Mitochondria play a central role in cell life and cell death. An increasing number of studies place mitochondrial dysfunction at the heart of disease, most notably in the heart and the central nervous system. In this article, I review some of the key features of mitochondrial biology and focus on the pathways of mitochondrial calcium accumulation. Substantial evidence now suggests that the accumulation of calcium into mitochondria may play a key role as a trigger to mitochondrial pathology, especially when that calcium uptake is accompanied by another stressor, in particular nitrosative or oxidative stress. The major process involved is the opening of the mitochondrial permeability transition pore, a large conductance pore that causes a collapse of the mitochondrial membrane potential, leading to ATP depletion and necrotic cell death or to cytochrome c release and apoptosis, depending on the rate of ATP consumption. I discuss two models in particular in which these processes have been characterized. The first is a model of oxidative stress in cardiomyocytes, in which reperfusion after ischemia causes mitochondrial calcium overload, and oxidative stress. Recent experiments suggest that cardioprotection by hypoxic preconditioning or exposure to the ATP-dependent K⁺ channel opener diazoxide increases mitochondrial resistance to oxidative injury. In a second model, of calcium overload in neurons, the neurotoxicity of glutamate depends on mitochondrial calcium uptake, but the toxicity to mitochondria may play a key role as a trigger to mitochondrial pathology, especially when that calcium uptake is accompanied by another stressor, in particular nitrosative or oxidative stress. The major process involved is the opening of the mitochondrial permeability transition pore, a large conductance pore that causes a collapse of the mitochondrial membrane potential, leading to ATP depletion and necrotic cell death or to cytochrome c release and apoptosis, depending on the rate of ATP consumption. I discuss two models in particular in which these processes have been characterized. The first is a model of oxidative stress in cardiomyocytes, in which reperfusion after ischemia causes mitochondrial calcium overload, and oxidative stress. Recent experiments suggest that cardioprotection by hypoxic preconditioning or exposure to the ATP-dependent K⁺ channel opener diazoxide increases mitochondrial resistance to oxidative injury. In a second model, of calcium overload in neurons, the neurotoxicity of glutamate depends on mitochondrial calcium uptake, but the toxicity to mitochondria also requires the generation of nitric oxide. Glutamate toxicity after activation of N-methyl-D-aspartate (NMDA) receptors results from the colocalization of NMDA receptors with neuronal nitric oxide synthase (nNOS). The calcium increase mediated by NMDA receptor activation is thus associated with nitric oxide generation, and the combination leads to the collapse of mitochondrial membrane potential followed by cell death. Diabetes 53 (Suppl. 1):S96–S102, 2004
of mitochondria plays a central role as a heat-generating mechanism in non-shivering thermogenesis in young mammals. It has also been suggested that the production of free radical species by mitochondria may play a key role as a signaling mechanism, for example, in the regulation of ion-channel activities and also in initiating cyto-protective mechanisms in stressed cells.

Damage to mitochondria inevitably leads to disease. The disease processes in which mitochondrial dysfunction has been identified and seems to play an important part generates a list that is growing rapidly. Mitochondrial damage in β-cells causes diabetes. Mitochondria are damaged during ischemia itself or during the reperfusion of ischemic tissue, best characterized in heart, brain, and kidney. Mutations of mitochondrial proteins give rise to a range of ill-understood patterns of disease; mitochondrial dysfunction has been implicated in all the major neurodegenerative diseases—Parkinson’s, Alzheimer’s, motoneurone disease (Lou Gehrig’s disease or amyotrophic lateral sclerosis), and possibly in multiple sclerosis (1,2). Mitochondrial dysfunction in the heart may give rise to cardiomyopathy (3). Recent evidence points to a major role for mitochondrial dysfunction in the condition of multi-organ system failure in sepsis (4). Accumulations of mitochondrial defects have been implicated as a mechanism of aging and age-related disease (5). Indeed, the production of free radicals by mitochondria has been considered as many to play a central role in the degradation of cellular function that appears to underlie the process of aging, whereas some of the genes identified in the control of longevity appear to target mitochondria or at least to alter antioxidative defenses of the cell (5,6).

Over the last few years, mitochondria have emerged as central players in the regulation of organized or programmed apoptotic cell death (7). The centrality of apoptosis in development and in disease is extraordinary. The inappropriate activation of apoptotic pathways leads to tissue dysfunction and damage, and the failure to activate apoptotic pathways appropriately in response, for example, to subacute injury causes cancers, making this area critical in understanding the role of mitochondria in disease.

Mitochondrial function and dysfunction have been implicated in many different aspects of health and disease. In this article, I will therefore consider some of these fundamental attributes of mitochondria and consider the role of mitochondrial dysfunction in generating disease.

MITOCHONDRIAL BIOENERGETICS AND CELLULAR CALCIUM SIGNALING

One of the major physiological features of mitochondria is the generation of a large transmembrane potential across the mitochondrial inner membrane. This is a direct consequence of the biochemical reactions that constitute the respiratory chain. Thus, substrates supplied to mitochondria such as pyruvate, some amino acids, and products of β-oxidation of fatty acids enter the tricarboxylic acid (TCA) cycle and maintain the reduced state of the NADH/NAD + and FADH 2/FAD couples. These supply electrons to the respiratory chain, which eventually are transferred to oxygen. The process also transfers protons across the mitochondrial inner membrane, generating a proton gradient—a proton motive force that is largely expressed as a membrane potential usually estimated as some 150–180 mV negative to the cytosol. This is the stored force that underpins the production of ATP, which is driven by the movement of protons down this gradient, driving the turbines of the F F0-ATP synthase to phosphorylate ADP to generate ATP. ATP must then be transported into the cytosol by the adenine nucleotide translocase (ANT).

As well as driving ATP synthesis, the mitochondrial potential, usually referred to as ΔΨm, also drives the accumulation of calcium into mitochondria. The pathway for calcium influx into the mitochondrion involves an electrogenic uniporter, which carries calcium into the mitochondrial down an electrochemical potential gradient whenever the concentration of extramitochondrial calcium rises. The molecular identity of the uniporter remains to be established. It behaves apparently more like a channel than like a carrier and is blocked by ruthenium red, which blocks many classes of calcium-permeant channels. The influx pathway also seems to show some cooperativity, conferring on the pathway some threshold-like features (8). Calcium that is accumulated by mitochondria must be removed. This is achieved largely through the action of an Na/Ca exchanger.

For many years, the physiological significance of this pathway was debated (9). However, it is now clear that, in almost every cell type studied, whenever cells engage in calcium signaling activity, then some of that calcium signal is transferred to the mitochondria. The primary functional role of this pathway is generally agreed to be the calcium-dependent metabolic regulation of the TCA cycle, which is upregulated by a rise in the intramatrix calcium concentration. The increased activity of the TCA cycle can be measured as an increase in the fluorescence of NADH (10), reflecting an increase in the provision of reducing equivalents to the respiratory chain, followed by a modest increase in mitochondrial potential (11) and an increase in ATP generation (12). This provides an eminently simple but elegant pathway that inevitably coordinates the rate of energy supply with use, because calcium signals in almost all cells are associated with work, and an increase in energy demand (secretion, contraction, etc.). Detailed studies of the interaction between calcium-signaling pathways and mitochondria have suggested that there may be privileged pathways of communication between mitochondrial and calcium-transmitting pathways—the endoplasmic reticulum (ER) (13) or sarcoplasmic reticulum (SR) (14)—or perhaps even at the plasma membrane in excitable cells (15), in which the primary route of calcium signaling operates through voltage-gated calcium channels.

Mitochondrial calcium uptake may also have an impact on the calcium signal, because mitochondria may effectively act as a fixed spatial buffering system, removing calcium locally, modulating the local calcium concentration, and so regulating the activity of calcium-dependent processes. The latter include the rate of calcium release through inositol triphosphate–gated channels (16) and the influx of calcium through capacitative influx channels (known as I tetras ) (17), because all these channel types are strongly regulated by local calcium concentration. The effect may be to blunt or slow the progression of calcium...
mitochondria (16), to limit the spread of local calcium release (18), and so to dampen the excitability of calcium signaling in the cytosol. However, in the case of $I_{\text{mPTP}}$, the effect is the opposite; by limiting local calcium concentration, mitochondria allow the channels to stay open longer, therefore enhancing calcium influx through the pathway.

Our interest in this pathway in the present context lies in its potential role in cell pathology, because mitochondrial calcium “overload” has been implicated repeatedly in a variety of different disease processes.

MITOCHONDRIA AS GENERATORS OF FREE RADICAL SPECIES

One consequence of mitochondrial respiration is the generation of unpaired electrons. The interaction of these electrons with $O_2$ results in the generation of superoxide ions—highly reactive free radical species (or reactive oxygen species [ROS]). Furthermore, other radical species, such as hydroxyl ions (OH·) and $H_2O_2$, may also be present in reasonably high concentrations, posing a risk of lipid peroxidation and damage to cell membranes and DNA. It is worth remembering that this includes mitochondrial DNA (mtDNA), which has no associated histones and is less protected from radical damage than nuclear DNA. Mitochondria represent the major source of free radical species in all cells except those specialized to generate free radicals such as macrophages and neutrophils. Mitochondria are equipped with an armamentarium of antioxidant defenses. They contain a high concentration of glutathione, a variant of superoxide dismutase, and catalase to remove the potentially harmful peroxide that is produced by superoxide dismutase.

The specific sites of free radical generation along the electron transport chain are controversial. Thus, in some tissues, it seems that inhibition of electron transfer at complex I (typically with rotenone) may generate an increase in radical formation, whereas in others, rotenone will reduce radical generation by preventing passage of electrons further into the distal chain. Methodological problems may underlie such differences, but in one study in which the same techniques were used side by side in mitochondria from kidney and from pulmonary vascular smooth muscle cells, the two populations of mitochondria responded in opposing ways to rotenone (19). The basis for such a difference remains obscure. The bulk of mitochondrial radical generation is believed to come from leak of electrons to oxygen at the interface between complex II and III. In general, the leak of electrons seems to be increased by an increase in mitochondrial potential and decreased with mitochondrial depolarization (20). Indeed, it has been suggested that a mild depolarization of the mitochondria by uncoupling proteins may serve as a protective mechanism to limit mitochondrial ROS generation in some instances. Inhibition of respiration distal to the point of radical generation (e.g., at complex IV with cyanide) is expected to increase ROS production, whereas uncouplers will tend to reduce electron leakage by increasing the respiratory rate, favoring the transfer of electrons to oxygen at complex IV and generating water. Nevertheless, much of this field remains poorly understood, highly controversial, and full of misleading and confusing literature. This is perhaps partly because the methods available to measure rates of radical production from cells are insecure and prone to artifact and so very difficult to use to give absolutely secure and unambiguous data.

One interesting issue is whether charged radical species such as superoxide anion can actually escape from the mitochondrial matrix at all, because there is no obviously membrane-permeant pathway through which such species can cross the membrane. Perhaps the main route through which mitochondrially generated ROS may escape is through conversion to $H_2O_2$, which probably can cross membranes. This argument does have ramifications in discussing the cell injury that results from mitochondrial oxidative stress.

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE

The major mechanism thought to mediate calcium-induced mitochondrial injury is the mitochondrial permeability transition pore (mPTP) (21). This is a large conductance pathway in the inner mitochondrial membrane, generally generated by an alteration in the conformation of membrane proteins that normally perform other roles—the ANT and possibly the voltage-dependent anion channel, which is sited in the outer membrane. The mPTPs seem to be found mostly at contact sites between the inner and outer mitochondrial membranes, where the voltage-dependent anion channel and the ANT are concentrated. In addition, a further protein, cyclophilin D, is associated with the pore complex. This is a cyclosporine-binding protein whose function and role remain uncertain, but which confers sensitivity of the complex to cyclosporin A, which blocks or prevents the opening of the mPTP. It is not really clear as yet whether the pore complex has a normal role in cell physiology. It has been suggested by some that the pore may represent a route for calcium to escape from overloaded mitochondria under physiological conditions, and some experimental observations suggest that the pore may flicker open in a reversible transient phenomenon, possibly in a subconductance state (22). It has also been suggested that the pore may play an important role in some forms of apoptotic cell death, because pore opening causes mitochondrial swelling and may cause rupture of the outer membrane with the release of cytochrome c (23). Certainly, if the pore opens irreversibly, cells will die, most likely through a necrotic route to cell death, because the mitochondria cannot maintain a potential and will at best fail to generate ATP; at worst, they will actively consume ATP (through the reversal of $F_1F_0$-ATP synthase) (24). That pore opening normally plays a role in other forms of coordinated programmed cell death seems unlikely, because in many cases of apoptosis, mitochondrial membrane potential can be maintained, at least through the early phases of the process, and maintenance of potential in the face of pore opening is most unlikely (25).

The most effective mechanism to open the mPTP is to expose mitochondria to a combination of high matrix calcium together with a pro-oxidant. Oxidative stress increases the probability of pore opening by modulating sulfhydryl groups, which govern pore opening. The site of calcium action remains uncertain, although ANT alone
may form pores in the presence of calcium. Pore opening is also inhibited by ATP and ADP and promoted by high inorganic phosphate; therefore, conditions of ATP depletion will favor pore opening. It was proposed many years ago that these properties make the pore a perfect candidate for the cell death associated with cardiac reperfusion injury. Thus, during a period of ischemia, the intracellular calcium concentration rises slowly but progressively while the mitochondria gradually depolarize (and so will not accumulate much of the calcium, because calcium uptake is potential dependent), whereas the mitochondria also consume ATP and, hence, ATP levels fall and the concentration of inorganic phosphate rises. At reperfusion, the restoration of oxygen causes a rapid repolarization, so the mitochondria take up calcium, mitochondrial ROS generation is also increased with the high potential and higher oxygen tension, inorganic phosphate is high, ATP is low, and these are ideal conditions to promote mPTP opening (26). In keeping with this, it has been shown that pore inhibition using cyclosporin A delivered at the time of reperfusion may dramatically reduce infarct size in the heart and also that labeled deoxyglucose enters mitochondria during this period (27). This makes the pore an attractive pharmacological target for cardioprotection. The role of the pore in equivalent forms of cell injury in other tissues has been less clearly demonstrated, but it is likely involved in neuronal injury during periods of ischemia and glutamate toxicity, and, again, cyclosporin A and pore inhibition has been shown to be protective in models of cortical ischemia (28).

**STUDYING MITOCHONDRIAL FUNCTION WITHIN CELLS**

To study all of the phenomena discussed above, it is necessary to have experimental models and to find ways in which the responses of mitochondria and related cellular functions can be studied in situ. Characterization of mitochondrial function from preparations of mitochondria isolated from major tissues (heart, liver, and brain) has provided the mainstay of our knowledge base. However, if we wish to understand the ways in which mitochondria behave in relation to other aspects of cell physiology, and if we wish to understand how other cell functions respond to changes in mitochondrial function, then it is necessary to have experimental models in which these processes can be studied in intact cells. Digital fluorescence imaging has made a major contribution in resolving these issues, because it allows detailed investigation of intracellular processes within single identifiable cells. This approach also has the advantage that tissues where the sample size might be too small for the preparation of isolated mitochondria for conventional biochemical studies become accessible for study. A wide range of fluorescence indicators are available that report changes in intracellular biochemical parameters; in particular, in relation to mitochondrial function, we can routinely measure changes in mitochondrial potential, mitochondrial redox state (the fluorescence of endogenous NADH and FAD), mitochondrial calcium concentration, cytosolic calcium concentration, pH, and so on. Dyes staining mitochondria have been used to study changes in mitochondrial morphology, localization, and distribution in cells. Transfected cells with fluorescent-tagged proteins to study their distribution or their redistribution with stimulation has also proven extremely instructive. The simultaneous measurement of multiple variables within single cells allows us to establish the direct relationships between changes in mitochondrial function and other cellular parameters on a cell-by-cell basis and has proven extremely important in a number of experimental models. Detailed descriptions of these approaches have been published recently (29).

**EXPERIMENTAL MODELS TO STUDY CALCIUM- AND ROS-RELATED CELL INJURY**

**Oxidative stress and models of cytoprotection.** To study the consequences of mitochondrial ROS generation for mitochondrial and cell function as directly as possible, we developed a model in which we can systematically increase the specific mitochondrial generation of ROS (30,31). This involves introducing a mitochondrial-targeted photosensitizing agent, a fluorescent indicator, which accumulates selectively within mitochondria as a direct response to the mitochondrial membrane potential. Illumination of the dye generates superoxide anion within mitochondria, and the degree of oxidative stress can be graded simply by varying the intensity of illumination. We have used a lipophilic fluorescent cationic dye, tetramethyl rhodamine methyl (or ethyl) ester—a dye that can be used at the same time to monitor changes in mitochondrial membrane potential. Because the accumulation into mitochondria is potential dependent, a collapse of mitochondrial potential causes the redistribution of the probe from mitochondria to cytosol, a process that can be readily followed using fluorescence imaging techniques.

It turns out that this model initiates a stereotypical sequence of events that culminates in cell death. We have explored the system in some detail in two cell types: an excitable cell, the cardiomyocyte (31), and a nonexcitable cell, the cortical astrocyte (30). In both systems, the first response to illumination of the dye consists of rapid short-lived transient and localized depolarizations of mitochondria. It is not yet clear whether such events occur spontaneously anyway or whether they result from the oxidative stress generated by illumination. What is clear is that the frequency of these events increases progressively with time and that the progression is hastened by increased illumination intensity. Finally, the whole population of mitochondria shows a complete and irreversible depolarization, and this will be followed by ATP depletion and cell death. In cardiomyocytes, but not the astrocytes, the progression usually terminates with a wave of depolarization that propagates slowly along the cell until there is a global collapse of mitochondrial potential, at which point ATP depletion is signaled by the progression to rigor. The role of ROS in this phenomenon is readily demonstrated both through increases in ROS production with illumination measured directly with the dye dicarboxyfluorescein diacetate and by the protection offered by antioxidants.

It seems that the whole phenomenon also requires the release of calcium from internal stores of the cell. Thus, the events could be inhibited by using antioxidants or by powerful calcium buffers (BAPTA, introduced into the cell as the AM ester) and/or by depletion of ER/SR calcium stores (31). Similarly, ER/SR calcium accumulation into...
mitochondria can be demonstrated with illumination using mitochondrially targeted calcium-sensitive fluorescent indicators. Thus, both calcium and ROS seem essential for the illumination-induced mitochondrial depolarizations. We have also found that illumination causes the progressive oxidation of NADH, measured as the decrease in endogenous blue fluorescence elicited by ultraviolet light. The global depolarization occurs when NADH is maximally oxidized.

Our working model to explain this phenomenon suggests a sequence of events triggered by mitochondrial ROS species, whereby locally produced ROS increase the probability of calcium release from ER/SR, probably through the modulation of thiol groups on the ryanodine or inositol triphosphate receptors. Calcium released locally is taken up by nearby mitochondria, which therefore begin to accumulate calcium. The transient depolarizations may represent either transient depolarizations after mitochondrial uptake of local pulsed release of calcium (which is an electrogenic process) or transient openings of the mitochondrial permeability transition pore, which opens in response to the combination of an increased intramitochondrial calcium concentration and oxidative stress. Such openings are transient until NADH (which regulates mPTP opening) is fully oxidized and ATP levels (which inhibit pore opening) are sufficiently low that the pore opens fully and irreversibly (M.R.D., unpublished data), after which the cell becomes rapidly depleted of ATP and dies.

The role of mPTP in this process is readily demonstrable because the whole phenomenon is largely suppressed by a variety of pharmacological agents that suppress mPTP opening—cyclosporin A, methyl valine Cs, and sanglifehrin. What perhaps surprised us most was that the transient openings of the mPTP appear to be completely innocuous and cause no evident harm to the cell, with no sign of apoaptosis or cytochrome c release in illuminated cells even 24 h later (30).

We have wondered to what extent this system represents a model for the events at the reperfusion of anoxic or ischemic tissue, in which ATP is depleted, ROS generation is high, and intramitochondrial calcium content is high, as discussed above. If the phenomenon requires this interesting interplay between ER/SR and mitochondria, we have also wondered how these responses may differ in different tissues and cell types in which different calcium release mechanisms predominate and in which different spatial relationships may be found between mitochondria and ER or SR. One fascinating phenomenon is that of ischemic preconditioning. This describes a situation in which a prior period of sublethal ischemia initiates mechanisms that protect the heart or brain from a second more severe episode of ischemia. Recent studies have suggested strongly that mitochondria may play a critical role in the pathway that protects the tissues, especially focusing on the possible role of mitochondrial ATP-dependent K⁺ (K\textsubscript{ATP}) channels. It is not yet clear whether the mitochondria are the final target of the protective mechanism, such that mitochondrial injury is reduced and so the cells are protected, or whether mitochondria lie on a pathway, generating a signal that then influences further downstream signaling pathways to protect the cells. Our recent data show that when we use the model described above, with a standardized set of illumination conditions, the mPTP will open in cardiomyocytes after a remarkably reproducible time period (~20 min). A number of manipulations that cause ischemic preconditioning, including a period of hypoxia or exposure to diazoxide or nicorandil, which are K\textsubscript{ATP} channel openers and which have been shown many times to mimic ischemic preconditioning, all prolong the time before the mPTP opens by as much as two- to threefold, increasing the time from some 20 min to 45–60 min (D. Hausenloy, S. Mani-Babu, D. Yellon, M.R.D., unpublished data). Whether or not these agents actually act at a mitochondrial K⁺ channel remains highly controversial. We have seen very little to suggest that they have any direct action on mitochondrial membrane potential or redox state. In contrast to the findings of Liu et al. (32), who showed that diazoxide could cause massive changes in flavoprotein autofluorescence, we have seen no effect at all of diazoxide on either flavoproteins or NADH autofluorescence, although the drug is clearly still significantly cardioprotective. We still do not adequately understand the association between the actions of these drugs and the reduced probability of mPTP opening. Most likely, the drugs act to inhibit complex II and increase free radical generation, and perhaps this upregulates intrinsic antioxidant mechanisms, limiting the damage induced by a second period of oxidative stress.

It seems that this model may be widely applicable to understanding the cell injury that develops after damage to the respiratory chain and the resultant increase in ROS generation and oxidative stress. For example, in sepsis, the increased generation of nitric oxide (NO) apparently reaches sufficient concentrations to compete with oxygen at complex IV and cause inhibition of mitochondrial respiration. This leads to increased ROS generation, and the superoxide that is the major species formed may in turn react with the NO, producing the more aggressively reactive peroxynitrite. This in turn may react with components of the respiratory chain, causing irreversible mitochondrial damage, which has been documented both in animal models and in patients with sepsis (4,33).

Perhaps a similar positive feedback loop occurs in patients with inherited mutations of mitochondrial DNA causing respiratory defects. The defects may not be sufficient to cause major mitochondrial impairment, but the modest impairment may be sufficient to increase ROS generation. Perhaps in many tissues and even for many years, antioxidant defenses may deal with the problem, until they are eventually overwhelmed and the increased ROS cause damage to the respiratory chain, which will increase ROS generation and so on to a slippery slope of disease manifestation and progression.

**Neuronal calcium overload and mitochondrial dysfunction in glutamate toxicity.** A model in which mitochondrial calcium overload has been shown unequivocally to play a central role in initiating cell injury is that of glutamate-induced neurotoxicity. The essence of this model is that glutamate, the major excitatory neurotransmitter of the mammalian central nervous system, accumulates in the extracellular space during periods of anoxia or ischemia. It has been clear for many years that glutamate can kill neurons in culture and that this neurotoxicity is
calcium dependent (34,35). Glutamate acts at the N-methyl-D-aspartate (NMDA) subclass of receptors and admits large amounts of calcium into the neurons. What has been less clear until recently has been the mechanism whereby a large calcium load causes cell death. Recently, it has become clear that calcium accumulation into mitochondria plays a central role in triggering the onset of calcium-dependent cell death (36,37). Indeed, inhibition of mitochondrial calcium uptake, if anything, increases the rise in cytosolic calcium concentration and yet protects the cells from death.

We were interested in the relationships between changes in intracellular calcium concentration and mitochondrial function and so measured calcium and mitochondrial potential simultaneously within individual neurons (38,39). Glutamate clearly caused a major collapse of mitochondrial membrane potential over the course of about 10 min following application at concentrations that would cause ~70% of cell death when measured 24 h later. However, it became clear very quickly that there was no apparent simple relationship between the collapse of potential and the change in intracellular calcium concentration (39). The change in calcium was essentially uniform throughout the population, whereas the change in mitochondrial function was remarkably heterogeneous in both time course and amplitude. It was also clear that the mitochondrial response, while calcium dependent, was not simply a function of the change in calcium because raising calcium to a similar degree simply by depolarizing the membrane to activate voltage-gated calcium channels never gave rise to any significant change in mitochondrial potential other than the small transient depolarization associated with a period of high calcium influx and mitochondrial uptake (39).

The simplest explanation for these observations was that the mitochondrial response depends not only on calcium uptake, but also on another factor or factors. One obvious candidate was ROS, because there is substantial literature showing that antioxidants can protect cells from glutamate-induced damage. In brief, we found no evidence for a role of ROS in glutamate-induced mitochondrial depolarization (40), and in fact we suggest that many of the protective effects of antioxidants in this model have nothing to do with ROS generation but rather with the redox modulation of the NMDA glutamate receptor, which is altered directly by the antioxidants, therefore reducing the calcium load (40,41). However, we did find that the mitochondrial response was suppressed by inhibition of nitric oxide synthase, without altering the change in intracellular calcium in response to glutamate (39). Recent work by Tymianski and colleagues (42,43) demonstrates that nNOS is held close to the NMDA receptor by the postsynaptic density anchoring protein PSD-95) in an animal model (43). In these experiments, disrupting the association between the activation of glutamate receptors and NO generation did not change the calcium load experienced by the cells when measured in culture but significantly attenuated the cell death that followed. Mitochondrial function was not examined in that study, but the association between calcium, NO, and mitochondrial damage seems clear enough.

The message therefore from these studies of two very different tissues seems to be that mitochondria may act as coincidence detectors, responding without harm to modest levels of stressors (calcium, ROS, and NO), whereas any of these in combination may cause catastrophic loss of function and initiate pathways to cell death. A similar conclusion was reached by Szalai et al. (44), who showed a similar relationship between exposure of mitochondria to calcium and to the proapoptotic ceramide. The physiological pathway of calcium uptake that has a fundamental importance for normal coordination of mitochondrial function switches into a destructive mode that leads eventually to cell death under conditions in which the calcium load combines with some other stressor.

ACKNOWLEDGMENTS
The work discussed here was supported by the Wellcome Trust, the Royal Society, and the British Heart Foundation. Work described was carried out by Julie Keelan, Olga Vergun, and Derek Hausenloy.

REFERENCES

DIABETES, VOL. 53, SUPPLEMENT 1, FEBRUARY 2004


28. Lipton SA, Sucher NJ, Pan ZH, Stamler JS: Redox state, NMDA receptor-PSD-95 protein interactions. *Cell Calcium* 31:133–141, 2002
