REVIEW ARTICLE

Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion

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Heart tissue is remarkably sensitive to oxygen deprivation. Although heart cells, like those of most tissues, rapidly adapt to anoxic conditions, relatively short periods of ischaemia and subsequent reperfusion lead to extensive tissue death during cardiac infarction. Heart tissue is not readily regenerated, and permanent heart damage is the result. Although mitochondria maintain normal heart function by providing virtually all of the heart's ATP, they are also implicated in the development of ischaemic damage. While mitochondria do provide some mechanisms that protect against ischaemic damage (such as an endogenous inhibitor of the F_1F_o-ATPase and antioxidant enzymes), they also possess a range of elements that exacerbate it, including ROS (reactive oxygen species) generators, the mitochondrial permeability transition pore, and their ability to release apoptotic factors. This review considers the process of ischaemic damage from a mitochondrial viewpoint. It considers ischaemic changes in the inner membrane complexes I–V, and how this might affect formation of ROS and high-energy phosphate production/degradation. We discuss the contribution of various mitochondrial cation channels to ionic imbalances which seem to be a major cause of reperfusion injury. The different roles of the H^+, Ca^{2+} and the various K^+ channel transporters are considered, particularly the K^+_{ATP} (ATP-dependent K^+) channels. A possible role for the mitochondrial permeability transition pore in ischaemic damage is assessed. Finally, we summarize the metabolic and pharmacological interventions that have been used to alleviate the effects of ischaemic injury, highlighting the value of these or related interventions in possible therapeutics.

Key words: F_1F_o-ATPase, ischaemia, mitochondria, reactive oxygen species (ROS), reperfusion.

INTRODUCTION

The heart is a pump that converts chemical energy into mechanical work. The power produced by cardiac muscle is generated almost entirely by the oxidation of carbon fuels with oxygen, and to a great extent the fuels are provided by coronary (myocardial) blood flow. Oxidative metabolism is the function of mitochondria within the cell, and thus the bulk of cardiac energy is supplied by oxidative phosphorylation within cardiac mitochondria.

Like many cells, when deprived of oxygen (anoxia), cardiac cells can maintain ATP levels by glycolytic ATP production, and can then revert smoothly to oxidative metabolism on reperfusion [1]. However, if blood flow is restricted, as in myocardial infarct, the cells accumulate glycolytic by-products (lactate, H^+) in addition to suffering from oxygen deprivation [2]. This is a condition known as ischaemia and can damage cardiac cells irreversibly. Paradoxically, however, the major damage to ischaemic cells comes on the re-introduction of oxygen (reperfusion). During reperfusion, the cells typically undergo further contraction (hypercontracture) and membrane damage, followed by cell death [3,4].

It is widely acknowledged that ischaemia and reperfusion lead to mitochondrial, as well as cellular, damage in cardiac cells [5–9]. Furthermore, the phenomenological description of the mitochondrial changes that occur in ischaemia and reperfusion, together with pharmacological studies on agents that protect against such changes, suggest that mitochondrial dysfunction might be important as a major causative agent in tissue injury during ischaemia and reperfusion. The aim of the current review, therefore, is to discuss the roles of the mitochondrion in the pathology of ischaemia and reperfusion injury, and as a potential site for intervention to limit the damage. In particular, we discuss the roles of the electron transfer complexes, the F_1F_o-ATP synthase, and mitochondrial ion channels, all of which normally mediate healthy mitochondrial function, as potential sites for promoting cell damage in this pathological situation. Other reviews have dealt with downstream events, such as the apoptotic pathway [10], the roles of ROS (reactive oxygen species) [11] and the long-term events of preconditioning [12].

NORMAL CARDIOMYOCYTE METABOLISM

Fatty acids provide the main energy source for the healthy heart, supplying 60–80 % of its energy requirement. The energy balance arises from the oxidation of lactate and glucose in approximately equal proportions [13]. Substrate availability largely controls fuel use: when the concentration of non-esterified 'free' fatty acids is high in plasma, intra-mitochondrial NADH and acetyl-CoA levels rise, agents that inhibit pyruvate dehydrogenase and limit the use of 3-carbon fragments.

Abbreviations used: $[Ca^{2+}]_c$, concentration of free cytoplasmic Ca^{2+}; $[Ca^{2+}]_m$, concentration of intramitochondrial free Ca^{2+}; HSP, heat-shock protein; IF_1, natural inhibitor protein of the mitochondrial F_1F_o-ATPase; iNOS, inducible nitric oxide synthase; K^+_{ATP}, ATP-dependent K^+ channel; mK^+_{ATP} mitochondrial K^+_{ATP}; MPT, mitochondrial permeability transition; $[Na^+]_c$, concentration of free cytoplasmic Na^+; PDH, pyruvate dehydrogenase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; UCP, uncoupling protein; VDAC, voltage-dependent anion channel.

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Over 90 % of heart metabolism is aerobic, and the heart is a highly oxidative tissue, with an oxygen utilization rate of 60–150 mmol/min in humans [14]. Even at ambient oxygen concentrations, some of this oxygen is partially reduced to ROS, which are potentially damaging to the cells. Estimates from *in vitro* preparations put this value at 1–2 %, although it may be lower *in vivo*. At these low levels, ROS are rapidly destroyed by mitochondrial and sarcoplasmic SODs (superoxide dismutases) and related enzymes [15]. The metabolism of normoxic heart cells is summarized in Figure 1(a).

Because of this high oxidative metabolism, heart cells have a high oxidative capacity. It has been estimated that 25–35 % of total cardiomyocyte volume is occupied by mitochondria [16]. Two forms of mitochondria have been recognized in heart cells on the basis of position, and ease of extraction, and these are termed 'interfibrillar' and 'subsarcolemmal' [17]. It is thought that interfibrillar mitochondria provide most of the ATP for the contractile apparatus, although it is unclear whether there is a significant functional difference between these classes of mitochondria.

Use of ATP by the heart is linked closely to contraction. Approximately two-thirds of the ATP hydrolysed in the cardiomyocyte is used by the contractile apparatus (actomyosin) while the remaining third is used by pumps which maintain ion balance, in particular by the sarcoplasmic reticulum Ca^{2+}-ATPase and the sarcolemmal Na^+/K^+-ATPase [18]. The ATP balance of normoxic cardiac cells is represented in Figure 2(a). The ATP used in these processes is regenerated by the mitochondrial F_1F_o-ATP synthase using the energy liberated by the respiratory chain. Oxygen utilization by the heart is correlated with contraction [19]. The connecting link is the proton-electrochemical gradient across the mitochondrial membrane, comprising a concentration gradient of approx. 1 pH unit (60 mV) and an electrical potential ($\Delta\psi$) of 140 mV (negative inside), giving a maximal $\Delta\mu_{H^+}$ (electrochemical gradient of protons) of approx. 200 mV in the aerobic heart [20].

ATP in cardiac cells is in rapid equilibrium with creatine phosphate, which acts as a temporary store of 'high-energy' phosphate bonds. The enzyme involved is creatine kinase, which is present in the mitochondrial intermembrane space and in the sarcoplasm. There have been some claims that, aside from a store of energy, this system may also promote the apparent diffusion rate of ADP to the mitochondria and thus this organization is important for the efficient functioning of oxidative phosphorylation [21].

In the short-term, the contractile mechanism of the cell is controlled by the $[Ca^{2+}]_c$ (concentration of free cytoplasmic Ca^{2+}). Beats are characterized by $[Ca^{2+}]_c$ transients, with $[Ca^{2+}]_c$ rising from approx. 100 to 500 nM [22], due to release of Ca^{2+} from the sarcoplasmic reticulum and uptake from outside the cell via the slow sarcolemmal Ca^{2+} channels (both uniports). The low level of $[Ca^{2+}]_c$ is then restored owing to the action of the sarcoplasmic Ca^{2+}-ATPase and the sarcolemmal Na^+/Ca^{2+} exchanger. These transients repeat with a period of approx. 1 s, depending on the organism.

$[Ca^{2+}]_m$ (free intramitochondrial Ca^{2+}) responds to $[Ca^{2+}]_c$ via the inner membrane Ca^{2+} uniport [23], which mediates Ca^{2+} entry, and the mitochondrial Ca^{2+}/Na^+ exchanger, which mediates Ca^{2+} exit. However, these act slowly relative to the above-mentioned transients, and the rapid changes in $[Ca^{2+}]_c$ are damped, leaving a $[Ca^{2+}]_m$ that reflects the time-averaged level of $[Ca^{2+}]_c$. These changes in $[Ca^{2+}]_m$ are known to regulate dehydrogenases of the tricarboxylic acid cycle, and certainly play a role in regulating heart metabolism [24]. However, the large Ca^{2+} capacity of the mitochondria in heart cells has led to the suggestion that,

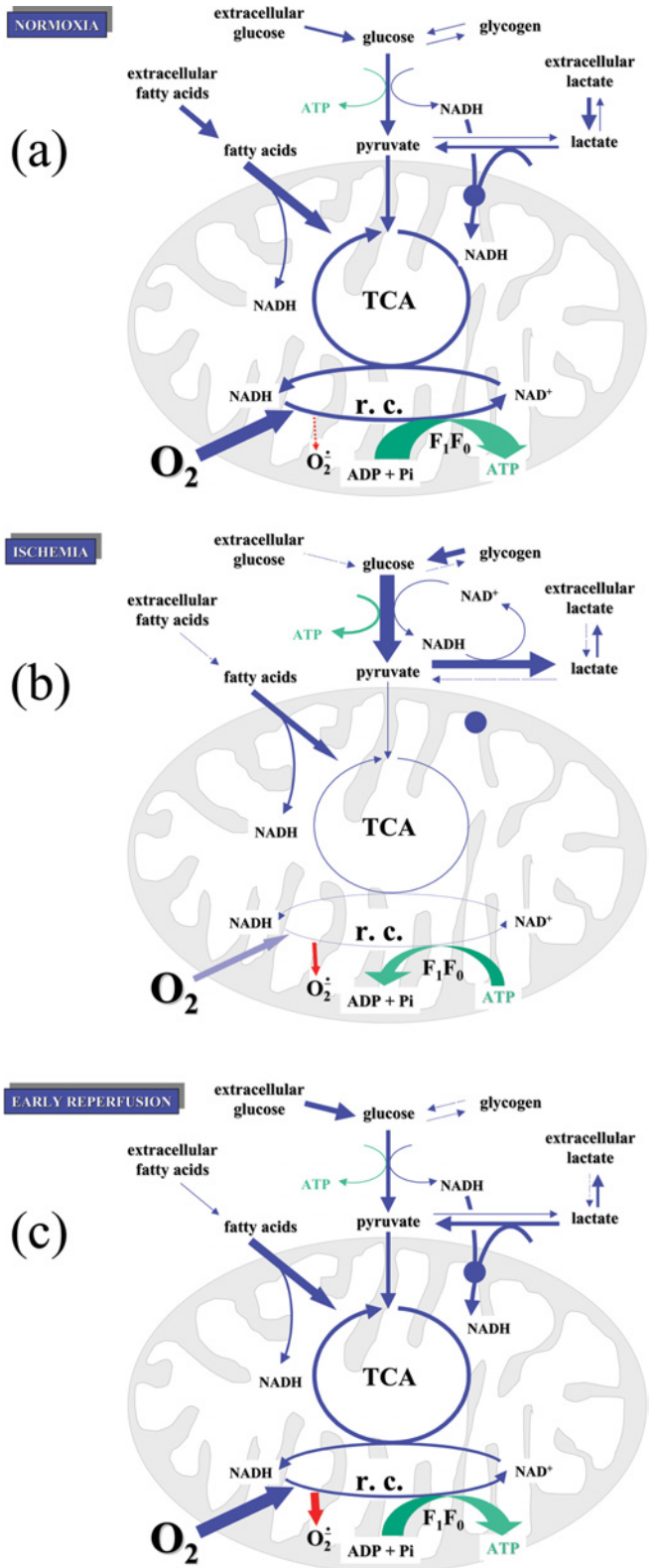

Figure 1 Fuel utilization in heart mitochondria

(**a**) Normoxia, where fatty acid oxidation provides the bulk of ATP. (**b**) Ischaemia, where glycolysis is the major ATP generator. (**c**) Early reperfusion, showing an increase in ROS production. Blue arrows indicate metabolic fluxes; green arrows indicate ATP production/hydrolysis; red arrows indicate ROS production; r.c., respiratory chain activity; O_2^- indicates all ROS. The thickness of arrows indicates the relative magnitudes of the fluxes involved. For a discussion, see the text.

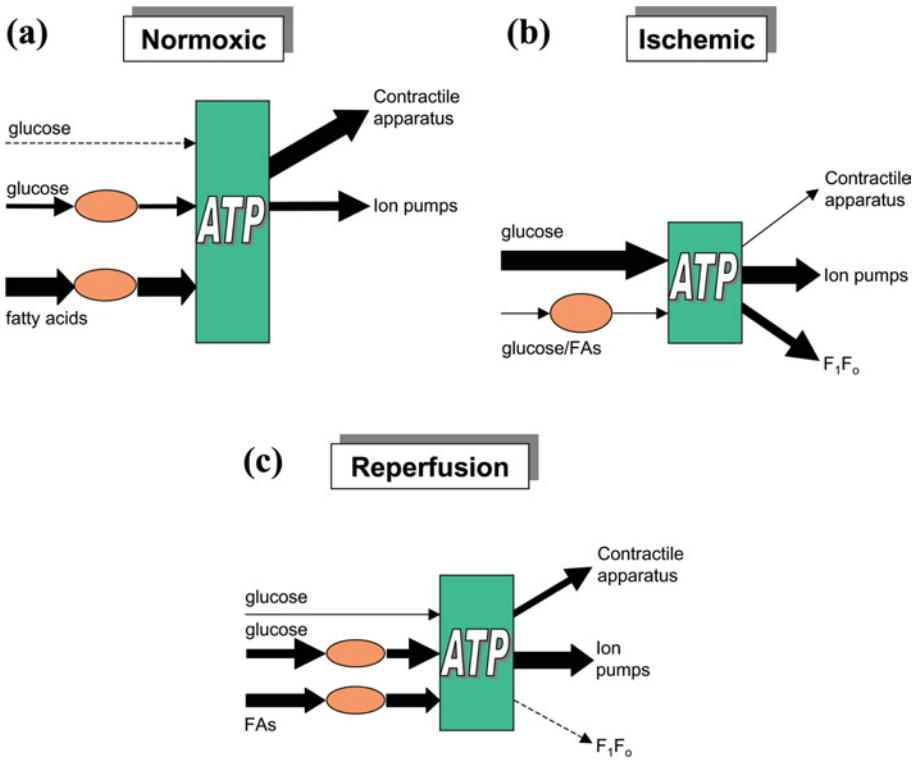

Figure 2 ATP balance in heart mitochondria

The height of the green box indicates intracellular ATP concentrations under conditions of (**a**) normoxia, (**b**) ischaemia and (**c**) reperfusion. The sources and sinks for ATP are indicated as black arrows, with the relative fluxes indicated by the thickness of the arrows. The pink ovals indicate respiratory chain function. For a discussion see the text. FAs, fatty acids.

in addition cardiac mitochondria might themselves be involved in controlling $[Ca^{2+}]_c$ [24a].

METABOLIC CHANGES IN ISCHAEMIA AND REPERFUSION

Cardiac muscle is a highly aerobic tissue. As noted above, under normal conditions, it obtains virtually all its energy from oxidative metabolism. Consequently, restriction of the blood supply to cardiac muscle has serious pathological consequences, leading to cell death in the oxygen-depleted region (infarct).

During hypoxia or ischaemia, the supply of O_2 to the respiratory chain fails. Non-esterified fatty acid levels rise, although probably as a result of lipid breakdown rather than the concomitant cessation of fatty acid oxidation [25,26]. The tricarboxylic acid cycle is blocked, and no energy is available from oxidative phosphorylation. This leads to an accumulation of cytoplasmic NADH, with the NADH/NAD^+ ratio increasing severalfold. In anoxia, ATP levels can still be maintained by glycolysis [1], but in ischaemia this is accompanied by an accumulation of lactate and a decrease in cytoplasmic pH (5.5–6 after 30 min of ischaemia) [27–29], and glycolysis is also inhibited.

The energy charge of the cardiomyocyte during ischaemia has been investigated by a number of researchers, using both classical chemical analyses, and real time analyses using ^{31}P NMR. Typically, creatine phosphate concentration falls precipitately (to less than 10 % after 10 min of ischaemia), reflecting a sharp increase in free [ADP]. ATP levels fall rather more slowly, with 40–50 % of [ATP] remaining after 30 min of ischaemia [30]. During ischaemia, the levels of total pyridine nucleotides seem to be roughly maintained, although there have been reports of significant loss (up to 30 %) of total nucleotides from the cell [31–33]. Their redox state, however, changes markedly, with [NADH] increasing sharply (as described above) [34,35]. The cytoplasmic [NADPH], in contrast, declines by approx. 30 %, resulting in a significant decrease in the NADPH/$NADP^+$ ratio. While at first this may appear surprising, the fall in [NADPH] could be due to the action of glutathione reductase, which is particularly active under conditions of oxidative stress. In addition, Hwang et al. [36] suggest that a contributory effect may come from the activation of aldose reductase, a member of the aldo-keto reductase family that utilizes NADPH to reduce carbonyl compounds, including glucose, in the metabolism of polyols. Inhibition of this enzyme promotes glycolysis and improves recovery from ischaemia.

The ionic content of the sarcoplasm also changes markedly in ischaemia. Owing to low [ATP], the sarcolemmal Na^+/K^+-ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase become ineffective, and cytoplasmic $[Na^+]$ and $[Ca^{2+}]$ rise [4]. Prolonged lack of mitochondrial oxidation will lead to abolition of $\Delta\mu_{H^+}$, and this leads to (i) a decreased activity of the mitochondrial Ca^{2+} uniport, with decreased uptake of Ca^{2+} into mitochondria, and (ii) the operation of the ATP synthase, in reverse, as an ATPase. This ATPase activity is thought to contribute significantly (35–50 %) to ATP loss in ischaemia [37], although this is disputed by some researchers [38]. The metabolism of ischaemic heart cells is summarized in Figure 1(b), and their ATP balance in Figure 2(b).

Over longer periods of ischaemia, DNA and protein synthesis are suppressed [39], although some specific proteins e.g. HSP (heat-shock protein)70, PKC (protein kinase C) ε, and iNOS (inducible nitric oxide synthase) may be induced [40,41] or repressed, e.g. ATPase ε [42]. On reperfusion, electron transfer and ATP synthesis restart, and the internal cytoplasmic pH is restored to 7.0 [2,43]. However, this leads in some way to a further deterioration of cell function. While ATP and creative phosphate levels recover to some extent, the myocytes undergo

further shortening (hypercontracture) and membrane damage, followed by cell death [4], which can be measured in beating heart preparations by the release of the intracellular enzyme, lactate dehydrogenase, into the perfusate. From studies on simulated ischaemia in cultured cardiomyocytes, it appears that both ATP depletion and the low pH are required for cell death to occur. Cell death may be due to either necrosis or apoptosis, although, using markers such as DNA fragmentation, the latter appears to be the dominant contributor [44].

Many explanations for this deterioration are linked to abnormal Ca^{2+} movements. $[Ca^{2+}]_c$ rises further, as indicated by hypercontracture [43] probably because of the reverse of the normal direction of the sarcolemmal Na^+/Ca^{2+} exchanger. This increased cytoplasmic Ca^{2+}, coupled with the restoration of mitochondrial membrane potential, leads to the accumulation of mitochondrial Ca^{2+} via the electrophoretic uniport, which has highly deleterious effects on mitochondrial function. The interaction between ions and mitochondrial function in ischaemia/reperfusion are discussed in more detail below. The metabolism of reperfused heart is summarized in Figure 1(c), and its ATP balance is shown in Figure 2(c).

Other researchers have emphasized the overproduction of ROS on reperfusion as a source of cell damage [45], and it is notable that approx. 50 % of free protein SH groups disappear, presumably owing to interference with the glutathione redox system [35]. Although cytosolic NADPH can be involved in maintaining reduced glutathione (as described above), the balance may shift towards the production of ROS by cytosolic NADPH oxidase; blocking NADPH production by inhibiting glucose-6-phosphate dehydrogenase, as well as inhibiting its re-oxidation [by NADPH oxidase or NO (nitric oxide) synthase] is, unexpectedly, protective against reperfusion injury [46].

A rather surprising observation in these systems is that reperfusion injury can be decreased by pre-treating the heart with a brief ischaemic episode (3–5 min), followed by a short recovery period, before prolonged ischaemia. This is known as 'preconditioning' the heart. Protection is maintained over a period of several hours, between the preconditioning and the prolonged ischaemic insult and in the long-term it results from the activation of PKC, and induction of proteins within the heart [47]. The link between the initial brief ischaemia, in which the metabolic changes are as described above, and the pre-conditioned state are unclear. However, there is sufficient evidence to implicate mitochondria in this process, with ROS and the mK^+_{ATP} (mitochondrial K^+_{ATP}) channels as candidates in a triggering mechanism [48–50].

MITOCHONDRIAL OXIDATION

General observations

It is widely accepted that during prolonged ischaemia, the maximum capacity for respiratory chain oxidation is decreased, with the severity of damage depending on the length of exposure. This was first shown by Jennings et al. [51], who associated morphological changes in mitochondria, with the transition from reversible to irreversible injury of heart tissue, and with the loss of ability of mitochondria to oxidize NAD-linked substrates. These findings have been confirmed by many other researchers (for a review, see Piper et al. [8]). In accordance with the decreased respiratory capacity, oxidative phosphorylation was also found to be depressed in experimental models of ischaemia and reperfusion [52–54].

Not all studies, however, show a clearcut decrease in the activity of the respiratory chain itself. Lucas and Szweda [55] reported that in mitochondria isolated from perfused Langendorff rat hearts, respiration of NAD-linked substrates was decreased after ischaemia or reperfusion. However, they attributed the decrease, not to the modest decrease in activity of the respiratory complexes, but to inactivation of matrix 2-oxoglutarate dehydrogenase and the consequent decrease in NADH supply to the respiratory chain.

Several *in vitro* studies seem to indicate that there is no decrease in respiratory activity in mitochondria after exposure to 30 min of ischaemia [30,56,57], and NMR studies of flux indicate that any damage is not sufficient to slow down coupled electron flow [58]. Indeed, recent work indicates that rates of coupled [ADP-stimulated-state III] and uncoupled 2-oxogluatarate and succinate oxidation increase following 30 min of ischaemia and 30 min of subsequent reperfusion [59]. These enhancements were attributed to increased mitochondrial matrix volume.

Minners et al. [60] reported increased rates of oxidation of endogenous substrates in various cell types after ischaemia/reoxgenation. This seems to be attributable to increased proton leakage in the mitochondria after an ischaemic insult. Taniguchi et al. [61] reported that at state IV (maximal attainable) membrane potential was approx. 10 mV lower in mitochondria isolated from hearts that were exposed to 30 min of ischaemia followed by 60 min of reperfusion, than in those isolated from control hearts (175 mV compared with 185 mV), and O_2 consumption rate (state IV respiration) was correspondingly greater in the former, 128 nmol O_2/mg per min, as compared with 77 nmol O_2/mg per min in the latter. An increased proton leak in ischaemia-damaged mitochondria was also deduced by Borutaite et al. [62] using a detailed kinetic analysis of respiration rates. Thus, it has been shown that after ischaemia/reperfusion in heart, respiration may fall, rise or remain the same. This is explicable in terms of the balance between three possibly limiting factors: (i) activity of the respiratory chain complexes themselves, (ii) proton leak at the mitochondrial inner membrane, and (iii) supply of respiratory substrates, as discussed below.

Ischaemia/reperfusion does cause some damage to respiratory chain complexes, but as these complexes are normally present in excess (having a low flux control coefficient), this damage has little effect on normal respiratory rates. Indeed, respiration rates may be observed to rise owing to an increased proton permeability of the inner-mitochondrial membrane (decreased respiratory control). This rise, however, will be dependent on an ample supply of oxidizable substrate, which may, in some conditions, be restricted and itself limit the respiratory rate observed.

Further complications in identifying a particular source of damage arise from the variety of assay methods used. Oxygen uptake, using a Clark electrode, has typically been measured either in mitochondria isolated from ischaemic heart or in skinned muscle fibres [10,63]. In the case of isolated mitochondria, the preparation may not represent the population of mitochondria in the original tissue. First, mitochondria may change, particularly in substrate and ion content, during isolation. Secondly, Jennings et al. [51] have shown that mitochondria isolated from ischaemic heart are more fragile that those isolated from normal heart. Thirdly, typical mechanical isolation procedures appear to yield largely subsarcolemmal mitochondria, while the interfibrillar mitochondria, which provide most of the energy for the contractile apparatus, are under-represented [64]. In skinned fibres, on the other hand, there may be problems with accessibility of substrates to the mitochondria [65], and respiration rates may be limited by diffusion rather than by the intrinsic activities of the enzyme involved.

In an alternative approach, Ozcan et al. [66] attempted to mimic conditions of ischaemia and reperfusion on a sample of mitochondria isolated from the heart. These conditions led to a sharp decrease of ADP-induced respiration after reperfusion, larger than

is known to occur within the tissue, owing to high levels of ROS being produced at reperfusion. These observations highlight the problems in carrying out experiments with minimal *in vitro* preparations. The observations show how respiration and ATP synthesis might be affected without the endogenous mechanisms which protect mitochondrial function in the cell; they give little indication of what actually does occur within the cell milieu.

NADH dehydrogenase (complex I)

Many researchers have identified complex I as a major site of damage to the respiratory chain in ischaemia [56,67–69]. They observed a reduction in oxidation rate for NADH-linked substrates by up to 60 %. Typically, oxidation rates with succinate were unchanged, suggesting that damage was restricted to complex I. Damage may increase on reperfusion [70].

Direct measurements of complex I activity in dog-heart mitochondria were made after ischaemia by total ligation of the left anterior descending coronary artery [67,71,72]. Activity was markedly decreased after 20–30 min of ischaemia. This may be due to effect of low pH on the enzyme, since Rouslin [73] observed that *in vitro* exposure of the enzyme to pH 6 for 1 min reduced enzyme activity by 40 % in the absence of ATP. However, the relevance of this observation to the *in vivo* situation is unclear, since 1–2 mM ATP protected complex I against this inactivation through preservation of $\Delta\psi$ and by a direct effect of the nucleotide upon the complex.

Similar effects are seen in rat heart. Veitch et al. [68] found a significant reduction in complex I activity in perfused rat hearts exposed to 20 min of global ischaemia, and Cairns et al. [69] showed that this damage was exacerbated by reperfusion. Maklashina et al. [74] reported a 40 % reduction in complex I activity upon exposure of cardiomyocytes to 30 min of nitrogen perfusion (hypoxia), and that this change was reversed on reperfusion. In contrast, Hardy et al. [56], found a specific decrease in complex I activity only upon reperfusion subsequent to hypoxia, both in mitochondria isolated from hypoxic-perfused hearts and in isolated cardiomyocytes subjected to a similar protocol of hypoxia/reperfusion.

The cause of complex I inhibition is unknown. Paradies et al. [75] associated the decrease in complex I activity under conditions of oxidative stress with the destruction of mitochondrial cardiolipin by ROS. Other researchers have suggested that NO might be the causative agent, possibly via peroxynitrite intermediates [76,77], since inhibitors of iNOS prevent the inactivation. This view is supported by Jekabsone et al. [78], who showed that complex I activity declines in isolated mitochondria exposed to NO, and that this decline is prevented by SOD. Thus either ROS or NO (or both) at the concentrations reachable during ischaemia and reperfusion could inactivate the enzyme directly, or via an effect on cardiolipin.

Thus it seems likely that the oxidative capacity of complex I does fall during ischaemia and especially during reperfusion. While some researchers have shown that NADH dehydrogenase can have a significant flux control coefficient (≈ 0.2) in isolated heart mitochondria (at limiting pyruvate/Ca^{2+} concentrations) [78a,78b], studies indicate that any such fall in complex I activity does not limit respiration *in vivo*. Sako et al. [79], using NMR measurements of ATP synthesis in isolated perfused hearts, and using pyruvate as sole energy source, found that the rate of myocardial oxygen consumption was, higher in reperfused ischaemic than in control hearts. Neubauer et al. [80] also observed high oxygen consumption rates following reperfusion after prolonged ischaemia, and recent work by Lim et al. [59], reporting studies on mitochondria rapidly isolated from perfused Langerdorff-rat hearts, showed an increase in respiration rate with both NADH-linked and succinate substrates. These researchers explained the increase in complex I turnover as a defect in coupling energy production to utilization in ischaemic/reperfused hearts, rather than as a feature of complex I itself.

Succinate dehydrogenase (complex II) and cytochrome *c* reductase (complex III)

Both complex II and complex III seem to be relatively resistant to ischaemia and reperfusion. Rouslin [67], found that complex III activity did decline in ischaemic dog heart, but more slowly than complex I activity. Its decrease paralleled that of succinate-supported oxygen uptake, implying that complex II was not affected by either ischaemia or reperfusion. Similar results were reported by Piper et al. [81] and by Veitch et al. [68] in rats. Veitch showed that 5 min of reperfusion could cause significant (34 %) loss of complex III activity after 60 min of ischaemia, and this was associated with slightly decreased content of two components of complex III, cytochromes *b* and c_1. Hoppel et al. [82] confirmed loss of complex III activity in both subsarcolemmal and interfibrillar mitochondria, but showed that the decreased activity proceeded via impairment of the iron–sulphur subunit without the loss of the apoprotein. However Petrosillo et al. [83] showed that loss of complex III activity paralleled the loss of cardiolipin from mitochondria and proposed that a substantial contribution to respiratory inhibition should be ascribed to cardiolipin degradation owing to oxidative attack by oxygen free radicals. Guidarelli and Cantoni [84] showed that peroxynitrite caused inhibition of complex III activity in cultured cells, and this too may contribute.

The above researchers do not suggest any significant effect of ischaemia/reperfusion directly on the succinate dehydrogenase enzyme, and indeed Lim et al. [59] showed that this enzyme was not affected. However, Abe et al. [76] reported that rat hearts exposed to ischaemia (20 min) and subsequent reperfusion (20 min) had a significantly decreased complex II activity, which was less than, but in addition to, a decrease in complex I activity. This decrease was attributed to NO and ROS, generated within the myocytes, inactivating the enzyme.

Cytochrome *c* oxidase (complex IV)

The situation regarding complex IV is more complicated. Several reports indicate it to be virtually unchanged by ischaemia [57,67,68,72,81]. Veitch et al. [68] showed that the cytochrome aa_3 content of complex IV was unaffected by ischaemia for up to 60 min, and a further 30 min of reperfusion (although approx. 25 % seemed to be lost during 60 min of ischaemia and 60 min of reperfusion). Borutaite et al. [85] and Lesnefsky et al. [86] showed that the maximal catalytic capacity of cytochrome oxidase, and its apparent K_m for oxygen, was unchanged after an ischaemic insult.

However, there did appear to be reduced electron flow through complex IV both in permeabilized ischaemic tissue and mitochondria isolated from ischaemic heart [85,86]. This may be attributable to the loss of cytochrome *c*, which mediates electron transfer between complexes III and IV, from the intermembrane space in subsarcolemmal mitochondria during ischaemia. Borutaite et al. [85] claimed that cytochrome *c* oxidase activity decreased by nearly 30 % during ischaemia (from 0.156 to 0.111 nmol/mg of protein) and that full respiration rates are restored by external addition of cytochrome *c*. These authors also showed that cytochrome *c* is found in the perfusate of hearts, following ischaemia/reperfusion [85], suggesting it can indeed be lost from mitochondria in pathological situations. In contrast,

Veitch et al. [68] claim that cytochrome *c* content of mitochondria is not significantly changed after ischaemia.

Other researchers suggest complex IV may be damaged more directly [85,86]. Paradies et al. [87] showed that, even though cytochrome aa_3 levels did not change, the catalytic capacity of cytochrome oxidase was reduced by 25 % after 25 min of ischaemia, and fell further, to 51 %, after another 15 min of reperfusion. Other authors [69] observed that cardiac ischaemia and early reperfusion, induced an increased cytochrome aa_3(ox)/cytochrome aa_3 (red) ratio, indicating inhibition of electron input to this complex. Surprisingly, they showed that the correct redox balance could be restored by post-ischaemic administration of succinate, rather than by cytochrome *c*. Most damage sustained directly by complex IV seems to occur after ischaemia, and during reperfusion. Possible mechanisms include loss/oxidation of cardiolipin [87,88], or formation of 4-hydroxynonenal adducts [89]. Approx. 50 % of complex IV activity might be lost in this way.

The effect of inhibition of cytochrome oxidase, either by direct damage or by loss of cytochrome *c*, might not be expected to have a major effect on respiratory rates *in vivo*. This enzyme is normally present in excess of respiratory demand and has a very low flux control coefficient [78a,78b], thus limited inhibition would seem unlikely to be a factor in decreasing oxidative phosphorylation in post-ischaemic conditions. However, Gnaiger and co-workers [90,91] have pointed out that assays for cytochrome oxidase activity are normally carried out under conditions far from physiological. At the low oxygen tension which might prevail *in vivo*, cytochrome oxidase activity may in fact become a rate-limiting factor, as has been demonstrated by Kunz et al. [92]. Thus the restriction on the rate of respiration caused by loss of cytochrome oxidase activity, either directly or by cytochrome *c* depletion, may represent a pathological effect.

In this discussion of the effects of ischaemia/reperfusion on the respiratory chain, the reader will note that there is considerable variation between observations from different groups. Factors involved appear to include the species of animal involved, its age, the degree of anoxia in different models of ischaemia and even the different techniques used to collect data. This implies that the relative importance of several variables may change between preparations. Nonetheless, a consensus would indicate that exposing hearts to 15–60 min of ischaemia leads to a modestly impaired electron transfer chain, with rates reduced by approx. 30 % relative to the control value. Complex I appears to be damaged, and there is some restriction of electron flow through complex IV, possibly because of loss of cytochrome *c*. During reperfusion, further damage to electron transfer components occurs, in particular to complex IV, possibly due to the action of ROS (as described below), and a 'vicious cycle' in which further inhibition leads to further ROS production, and so on, is a possible cause of irreversible damage to mitochondria.

OXYGEN FREE RADICALS: ROS IN ISCHAEMIA AND REPERFUSION

Cardiomyocytes, like many other types of cell, produce ROS. It has been estimated that, under normal physiological conditions, 1–2 % of electron flow through the mitochondrial respiratory chain gives rise to ROS [91,93]. It might be expected that ischaemia, caused by low partial pressure of O_2, would decrease ROS production, but this is paradoxically increased [30,94,95], with a further increase occurring on reperfusion [30,96]. Cell damage can occur through mechanisms involving lipid peroxidation, covalent modification of protein (particularly on -SH groups) and mitochondrial DNA oxidation (for a review, see [97]). Certain protective responses are induced by low levels of ROS, notably gene transcription and cell proliferation [98–100], but higher levels ultimately lead to cell death by apoptosis or necrosis [101,102].

Under normal conditions, scavenging mechanisms operate swiftly to remove excess ROS. Superoxide (O_2^-) is removed by SOD in cytosol (Cu/Zn-SOD) and in mitochondria (Mn-SOD) [103], and the resultant H_2O_2 is removed by catalase, glutathione peroxidase and peroxiredoxin [104,104a,104b]. However, elevated levels of ROS can swamp the protective mechanisms of the cell, and are a significant cause of ischaemic/reperfusion damage. The importance of ROS in inducing ischaemic damage is shown by a protective effect in perfused hearts of transgenic mice overexpressing Mn-SOD [105] and by a study showing that post-ischaemic recovery of contractile function is impaired in Mn-SOD-knockout mice [106]. Direct measurements of SOD levels have, unfortunately, yielded equivocal results, with Ueta et al. [107] indicating a decrease in SOD in ischaemia, but more recent work indicating increased activity associated with mitochondrial protection and a reduction in apoptosis, possibly through a mechanism involving HSP72 [108].

In general, ROS may arise from the operation of cytoplasmic xanthine oxidase. In heart, however, mitochondria probably constitute the principal source of ROS, since the respiratory chain deals with most of the electrons potentially capable of reducing O_2 [95,109]. Redox components of the respiratory chain, particularly impaired complexes I and III in their reduced state, have been shown to produce ROS [110,111], possibly via ubisemiquinone radicals [112]. Complex I, in particular, is impaired during ischaemia/reperfusion (as described above), and may be considered as the source of the damaging radicals, although experiments using the electron transfer inhibitors amytal and myxathiazole suggest complex III as a major site of ROS production during ischaemia [95]. Recent studies indicate that complex II may also produce ROS [112a], particularly if certain mutations are present in regions of the CoQ (ubiquinone) or the FAD-binding sites [113,114].

In conclusion, cardiac ischaemia induces increased generation of ROS, and subsequent reperfusion can result in toxic ROS overproduction that possibly contributes to irreversible damage of mitochondrial function and consequent impaired recovery of physiological function and cell death.

Nitric oxide

NO is an endogenous mediator of several important physiological processes and it is involved in a number of protective mechanisms in cells [115]. However, overproduction of NO can occur and this can lead to cytotoxicity. The balance between protective and deleterious effects of NO has led to difficulties in assessing its role(s) in ischaemia/reperfusion. NO levels do rise during ischaemia and reperfusion and, under these conditions, NO can interfere with mitochondrial functions [76]. Thus NO can induce cell death by necrosis through inhibition of mitochondrial respiration [116,117], or trigger apoptosis mediated by the mitochondrial permeability transition and by cytochrome *c* release [118].

NO certainly inhibits respiration in mitochondria. It has a direct effect on cytochrome *c* oxidase, binding to the oxygen-binding site and hence inhibiting respiration. It is unlikely, however, that this type of inhibition is greatly damaging in ischaemia/reperfusion, since it is competitive and readily reversed when the inhibited enzyme is exposed to oxygen [119]. A more likely source of respiratory chain inhibition might come indirectly, by its forming covalent, peroxynitrite or S-nitrosothiol derivatives with proteins, and particularly with components of complex I or III [77,84]. Ubiquinol, which can reduce peroxynitrite, is believed to protect

mitochondria against such damage to some extent [120], but with increasing amounts of peroxynitrite, such modifications are likely to arise, and will be irreversible in the physiological milieu.

Whether these changes lead to apoptosis, or other cell death mechanisms, is, however, controversial [121,122]. Several other researchers have demonstrated protective effects of NO in ischaemia and reperfusion. Rakhit et al. [123] found that rat ventricular myocytes in culture were protected by NO donors when the cells were exposed to 1 h of simulated ischaemia and 1 h of reperfusion. Protective effects have also been demonstrated in perfused mouse heart subjected to ischaemia and reperfusion [124]. A protective role for NO against loss of mitochondrial $\Delta\psi$ and apoptosis was also shown by Beltran et al. [125], albeit in the largely glycolytic Jurkat cells.

The main site of NO synthesis in cells seems to be extramitochondrial, as the activity of mitochondrial NO synthase is low. Thus intramitochondrial NO presumably changes in response to cytoplasmic NO [126]. These levels appear to be buffered by the formation of nitrosyl–haem complexes which form in significant amounts in the ischaemic heart [127]. However, it has also been reported that the mitochondrial NOS is functionally upregulated (2-fold increase) after hypoxia in animal models [128], implying a role for the mitochondrial enzyme in tissue protection.

ATP SYNTHASE

Heart muscle is essentially an aerobic tissue and, under normal circumstances, over 90 % of its ATP is made by oxidative phosphorylation, and hence by the F_1F_o-ATP synthase. This is a complex enzyme, comprising a transmembrane H^+ channel (F_o), and a nucleotide-binding extrinsic sector (F_1). Two inhibitory proteins, the $H^+/\Delta\psi$-dependent IF_1 (natural inhibitor protein of the mitochondrial F_1F_o-ATPase), and the Ca^{2+}-binding ATPase-inhibitor protein, are believed to associate with the ATP synthase (for reviews, see [129,130]). Ca^{2+}-activation of the ATP synthase has been demonstrated with increased work load/respiratory rate in rat and pig heart [1,131], although the quantitative importance of this effect in normal function is uncertain [132,133].

In the context of the current review, however, an important feature of the F_1F_o-ATP synthase is its reversibility. The synthase can hydrolyse ATP and use the energy released to pump protons out of the mitochondrial matrix, increasing $\Delta\psi$. Using oligomycin as a specific inhibitor of the ATP synthase, it has been shown that, in ischaemia, a considerable fraction (30–50 %) of the decline in cytoplasmic high energy phosphates is due to hydrolysis by the ATP synthase working in reverse [37,67]. Although it is possible that limited reversal of the ATP synthase activity may have some useful (but unknown) role *in vivo*, this precipitous loss of, for example, over 95 % of creatine phosphate within 5 min is clearly deleterious to the cell.

The tendency of the F_1F_o-ATP synthase to reverse during ischaemia thus appears to be an inescapable consequence of the thermodynamics of the system, and in the absence of the energy-yielding reactions of electron transfer, ATP synthesis is unfavourable and hydrolysis is favoured. However, there does appear to be some endogenous regulatory mechanism restricting hydrolysis by the ATP synthase. Several groups have shown that the rate of ATP hydrolysis by mitochondria from ischaemic hearts or cardiomyocytes is decreased below that of mitochondria from normoxic hearts in dogs [134–137] and humans [132]. There has been some dispute as to whether the same inhibition was observed in rat heart, but this seems to be the case [1,27]. A study by Ylitalo et al. [132] shows that failure by some researchers to observe the effect in rat was probably due to lack of pH control in the isolation media.

Typically, ischaemia-induced inhibition of F_1F_o-ATPase activity *in vitro* is approx. 50 % of maximal activity (although the procedures used, involving extracting mitochondria from the heart muscle, may underestimate inhibition *in vivo*), with inhibition developing over 2–5 min. The kinetics and pH dependence of the effect suggests that inhibition is due to combination of the F_1F_o-ATPase with the IF_1 protein.

Inhibition of wasteful ATP hydrolysis during ischaemia might be assumed to have a beneficial effect on the heart. During reperfusion, when the ATP synthase should be working to synthesize ATP, however, inhibition of this enzyme would presumably delay recovery. This is explicable in terms of the properties of IF_1, which is known to bind to F_1F_o at low pH and low $\Delta\psi$ (such as occur in ischaemia), but to be released at high pH and high $\Delta\psi$ (such as occurs during electron transfer) [138], acting, effectively, as a one-way valve. Thus, during reperfusion, we would expect F_1F_o to be re-activated. This reversibility of inhibition, again on a 2–5 min time scale, has been demonstrated in dog, rat and goat hearts [37,129,132,139,140]. Thus the active/inactive transitions of the F_1F_o-ATP synthase do not contribute directly to the adverse effects of reperfusion, although it is likely that ATP hydrolysis by the synthase again becomes significant if the mitochondria undergo a permeability transition, and consequent depolarization, after reperfusion (see below).

Preconditioning is a phenomenon in which a brief period of ischaemia will protect the heart against subsequent prolonged periods of ischaemia/reperfusion. Preconditioned hearts show decreased tissue damage, and increased recovery of high-energy phosphates and contractile activity during reperfusion after an ischaemic insult. However, it is difficult to relate this unambiguously to changes in F_1F_o activity. There are conflicting observations even as to the fate of ATP in pre-conditioned hearts. Jennings and co-workers [136,141] and Hassinen et al. [142] show a decrease in ischaemic ATP loss after preconditioning in dog and rat heart respectively, while Green et al. [143] show a greater loss in rat heart.

In view of the time scale of changes in ATP synthase capacity, which occur over a 2–5 min time period, it seems unlikely that the F_1F_o-ATP synthase, or IF_1, are involved in the phenomenon of preconditioning. This view is supported by the work of Jennings and co-workers [136] and Green et al. [143]. However, Hassinen and co-workers found that the drop in the hydrolytic capacity of the F_1F_o-ATPase induced by ischaemia was greater after preconditioning than in untreated rat heart [27,144], and other researchers [136,140] have confirmed that, at least, a transient inhibition of F_1F_o is induced by preconditioning. Although the reason for this increased inhibition is uncertain, it may indicate that the F_1F_o-ATP synthase does have some role to play in the preconditioning phenomenon.

MITOCHONDRIAL MEMBRANE POTENTIAL AND ION CHANNELS

The inner-mitochondrial membrane has a limited, and highly selective, permeability to ions. There exists an extensive family of anion transporters that are largely antiports (P_i/OH^-, ATP/ADP, malate/aspartate exchanger, etc.) [145], which are involved with substrate import. It is probable that these, in their normal form, do not contribute specifically to ischaemic/reperfusion damage, although the ability of the ATP/ADP translocator, when modified, to participate in the mitochondrial permeability pore may be important in promoting cell death in the longer term (as described below). It is in the area of cation transport (H^+, Ca^{2+}, K^+) that most attention has been focused.

Since the energy released by the respiratory chain is stored as an $\Delta\mu_{H^+}$, the permeability of the mitochondrial membrane to H^+ is

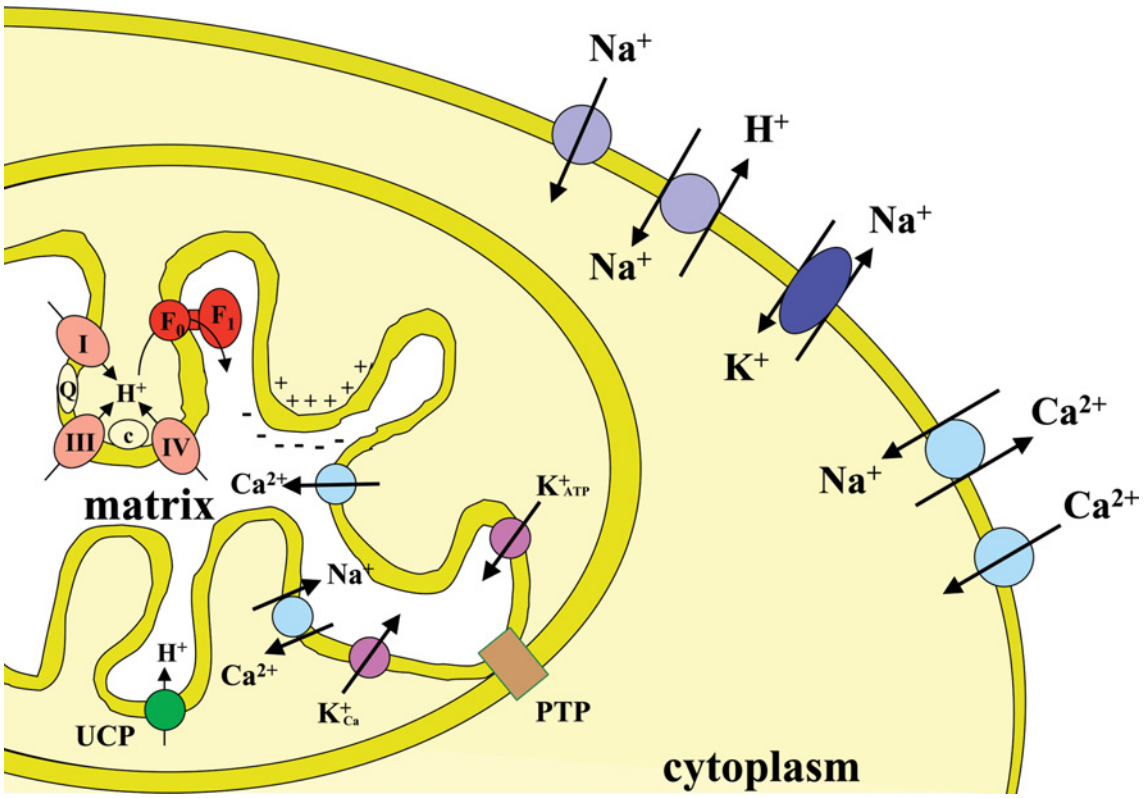

Figure 3 Cation transporters in heart cells

Physiological fluxes of ions are indicated by the arrows. Circles refer either to uniports or antiports. Ellipses indicate ATP-coupled transport systems. Inner membrane respiratory chain complexes are denoted I, III and IV; *c*, cytochrome *c* oxidase; Q, ubiquinone; PTP, permeability transition pore.

necessarily limited. Below dielectric breakdown, H^+ can enter the mitochondrion only via the F_o channel of the F_1F_o-ATP synthase or via controllable UCPs (uncoupling proteins) [146]. The other main routes for cation entry are the Ca^{2+} uniport (which is opposed by an Na^+/Ca^{2+} exchanger), the low capacity K^+_{ATP} channels and the recently described K^+_{Ca} channel [147] (Figure 3).

H^+ movements

On a simple model, it is expected that, during ischaemia (as mitochondrial electron transfer is abolished) $\Delta\mu_{H^+}$ will fall rapidly. It should then rise during reperfusion, when electron transfer is restored. While this seems in principle to be the case, investigations reveal a greater complexity. First, in ischaemia, a mitochondrial $\Delta\psi$ is maintained for some minutes [148] due to the hydrolysis of cytoplasmic ATP by the F_1F_o-ATP synthase acting in reverse as an ATP driven pump [37,67]. This hydrolysis will decrease cellular ATP levels, and is gradually switched off by a regulatory element at the level of the ATP synthase (as described above). It is unclear whether the maintenance of $\Delta\psi$ is a physiologically useful phenomenon (e.g. in maintaining ion distributions), or whether this ATP hydrolysis is an unavoidable pathological process.

Conversely, during reperfusion, we should expect $\Delta\mu_{H^+}$ to recover rapidly. Again, however, moderating factors seem to be operating. A number of researchers have reported that, after reperfusion, $\Delta\psi$ is depressed from its control value, and that mitochondria have an increased proton leak. This was originally demonstrated by analysis of flow force relationships in isolated heart mitochondria after ischaemia (and/or reperfusion) [56,85,149, 150]. However, it also seems to be the case in heart itself, as indicated by an increased rate of O_2 utilization relative to work output (i.e. a decreased cardiac efficiency) on reperfusion [151]. It is possible, again, that this change represents either unavoidable mitochondrial damage during ischaemia/reperfusion or the preferential use of oxidative energy to restore ion concentrations. However, a number of researchers have suggested that the depression of $\Delta\psi$ may play a protective role during early reperfusion, limiting the electrophoretic influx of Ca^{2+} and/or decreasing the production of ROS by facilitating electron transfer (see below). Agents that promote the leakage of protons into mitochondria, such as uncouplers [152,153], or transfection with UCPs [154,155] have been shown to protect against ischaemia/reperfusion injury.

Ca^{2+} movements

The Ca^{2+} uniport acts electrophoretically, and is thus dependent on the $\Delta\psi$ component of the electrochemical potential. Under normal conditions, $[Ca^{2+}]_c$ in the sarcoplasm, like $[H^+]_c$, is approx. 10^{-7} M. Under these conditions, the low affinity of the Ca^{2+} uniport for Ca^{2+} (and the operation of the Na^+/Ca^{2+} exchanger) means that $[Ca^{2+}]_m$ is slightly lower than $[Ca^{2+}]_c$ [156]. Under normal physiological conditions, $[Ca^{2+}]_c$ would rise with increasing work load, and $[Ca^{2+}]_m$ would mirror this change. The rise in $[Ca^{2+}]_m$ leads to an increase in activity of the dehydrogenases of the tricarboxylic acid cycle [157] and increased oxidation rates. In heart muscle, where mitochondria make up 25–35 % of cell volume, it is also believed that mitochondria can play an active role in modulating $[Ca^{2+}]_c$ [158].

Although total myocardial Ca^{2+} does not change during ischaemia [63], $[Ca^{2+}]_c$ increases significantly as ATP-driven

pumps (notably the sarcoplasmic Ca^{2+} pump) become less active. $[Ca^{2+}]_m$ will also tend to rise, but this rise is limited by the lowered mitochondrial membrane potential. The major increase in $[Ca^{2+}]_m$ occurs on reperfusion, when the mitochondrial membrane repolarizes [159]. A study in guinea-pig heart by Varadarajan et al. [160] showed that during ischaemia, $[Ca^{2+}]_m$ rises only by approx. 50 %, whereas the increase was 4–5-fold during reperfusion. They also showed that this high level was maintained for up to 1 h during perfusion, even though $[Ca^{2+}]_c$ returned to normal within 10 min, suggesting that ischaemia had induced a chronic problem in mitochondrial Ca^{2+} handling.

It seems likely that these abnormal Ca^{2+} distributions are a major contributor to ischaemia/reperfusion cell damage. A number of researchers have shown that if Ca^{2+} entry into mitochondria is blocked, cell damage (or infarct size, in heart preparations) is much reduced. Ruthenium Red, which blocks the mitochondrial Ca^{2+} uniport, has been shown to have a protective effect [161,162], and other, less direct effectors that have a protective effect, also oppose the mitochondrial Ca^{2+} uptake. These effectors include inhibitors of the sarcolemmal Na^+/H^+ exchanger [150,163], uncouplers [153] and low $[Ca^{2+}]$ media [164]. Arieli et al. [165] have recently shown that cardiac mitochondria from male rats are more susceptible to Ca^{2+}-induced depolarization than females, and suggest that this may account for the higher susceptibility of males to ischaemic heart damage.

K^+ movements

In recent years, there has been considerable interest in the mK^+_{ATP} in the context of ischaemia/reperfusion. Such channels allow K^+ entry and are blocked by ATP. Thus, in the absence of ATP (e.g. in ischaemia), mK^+_{ATP} should open, leading to a decrease in $\Delta\psi$ and an interior alkalinization of the mitochondrion ($\Delta\mu_{H^+}$ remaining constant). There is evidence for a K^+_{ATP} channel in heart mitochondria [166], and its molecular composition, although not yet clear, is different from other cytomembrane K^+_{ATP} channels [167]. Its role, however, is controversial, as it appears to have a low capacity, and it has been difficult to demonstrate significant changes in $\Delta\psi$ whether it is open or blocked [168].

Pharmacological evidence indicates that the mK^+_{ATP} channel in heart exerts its effects on the phenomenon of preconditioning. This is a protective effect, observed as a result of subjecting a heart to a brief period of hypoxia, before the major ischaemic insult. A number of researchers have reported that diazoxide (which opens the mK^+_{ATP} channel) will induce the same protection as preconditioning, while 5-hydroxydecanoate, (which blocks the channel), will abolish its protective effect [169–171]. It is proposed that these effects are due to the brief release of ROS, induced by some unknown mechanism, on opening the K^+_{ATP} channel, and this in turn triggers a variety of long-term protective responses, which are outside the scope of this review. ROS production is indeed induced in intact heart cells by diazoxide [172], although paradoxically this agent lowers ROS in mitochondrial preparations [173]. This work has recently been reviewed by O'Rourke [174].

Other researchers have questioned the role, or even the existence, of functioning K^+_{ATP} channels in heart mitochondria. Askenasy et al. [175] could not demonstrate an effect of K^+_{ATP} channel blockers in rat heart. Diazoxide and hydroxydecanoate have been shown to inhibit or activate mitochondrial electron transfer respectively [176,177], and it could be that these agents exert their effects on preconditioning independently of any effect on the mK^+_{ATP} channel. Diazoxide is also known to promote IF_1-binding to F_1, which could itself be cardioprotective [178]. Dos Santos et al. [179], and Ganote and Armstrong [180], have emphasized possible roles of the mK^+_{ATP} channel in maintaining mitochondrial volume in cells, while Das et al. [181] were unable to demonstrate any effects of a putative K^+_{ATP} channel on the volume of isolated rat heart mitochondria. As yet, therefore, any role of the K^+_{ATP} channel in ischaemia/reperfusion injury should be viewed with caution, especially in view of the recent report of a mitochondrial K^+_{Ca} channel, which might link K^+ movements with Ca^{2+} levels in heart cells [182] (Figure 3).

Na^+ movements

Abnormal levels of other ions are also known to contribute to ischaemia/reperfusion damage. The best known, perhaps, is Na^+. NMR and probe studies indicate that intracellular $[Na^+]$ roughly doubles in perfused heart during a 20–30 min period of ischaemia, with a further increase of 50–150 % during a 30–60 min period of reperfusion [163,183,184]. Reperfusion, in fact, induces periodic Ca^{2+} transients which lead to hypercontracture and mechanical damage to the myocyte [4]. Inhibitor studies indicate that the bulk of the Na^+ ions enter the cell through the sarcolemmal Na^+/H^+ exchanger [150,185], probably as a response to lowered internal pH during ischaemia and reperfusion [186], although there is some contribution from fast Na^+ channels, and a lowered Na^+/K^+ pump activity [173]. During reperfusion, Na^+ continues to move into the cell (through both the Na^+/H^+ exchanger and the Na^+/HCO_3^- symport) to relieve the acid load that has built up [187].

Although this increased $[Na^+]_c$ (concentration of cytoplasmic free Na^+) is damaging to heart cells, its action is not simple. Mitochondria may be affected directly by Na^+ acting on mitochondrial swelling and inducing abnormal mitochondrial permeability, and such effects of Na^+ can indeed be demonstrated in isolated mitochondria [163]. However, the observed damage is more likely due to the indirect effects of $[Na^+]_c$ on both proton and Ca^{2+} fluxes. After ischaemia, the raised $[Na^+]_c$ causes a reversal of the sarcolemmal Na^+/Ca^{2+} exchanger, leading to a raised $[Ca^{2+}]_c$ and hence to the problems of Ca^{2+} overload discussed above [188–190]. This view is supported by the observation that decreased levels of Ca^{2+} in the perfusate ameliorate the effects of high $[Na^+]_c$ [191]. However, too rapid a recovery of internal pH also seems to be damaging. Reperfusion damage can be reduced by slowing down the recovery of internal pH (i.e. keeping pH_i low), by inhibiting the Na^+/H^+ exchanger and the Na^+/HCO_3^- symport, possibly because this low pH inhibits the Ca^{2+} transients [4].

MPT (mitochondrial permeability transition)

When mitochondria are exposed to pathologically high levels of $[Ca^{2+}]_m$ and various synergistic effectors they abruptly develop permeability to a range of small molecules (< 1500 Da). This is known as the MPT. This leads to uncoupling, swelling, and loss of nucleotides (especially NAD^+ and ADP) and other small molecules from the mitochondrial matrix [192]. ROS and non-esterified fatty acids are also known to promote the MPT. If the transition is prolonged, it is presumably pathological, as mitochondria so permeabilized will be unable to retain substrates or $\Delta\psi$, ATP and NAD^+ will be hydrolysed, and the cell will die by necrosis.

The MPT pore seems to be formed from an assembly of otherwise innocuous mitochondrial proteins, including peptidyl prolyl isomerase (cyclophilin D) from the mitochondrial matrix, the adenine nucleotide carrier from the inner membrane and the VDAC (voltage-dependent anion channel) of the outer membrane [193]. However, it is not known how these components assemble to make the pore, nor whether other proteins are involved. Recent studies using microarray analysis have suggested that the

pro-apoptotic BNIP3 (Bcl2/adenovirus E1B 19 kDa protein interacting protein) might contribute to the pore [194]. However, none of the known components would be expected to confer Ca^{2+} sensitivity on the system. One possibility for modulating the pore in a Ca^{2+}-dependent manner is PKC. Baines et al. [195] have shown that PKCε might phosphorylate VDAC in the pore complex. Since the conditions of high $[Ca^{2+}]_m$ and raised levels of ROS and non-esterified fatty acids, which promote the MPT, are also conditions that promote cell death on ischaemia/reperfusion, it seems likely that this transition is the cause of cell death under these conditions.

This view is supported by the ability of cyclosporin, which specifically prevents the MPT, to protect against cell death in ischaemia/reperfusion [196–198]. In perfused heart, using [^{3}H]-deoxyglucose as a marker for MPT in mitochondria, Halestrap et al. [199] have shown that the mitochondrial pore remains largely closed during ischaemia, but opens during the reperfusion period, which is when most cell death occurs. While it seems likely that much cell death following ischaemia/reperfusion is due to the relatively disordered process of necrosis, apoptosis may also be important [200,201], and a precise estimate of the relative importance of each may well depend on the marker used for apoptosis [202]. Borutaite et al. [10,203] have shown that typical apoptotic changes (cytochrome *c* release, caspase activation, DNA strand breaks) begin during ischaemia in heart, and intensify during reperfusion. Again, these changes have been pharmacologically linked to the MPT via cyclosporin inhibition [204], although it is still uncertain how this transition may be linked to the early event of cytochrome *c* release from the intermembrane space. In an attractive model, Halestrap et al. [199] suggest that a transient opening of the MPT pore (e.g in the areas surrounding an infarct) might lead to apoptosis (which requires ATP) whereas a prolonged opening would lead to necrosis (which occurs in the absence of ATP).

METABOLIC AND PHARMACOLOGICAL INTERVENTIONS IN ISCHAEMIA AND REPERFUSION

The effects of ischaemia and reperfusion on the cardiac cell are profound. In the cytoplasm, there is a decrease in energy charge, an increase in the NADH/NAD^+ ratio and a general loss of nucleotides. The cytoplasmic pH falls, and $[Na^+]_c$ and $[Ca^{2+}]_c$ both rise, constituting serious ionic imbalances. There is an increased production of ROS and of long-chain non-esterified fatty acids, and the NADPH/glutathione system becomes more oxidized, leading to protein damage. All of these changes have an impact on mitochondrial function leading to inhibition and uncoupling of electron flow, activation of the mitochondrial permeability transition, and loss of intermembrane cytochrome *c*. We have argued above that the mitochondrion is a major participant in these effects, and also that it might be the final arbiter of cell death in the apoptotic or necrotic changes that occur in myocardial infarct.

Whatever the precise sequence of events leading to cell death, it is certainly the case that treatments that oppose the above changes can be usefully employed to correct against ischaemic/reperfusion injury. Actions aimed to increase the energy charge, to render the relatively high negative redox potential less negative, to increase pH, and to restore the physiological concentration of ions in cell compartments are helpful to promote cardiac cell survival and recovery. Similarly, agents which minimize the cytotoxic effects of harmful agents such as ROS and long-chain fatty acids may be protective. In many systems, studying the effects of such agents (e.g. specific inhibitors) has helped us to understand, as well as to treat the defect, although such studies in heart have on balance arguably been confusing rather than aiding an understanding of the primary cause of ischaemia/reperfusion injury. Various treatments that have been applied are summarized in Table 1.

Metabolic interventions

Under normal circumstances, the mammalian heart relies largely on mitochondrial fatty acid oxidation for energy (for a review, see [14]). However, it exhibits a dynamic plasticity related to energy substrate preferences and can switch seamlessly to glycolytic production of ATP under hypoxic conditions [1,247]. Since mitochondrial function is impaired at the start of reperfusion, metabolic interventions that promote glucose catabolism over fatty acid oxidation should be beneficial.

Clinical and experimental evidence clearly indicate the beneficial effect of glucose–insulin–potassium administration in reducing ischaemic damage [248]. Glucose can generate ATP glycolytically and will promote the production of malonyl-CoA, which in turn inhibits carnitine palmitoyltransferase-1 and hence fatty acid oxidation in mitochondria [217]. Correspondingly, increased myocardial glycogen will also protect against ischaemia/reperfusion damage [249], and species such as the harp seal, with myocardial glycogen levels at 10 times that in the rat, are resistant to ischaemic insults [250].

The seemingly paradoxical observation that fasting is protective against ischaemic/reperfusion damage [207,251] can be explained by the fact that fasting, in fact, increases heart glycogen levels [252]. Insulin itself may also have a direct protective effect, since it is known to protect cardiac cells from apoptosis [253]. Other important metabolic approaches to the treatment of ischaemic heart disease are reviewed by Wolff et al. [254] and by Stanley [13]. An interesting study by Weinberg et al. [255] indicates that in kidney, at least, deleterious effects of hypoxia/reperfusion can be alleviated by perfusion with α-oxoglutarate and aspartate (generating ATP via anaerobic operation of the tricarboxylic acid cycle), and the authors suggest that this might be applicable to heart. Along these lines, oral supplementation with mixed essential amino acids appears to protect rat heart exposed to ischaemia and reperfusion [256]. It is possible that the preservation of mitochondrial production of high-energy phosphates plays a role in the attenuation of ischaemia and reperfusion injury.

A particularly intriguing metabolic intervention, which clearly protects the heart against ischaemia/reperfusion injury, is a brief period of ischaemia itself. This is the phenomenon of preconditioning, discussed above. Although a variety of metabolic changes occur during the preconditioning period, they mirror the changes occurring during a damaging bout of ischaemia, and it is difficult to identify factors generated during a short period of ischaemia that would protect against a longer one. Acute factors, such as K^+_{ATP} channel opening and F_1 inhibition, are discussed above, but there are, in addition, some long-term effects involving protein induction and possible humoral effects (as discussed below).

Pharmacological interventions

A range of inhibitors of mitochondrial activities have been reported to prevent ischaemia/reperfusion damage. Electron-transfer inhibitors, acting in the first stages of the respiratory chain are believed to act by inhibiting the production of ROS (which are largely generated at complexes I and III). The action of ATP synthesis inhibitors is, however, complex. Uncouplers such as dinitrophenol (and endogenous UCPs) are protective, presumably by decreasing $\Delta\psi$ and decreasing the activity of the mitochondrial

Table 1 Pharmacological and biochemical targets for cardioprotection involving mitochondrial activities

CPT-1, carnitine palmitoyl transferase-1; DEVD-CHO, acetyl-Asp-Glu-Val-Asp-CHO; PDH, pyruvate dehydrogenase.

Treatment	Effect(s)	Comment(s)	Reference(s)
General Treatments			
Fasting and diet	Complex	Lowers glycogen stores	[205–207]
Brief incubation at low O_2	Complex	Ischaemic preconditioning	[208]
Glucose/insulin	Promote glycolysis	May induce protein phosphorylation	[209–211]
Pyruvate	Metabolic substrate, inhibits fatty acid oxidation	Also protects against ROS	[212,213]
Inhibitors of fatty acid metabolism			
Trimetazidine, ranolazine	Inhibit fatty acid dehydrogenases		[214]
Oxfenicine and other CPT-I inhibitors	Inhibit fatty acid entry into mitochondria		[206,215]
Malonate	Inhibits fatty acid oxidation		[216, 217]
Dichloroacetate	Inhibits PDH kinase	Promotes glucose utilization	[218]
Carnitine and its derivatives	Lowers acetyl-CoA/free CoA, increasing free CoA	Promotes glycolysis by decreasing inhibition of PDH	[219,220]
Niacin	Reduces plasma non-esterified fatty acids	May help NAD recovery	[29,221]
Inhibitors of mitochondrial respiration			
Amytal, rotenone	Inhibits electron flow, complex I	Decreases ROS production	[30,222]
Myxothiazole	Inhibits electron flow, complex III	Antimycin and CN^- not protective	[223]
Uncouplers (dinitrophenol)	Decrease mitochondrial membrane potential	Time/dose dependence, to avoid energy wastage	[223]
Oligomycin	Inhibits F_1F_0-ATPase	Time/dose dependence, to avoid ATP depletion	[224]
Inhibitors of ion movement			
Ruthenium Red	Blocks mitochondrial Ca^{2+} import	Can also block the sarcoplasmic reticulum Ca^{2+}-channel	[225,226]
Nisoldipine; gallopamil	Block Ca^{2+} entry into cytoplasm		[227–229]
Tetrodotoxin	Blocks Na^+ entry into cytoplasm	Damages mitochondria during ischaemia	[63]
Amiloride	Blocks cytoplasmic Na^+/H^+ exchanger	Prevents Ca^{2+} overload, therefore protects against reoxygenation-induced hypercontracture	[150,230,231]
Diltiazem; SEA0400 (5-ethoxyaniline derivative)	Inhibits mitochondrial Na^+/Ca^{2+} exchange		[232–234]
Diazoxide; nicorandil	K^+-channel opener	Reversed by 5-OH decanoate, decreases ROS and plays roles in other undefined mechanisms	[59,66]
Inhibitors of cell damage mechanisms			
Cyclosporin	Prevents MPT opening	May also inhibit Ca^{2+}/CAM-dependent protein phosphatase (calcineurin)	[197,235]
Propofol	Traps ROS	ROS promote MPT	[199,236]
DEVD-CHO	Caspase 3 inhibitor (prevents downstream steps in apoptosis)	Only partially effective	[203]
Dipyridamole; carvedilol; natural compounds such as vitamin E and resveratrol; N-acetylcysteine	Antioxidants	Restores GSSG/GSH balance	[237–240]
Chloramphenicol	Inhibits cytochrome P450 mono-oxygenases	Also inhibits mitochondrial protein synthesis	[241]
Effectors of signalling pathways			
Sevoflurane and other anaesthetics	NADH increase on ischaemia is reduced, and total [NAD] + [NADH] is preserved	Uncontrolled effects on membrane permeability to ions	[242]
Bromoenol-lactone	Inhibits mitochondrial Ca^{2+}-independent phospholipases		[243]
Soluble factors released from preconditioned heart	Includes adenosine, catecholamines, NO, prostanoids, endorphins	See review	[244]
Adenosine	Many effects, including modulation of mK^+_{ATP} channel and protein kinase C	Other additional protective mechanisms include phosphorylation to 5′-AMP during reperfusion	[245,246]

Ca^{2+} uniport, while oligomycin (which blocks F_1F_o activity) presumably decreases wasteful ATP hydrolysis (as will the endogenous regulator protein, IF_1). However, these two agents will have complementary deleterious effects too: uncouplers will increase ATP hydrolysis, while F_1F_o inhibitors will increase $\Delta\psi$ and, presumably increase mitochondrial Ca^{2+} uptake. The observed outcome will depend on the relative importance of these various effects in the system under study.

The next group of protective agents listed in Table 1 are those which prevent ion movements, and in particular inhibit the abnormally high accumulation of Na^+ by the cytoplasm, and of Ca^{2+} by the mitochondrion, both of which seem to lead to irreversible damage. Also included are those which open the mK^+_{ATP} channels, a process which is implicated in protection via preconditioning. Like all inhibitors, however, their action will not be totally specific: Ruthenium Red and diazoxide, for example, inhibit sarcoplasmic reticulum ion channels, as well as mitochondrial channels for these respective ions, and diazoxide also inhibits electron transfer. As a result, the fact that a particular inhibitor prevents ischaemic/reperfusion damage cannot be taken as unequivocal proof for the participation of a particular ion channel in causing cell death.

There are many ways for a cell to die, and attempts to target known pathways in cell death are discussed next. The ordered apoptotic pathway (involving cytochrome *c* release from the intermembrane space of mitochondria and the activation of caspases, but requiring ATP) may contribute to ischaemia/reperfusion damage [10], and inhibitors of this pathway are partially protective. However, the importance of the contribution of the apoptotic pathway is unclear. Many researchers have suggested an important role for prolonged opening of the mitochondrial transition pore (reviewed in [9,199]), leading to depolarization of the

mitochondria and massive ATP depletion, and indeed cyclosporin, the classical inhibitor of this transition, is protective. However, as transient opening of this pore may be a step in the apoptotic pathway, the interpretation of the cyclosporin effect is not unambiguous. Finally, there are direct effects of high [Ca^{2+}] and ROS levels on other cell processes: both initiate lipid damage (activation of phospholipases and lipid peroxidation respectively) and protein damage (activation of proteases and oxidation of SH groups repectively), which might lead to irreversible loss of other essential cellular functions. Antioxidants in general have been shown to prevent oxidative damage to lipids, proteins and DNA following cardiac ischaemia and reperfusion [237–240], and these have been reviewed in [240a,240b].

Protective proteins

Using protein quantification and genetic manipulation techniques, several families of proteins have been shown to be protective against ischaemia/reperfusion damage. Unsurprisingly, these include HSPs, but also involved are the mitochondrial UCPs. Both groups are overexpressed in conditions of ischaemia/reperfusion [257–259] and can protect from cardiac injury. Endogenous mitochondrial HSP60 and HSP10 have been overexpressed experimentally and exert a protective effect on mitochondria exposed to simulated ischaemia [260]. This protective effect leads to increased recovery of ATP levels, and of the activities of complex III and IV [261]. Another protein of interest might be the HSP90-related protein, tumour-necrosis-factor-receptor-associated protein-1, which is expressed in mitochondria, and has a striking homology with (cytoplasmic) HSP90, although as yet its function is unknown [262].

Finally, it should be mentioned that the anti-apoptotic Bcl-2 family of proteins can reduce cell death, following ischaemia/reperfusion, by preventing reperfusion-induced apoptosis. They appear to inhibit cytochrome *c* release from the mitochondria and prevent activation of caspases 3 and 9 [263–265]. The importance of apoptosis in ischaemia/reperfusion damage is controversial as discussed above, and has been reviewed by Borutaite and Brown [10].

CONCLUSIONS AND PERSPECTIVES

In summary, while a number of processes have been shown to contribute to ischaemia/reperfusion damage in the heart, the relative importance of these processes is not yet clear. Further experiments with animal models, in which the variables (duration of ischaemia, oxygen tension, opening of ion channels) are more closely controlled, will be necessary to establish the primary or most damaging factor. Conclusive results on mitochondrial ischaemia/reperfusion injury, applicable across several different models of the state, were rare for several reasons. (i) Results on respiration rate, particularly under state IV conditions, were inconsistent. (ii) Direct comparison of studies was difficult due to the various methods used to perform myocardial perfusion, and to isolate the mitochondria. Other sources of variability include the use of different model species, and different tissue preparations (*in vivo* perfusion, isolated organ studies, isolated cell preparations). (iii) There is insufficient data on time dependence to allow correlations between the activity of ion channels, or the MPT pore and reperfusion injury, and protection. (iv) The role of NO was uncertain, as conclusions relied on the wide use of different concentrations of NOS inhibitors, and non-enzymatic NO production was not considered. Future advances in this field will depend on the identification of experimental factors that might influence respiration, ATP synthesis and cell death, and the standardization of conditions so as to remove these potentially confounding factors. Nonetheless, the work summarized above shows that there is substantial evidence to implicate impaired mitochondrial function in pathological changes associated with ischaemia and reperfusion injury in the heart. Hence manipulating mitochondrial function might prove to be useful in minimizing, or reversing, these changes.

Some of the changes appear to be due to global changes in energy charge, or redox state, and thus may be susceptible to general treatments with, for example, creatine, or coenzyme Q. However, a more fruitful approach might be to identify the specific systems that are implicated in causing, or contributing to, these changes, and which may therefore be appropriate for investigation as specific therapeutic targets. The work outlined above suggests a range of such potential targets, including the F_1F_o–ATPase complex (with or without its inhibitory protein), free radical generators (of both the respiratory chain and the cytochrome P450 systems), the MPT pore and mitochondrial ion channels, particularly mK^+_{ATP}.

Further work appears necessary to identify the relative importance of these various systems in pathogenesis, and indeed any cause/effect relationships between them. A variety of possible approaches is summarized below. (i) Significant advances in our understanding of metabolic and mitochondrial functional changes in ischaemia and reperfusion have come from *in vitro* studies. Clearly it would be advantageous to obtain more precise information *in vivo* from hearts. This could be accomplished with the development of highly resolving magnetic resonance spectroscopy and imaging. Work *in vivo* with probes should also include monitoring the time course of changes in mitochondrial membrane potential, ion fluxes and metabolite concentrations. (ii) The rapidly emerging field of proteomics will provide powerful tools to identify mitochondrial proteins differentially expressed in normal and disease states. This will allow the identification of proteins involved directly in ischaemia and reperfusion associated with, or caused by, mitochondrial dysfunction. Future efforts will focus on linking genomic array information to actual protein levels in mitochondria in relation to their functions. (iii) Developments are needed in characterizing protein–protein interactions, which profoundly influence metabolic control. The interaction surface between the inhibitor protein and the mitochondrial ATPase, for example, has now been identified [266]. Knowledge of the specific amino acid side chains that are involved in such interactions should allow pharmacological, or even genetic, interventions to increase the protection of cardiomyocytes from ischaemia and reperfusion.

Therapy is, in fact, mostly based on the administration of various compounds, alone or in combination. Controlled clinical investigations, on human subjects, will be needed to judge the efficacy and safety of drug regimens, but this should be informed by the basic scientific investigations outlined above. We suggest that clinicians and researchers in basic science set up common projects to design studies on patients, in parallel with animal models, paying particular attention to the first several hours after an ischaemic insult.

We hope that this review contributes to an understanding of the central role of mitochondria in energy metabolism and ion homoeostasis in heart ischaemia and reperfusion, and, paraphrasing the words of Lopaschuk and Opie [267], it might "... help to re-instate metabolism and bioenergetics as proper supports of cardiology".

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