the focus of research in many labs worldwide.

Perhaps, in the midst of all this activity, it is worth remembering that cell death in many disease processes is not apoptotic but necrotic, or perhaps some amalgam of the processes. There seems to be a tendency to regard necrotic cell death as boring – the inevitable consequence of energetic failure. However, there still must be a point of no return, and many intriguing questions remain. What defines the degree of energetic failure that can be tolerated and that which will inevitably proceed to cell death? Why do we have 3 min to start resuscitation at a cardiac arrest? What happens that makes the loss of function – signalled in the brain by a loss of consciousness – irreversible?

Key questions in apoptosis research must now be: how is cyt *c* release regulated? How is it initiated? Indeed, how is it prevented? Are there multiple routes and mechanisms that trigger release? As cyt *c* is central to both oxidative phosphorylation and apoptotic signalling, what determines whether the route to cell death upon cyt *c* release is apoptotic or necrotic? Are there yet more mitochondrial proteins involved in initiation and coordination of apoptosis? Several recent publications offer fascinating food for thought. We know now that cyt *c* is released from the mitochondrial intermembrane space (MIMS), possibly from a pool distinct from that committed to the respiratory chain.[3] Cyt *c* binds to Apaf-1 and activates procaspase-9, initiating the cascade of cellular self-destruction. But there is a problem. In many cells, there are other proteins, the Inhibitors of APoptosis (a family of proteins, the IAPs), presumably there to provide the belt and braces of security against accidental caspase activation. So, perhaps it is not sufficient just to release cyt *c*? There, also in the intermembrane space lies another protein, *diablo*, or SMAC (Second Mitochondria-derived Activator of Caspase), which inhibits the inhibitor, and so will permit activation of the apoptotic pathway to proceed. It turns out that other procaspases also lie quietly in the intermembrane space waiting their chance to participate in this process – procaspase-9 has been identified in the intermembrane space, as well as the flavoprotein apoptosis inducing factor, AIF. It turns out that this is not all. A recent paper from Larisch *et al*.[4] reveals yet another pro-apoptotic protein (Apoptosis Related Protein in the TGF-β Signalling pathway, ARTS) that seems to localise to mitochondria and translocates to the nucleus during apoptosis. This protein is a feature of the apoptotic pathway initiated in response to transforming growth factor, TGF-β. Larisch *et al*.[4] show that cells previously resistant to TGF-β apoptose in response to the 'growth' factor when over-expressing ARTS. Conversely, cells normally sensitive to TGF-β lose that sensitivity when ARTS expression is suppressed by expression of antisense to ARTS. Exposure of ARTS-expressing cells to TGF-β is associated with caspase-3 activation. It is not clear whether ARTS is involved in cyt *c* release or whether it operates as an independent pathway. ARTS belongs to the family of proteins known as septins, proteins with GTPase activity involved in cell motility and growth, not previously identified as mitochondrially localised. A scheme outlining these principles is set out in Figure 1C.

Interestingly, at least some of the other major mitochondrial proteins involved in apoptosis also seem to have a 'day job' – cyt *c* and AIF are both involved in redox reactions, although a specific role for AIF has yet to be identified. The voltage-dependent anion conductance, (VDAC) and the adenine nucleotide translocase (ANT) are both necessary for transport of molecules across mitochondrial membranes. Does this apply also to ARTS? What else might it do? Is it involved in mitochondrial movement – itself such a curious phenomenon to watch and so little understood (see [5])? And from where does ARTS come? Is it the intermembrane space again? Larisch *et al*. suggest that EM immunolocalisation shows that the

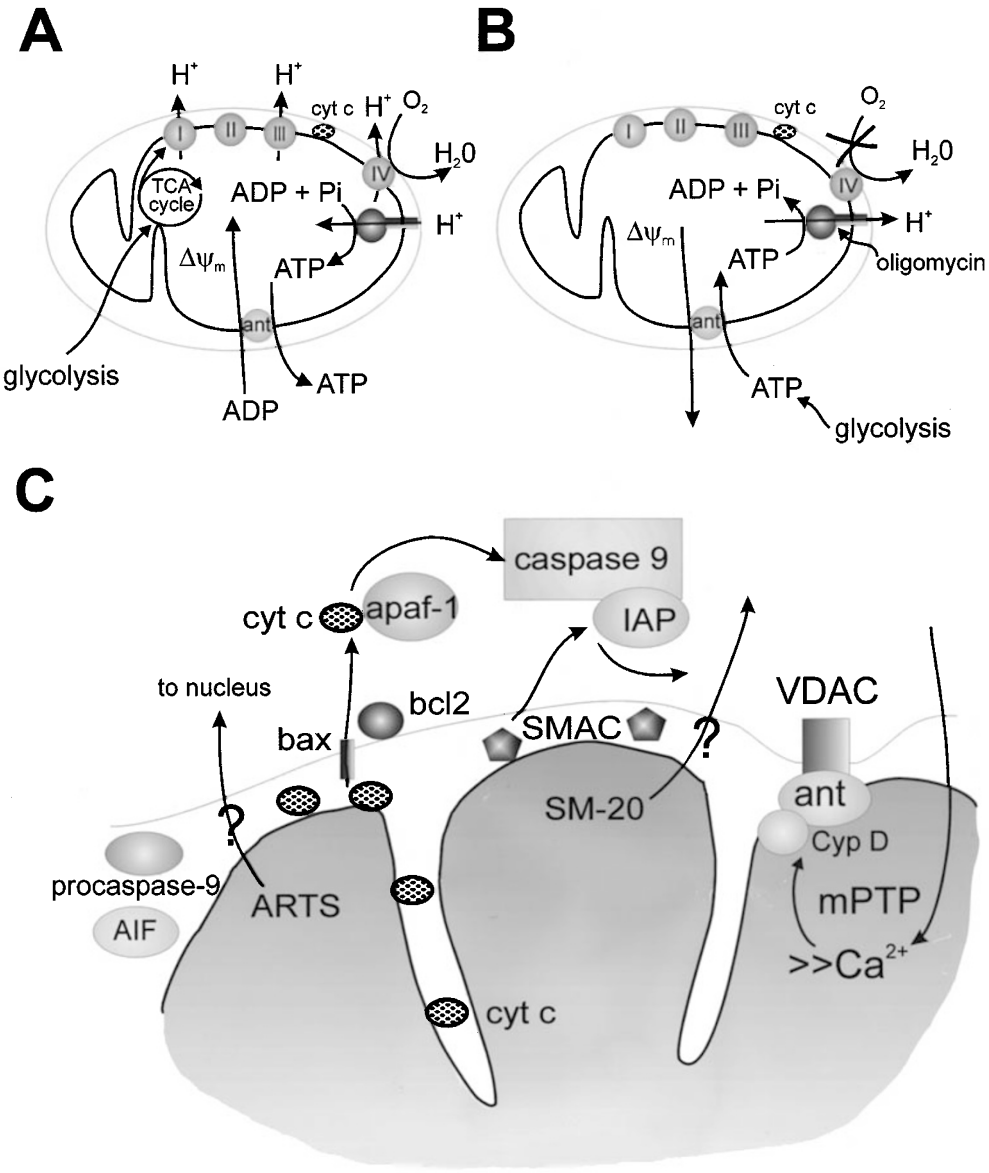

Figure 1 (**A**) In energised mitochondria, metabolic intermediates from glucose of fat metabolism enter the tricarboxylic acid (TCA, or citric acid) cycle, which maintains a supply of reduced NADH and flavoproteins to the four enzyme complexes of the respiratory chain. At Complex IV, electrons are transferred to molecular oxygen. Electron transfer between respiratory complexes translocates protons across the inner mitochondrial membrane and so develops and maintains a potential ($\Delta\Psi_m$), estimated at about -150 mV relative to the cytosol. That potential is used to drive ATP synthesis by the oligomycin sensitive F_1F_0-ATP synthase. If respiration is inhibited or is somehow inactive (as in the eosinophils[19]) then $\Delta\Psi_m$ may be maintained by reversal of the ATP synthase, acting as a proton translocated ATPase (scheme **B**). Now, glycolytic ATP is used to drive proton movement and so maintain a potential in the absence of oxygen consumption. (**C**) Mitochondria, and particularly the intermembrane space, are a storehouse for potentially lethal pro-apoptotic compounds, including cytochrome c, apoptosis inducing factor (AIF), procaspase 9, SMAC (or diablo), ARTS[4] and SM-10[6]. The precise localisation of ARTS or SM-20 remains uncertain. The routes for the release of these compounds from the mitochondria remain controversial. It seems likely that oligomerisation of bax, regulated by bcl-2, represents one route for movement of compounds from the intermembrane space, perhaps involving the outer membrane channel VDAC. VDAC may also play a role as a component of the mitochondrial permeability transition pore (mPTP). The latter is concentrated at contact sites between inner and outer mitochondrial membranes, spanning both membranes. Opening of the mPTP therefore promotes efflux of compounds from the matrix but can only allow loss of compounds from the intermembrane space by causing mitochondrial swelling and rupture of the outer membrane

protein is matrix, but in truth the published images make this hard to judge. The localisation of the protein has important implications for the likely route of escape.

Yet another proapoptotic protein seems to reside in mitochondria, but here the functional significance of the mitochondrial localisation is not yet clear. SM-20 is a protein implicated in cell growth and division in a number of models. SM-20 expression is up-regulated in sympathetic neurons induced to undergo apoptosis by NGF deprivation or by exposure to anti-tumour agents. Lipscomb *et al*.[6] noticed that the protein has a mitochondrial targeting consensus sequence and showed that expression in

sensory neurons resulted in a mitochondrial localisation of the protein which was prevented by truncation of the consensus sequence. Overexpression of the protein also initiated apoptosis, but here it did not seem to matter if the protein was sequestered in mitochondria or not – expression of both the native protein and a truncated protein caused apoptosis. SM-20 over-expression caused caspase-3 activation, but not widespread, global cytochrome *c* release. And so, in this case, the questions remain – is this another mitochondrial protein locked carefully away until it is needed, or is it manufactured on demand? And what else is it doing? Why target it and transport it to mitochondria unless it has a function there?

One of the key questions is, 'how do the proteins escape from the intermembrane space?' For some years, the focus has been the mitochondrial permeability transition pore (mPTP). Discovered in the late 1970's, this was largely regarded as a biochemical oddity, ignored until the suggestion that it might play a role in apoptosis. The question still seems to be, does it? The pore seems to be a pathological configuration of mitochondrial proteins that span the two mitochondrial membranes – VDAC in the outer membrane, and ANT in the inner, together forming some kind of complex associated with and regulated by the mitochondrial cyclophilin D (Cyp D; see Figure 1A). It is important to understand that the open pore spans both membranes – it does not *per se* represent a route of exit for proteins from the intermembrane space except by inducing mitochondrial swelling and rupture of the outer membrane.[7,8] In most models (but not all) such swelling and rupture has not been seen (e.g. see [9]). The pore has a large conductance. Opening will inevitably cause a collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) required to drive oxidative phosphorylation. In many cases of receptor driven apoptotic cell death, the mitochondrial membrane potential seems to be maintained until after cyt *c* release (for review, see[2]), and so the consensus seems to be that mPTP opening is unlikely as a primary trigger to cyt *c* release in coordinated growth factor mediated apoptotic cell death.

One can, however, ask the converse question – if a pathway opens the mPTP – notably, cellular Ca^{2+} overload and oxidative stress, features of pathological states such as reperfusion injury in the brain and heart – will that then cause cyt *c* release and apoptosis? A vast literature argues strongly that it can – innumerable inducers of mPTP opening cause apoptosis.[10] What seems remarkable is that overexpression of bcl-2 could protect cells under these conditions. Does this mean that Bcl-2 associates with the mPTP? Bax seems to interact with VDAC to regulate cyt *c* release,[11] perhaps through the channel forming dimerisation of this erstwhile bacterial pore former. And VDAC appears after all to be a component of the mPTP, and yet it is also clear that bax mediated cyt *c* release can proceed without any apparent loss of mitochondrial membrane potential[12] . . . . The associations seem almost to taunt one, and yet do not quite fit.

One of the biggest problems in studying the mPTP is trying to find a tool that will allow unambiguous experimental verification that the mPTP is involved. Cyclosporin A (CsA) is used routinely, binding to Cyp D and inhibiting pore opening. But CsA binds with all cyclophilins and at least nine such proteins have been identified, although their functions remain uncertain. For example, one cytosolic cyclophilin (Cyp A) binds the ubiquitous signalling protein, calcineurin. Methyl-valine cyclosporin (mv-Cs) is supposed to be more selective as the mv-Cs:cyclophilin complex does not interact with calcineurin,[13] but in truth, we do not know what else mv-Cs might do – it is not as easy an answer as it might seem and indeed, it is not readily available anyway. In an elegant assay, Lemasters *et al*.[14] and Petronilli *et al*.[15] have each devised techniques to image mPTP opening based on the redistribution of the large fluorescent molecule, calcein, between mitochondrial and cytosolic compartments as a way to identify opening of a large conductance pathway. Lemasters' group follow the movement of calcein from the cytosol into mitochondria, seen as negatively stained structures that appear dark against the bright cytosolic calcein of hepatoyctes. This only works in cells with nice fat mitochondria that do not themselves accumulate calcein-AM, as simple optical constraints limit the ability to view fine mitochondrial structures in most cells by negative staining.[16] Petronilli *et al*. allow calcein AM to permeate throughout the cell, including the mitochondria, but quench the cytosolic dye with cobalt. The mitochondria are left brightly stained and mPTP opening causes calcein efflux (or cobalt influx?) with a loss of the mitochondrial signal. Anecdotally, we know of a number of labs, including our own, who have attempted these approaches in other cell types in which mitochondria have simply refused to load with calcein. Thus, imaging the pore opening in living cells may be possible, but modulating pore function with clean, selective, cell permeant pharmacological agents is less straightforward.

It has long been proposed that the pore can open in multiple subconductance states.[17] In a recent paper, Petronilli *et al*.[18] suggest that transient opening of the pore, perhaps in a low conductance state, (although this is impossible to verify experimentally in this model) will cause immediate quenching of mitochondrial calcein, while the mitochondrial membrane potential (measured with the potentiometric indicator TMRM) is more or less maintained. The latter is only feasible if the pore opening is brief and transient, so that transient depolarisations can recover. This process, induced by the calcium ionophore A23187, was not associated with cyt *c* release. However, another mPTP inducer, arachidonic acid, apparently caused full and irreversible opening of the pore, with the collapse of mitochondrial potential and the loss of calcein signal, followed by cytochrome *c* release. This appears to contrast with data from Szalai *et al*.[19] who showed that exposure of cells to sublethal concentrations of ceramide followed by a transient increase in $[Ca^{2+}]_c$ caused a transient mitochondrial depolarisation due to transient opening of the mPTP and cyt *c* release. They argued that the mPTP acts as a detector of the coincidence of a calcium signal with a propapoptotic stressor – oxidative stress, ceramide. Perhaps, in this instance, the pore opened fully but reversibly, while in the experiments of Petronilli *et al*. the pore opened in a flickering low conductance state? Indeed,

Pastorino *et al*. (1999)[20] also suggested that low concentrations of bax can induce mPTP opening in a low conductance state, release cyt *c* and initiate apoptosis. It is hard to see how a pore that spans both membranes can release proteins from the intermembrane space without causing mitochondrial swelling. Perhaps one needs to invoke some feedback process whereby bax causes cyt *c* release, perhaps also pro-caspase-9, activating caspase activity and perhaps then promoting mPTP opening? These questions are very hard to resolve within cells, and seem a little bewildering. In particular, it seems that we badly need more precise molecular or pharmacological tools to manipulate the mPTP rather than the miserably blunt tool provided by CsA.

The conceptual switch in our thinking from a focus on the energetic role of mitochondria to the regulation of death is perhaps most dramatically exemplified by a fascinating recent paper describing mitochondrial function in eosinophils. We think of mitochondria as the ultimate symbiont – a bacterial structure that has become so integral to eukaryotic cell life that its maintained function is essential to cell life, while the mitochondrion itself is no longer capable of independent existence. A recent publication by Peachman *et al*.[21] suggests that in some instances, perhaps the mitochondrion has become almost a parasite rather than a symbiont, or perhaps a high security storeroom maintained at some energetic cost to the cell – the place to put dangerous proteins. According to Peachman *et al*., eosinophils contain only a few dozen mitochondria compared to the thousand or so found in hepatocytes.[22] The cells appear to show almost no measurable oxygen consumption. Energetic demands are apparently met by glycolytic ATP production (see Figure 1A,B). Thus, inhibition of cytochrome oxidase with cyanide has no effect on ATP levels – the cells can survive in CN^- for 24 h without loss of ATP! The mitochondria of these cells seem functionally redundant, at least in terms of bioenergetics. However, it turns out that they maintain a membrane potential, as measured by the accumulation of lipophilic cationic fluorescent dyes. Normally, of course, mitochondrial potential is maintained by respiration (Figure 1A), but if oxygen consumption is barely detectable, how is $\Delta\Psi_m$ maintained? Remarkably, inhibition of the mitochondrial F_1F_0-ATP synthase with oligomycin or inhibition of the mitochondrial adenine nucleotide translocase (required for mitochondrial ATP transport; Figure 1B) caused a collapse of $\Delta\Psi_m$ and apoptotic cell death. This simply reflects the fact that the ATP synthase is a proton pumping ATPase that is normally driven as an ATP synthase by the proton gradient across the mitochondrial inner membrane. In the absence of respiration, it will run as an ATPase, consuming ATP, pumping protons out of the mitochondrial matrix and so generating a potential (Figure 1C). Incidentially, the same process should cause ATP depletion once the mPTP has opened, raising the question 'how is the ATP necessary for apoptosis sustained' if the mPTP is involved? However, the implication of these observations is that the mitochondria in these cells represent an energetic cost to the cell, consuming glycolytic ATP to maintain a potential. This occurs in other cell types when respiration is impaired – during anoxia for example – or if the mitochondrial respiratory chain is damaged or if mitochondrial DNA is absent but why in these cells where oxygen is freely available? It seems as though respiration is switched off in the eosinophils for some reason, perhaps as a means to give a cell in which apoptosis needs to be readily controlled more control over $\Delta\Psi_m$? Nor is it clear why the cells should apoptose when $\Delta\Psi_m$ collapses – perhaps mitochondrial swelling is then sufficient to release cyt *c*?

What is so remarkable is that here the mitochondria seem to have lost any useful function in terms of bioenergetics, and are present solely as a safe house in which to store away proapoptotic proteins, maintained by the cell at an energetic cost. Skulachev[7] has made the interesting point that the MIMS is evolutionarily a sort of no-man's land – the inner membrane is thought to have originated from bacterial membranes, the outer membrane as a delimiting membrane from the host cell, and this space is emerging as a fascinating microscopic intracellular compartment with it's very own microenvironment that is crucially controlled and involved in the regulation of cell life and death.

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