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Mitochondrial energy production and cation control in myocardial ischaemia and reperfusion

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Summary: In the heart mitochondria exert two roles essential for cell survival: ATP synthesis and maintainance of Ca^{2+} homeostasis. These two processes are driven by the same energy source: the H^+ electrochemical gradient ($\Delta\mu H$) which is generated by electron transport along the inner mitochondrial membrane.

Under aerobic physiological condition mitochondria do not contribute to the beat to beat regulation of cytosolic Ca^{2+}, although Ca^{2+} transient in mitochondrial matrix has been described. Increases in mitochondrial Ca^{2+} of μmolars concentration stimulate the Krebs cycle and NADH redox potential and, therefore, ATP synthesis.

Under pathological conditions, however, mitochondrial Ca^{2+} transport and overload might cause a series of vicious cycles leading to irreversible cell damage.

Mitochondrial Ca^{2+} accumulation causes profound alterations in permeability of the inner membrane to solutes, leading to severe mitochondrial swelling. In addition Ca^{2+} transport takes precedence over ATP synthesis and inhibits utilization of $\Delta\mu H$ for energy production.

These processes are important to understand the sequence of the molecular events occurring during myocardial reperfusion after prolonged ischaemia which lead to irreversible cell damage. During ischaemia an alteration of intracellular Ca^{2+} homeostasis occurs and mitochondria are able to buffer cytosolic Ca^{2+}, suggesting that they retain the Ca^{2+} transporting capacity. Accordingly, once isolated, even after prolonged ischaemia, the majority of the mitochondria is able to use oxygen for ATP phosphorylation.

When isolated after reperfusion, mitochondria are structurally altered, contain large quantities of Ca^{2+}, produce excess of oxygen free radicals, their membrane pores are stimulated and the oxidative phosphorylation capacity is irreversibly disrupted. Most likely, reperfusion provides oxygen to reactivate mitochondrial respiration but also causes large influx of Ca^{2+} in the cytosol as result of sarcolemmal damage. Mitochondrial Ca^{2+} transport is therefore stimulated at maximal rates and, as consequence, the equilibrium between ATP synthesis and Ca^{2+} influx is shifted towards Ca^{2+} influx with loss of the ability of ATP synthesis.

Key words: Heart mitochondria – Ca^{2+} metabolism – energy production – myocardial ischaemia – myocardial reperfusion

Introduction

Myocardial tissue is typically aerobic and its metabolism is closely dependent upon oxygen viability as confirmed by the abundance of mitochondria (30 % of the total volumes) and

myoglobin. The contractile process or, more precisely, myosin ATPase activity represents more than 75 % of myocardial energy requirements, the remainder being covered mostly by ionic homeostasis which, in turn, is essential for the cardiac contraction cycle. The high energy requirement is most exclusively met by mitochondria oxidative phosphorylation. This, in turn, leads to high sensitivity of myocardial cell to oxygen deficiency and mitochondrial function is likely to play a central role in the molecular events leading to the tissue damage occurring under condition of oxygen deprivation.

This is certainly true if we consider that the physiological role of mitochondria in the cell is related not only to energy coupling during electron transport but also to maintainance of ions homeostasis and in particular of calcium.

This review is focused on several aspects of cardiac mitochondria function and metabolism. Such as: mitochondria ATP production, Ca^{2+} transport and permeability to solutes, then it is examined in detail the relationship between ATP production and Ca^{2+} accumulation both under physiological condition or during ischaemia and reperfusion.

Mitochondrial ATP production and calcium accumulation

As stated in Mitchell's chemiosmotic hypothesis (60), mitochondria energy conservation is mediated by the formation of a H^+ electrochemical gradient ($\Delta\mu H$). This can be utilized as energy source for ATP synthesis via the F_1 F_0 ATPase and for ion and metabolite transport via specific system.

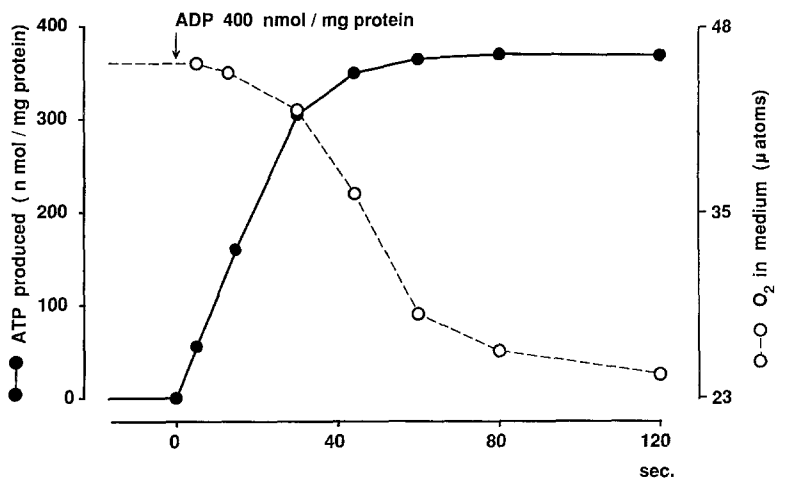

Fig. 1. Mitochondrial ATP production. Time course of oxygen consumption and ATP synthesis monitored using an NADH-coupled enzymatic analysis by isolated rabbit cardiac mitochondria respiring with glutamate as respiratory substrate. Mitochondria were isolated from freshly excised, non perfused rabbit heart by differential centrifugation (2). 2.5 mg of mitochondrial protein were added to a reaction medium consisting of 250 mM sucrose, 3 mM KH_2PO_4, 3 mM potassium glutamate, and 2 mM Hepes, pH 7.2, continuously stirred and maintained at 25 °C in a water-jacketed vessel. Following 1 min of equilibration and aliquot was removed for the determination of the mitochondrial endogenous ATP content; 400 nmol/mg protein ADP was then added to initiate oxidative phosphorylation and further aliquots removed over a period of 2 min. Samples were immediately mixed with 50 µl 10 % perchloric acid on ice to stop ATP production, and precipitated protein was separated by sedimentation at 3000 × g for 10 min. Following neutralization, the ATP content of the various supernatants was determined using hexokinase and glucose-6-phosphate dehydrogenase.

Mitochondrial ATP synthesis may be monitored using a number of procedures. In the early 1940 Ochoa made measurement of phosphate consumption during oxidative phosphorylation. In recent years this method has been replaced by direct measurement of ATP concentration, either using isotopic techniques or enzymatic determination. Figure 1 shows an example of the time course of ATP production and oxygen consumption by isolated rabbit cardiac mitochondria respiring with glutamate as respiratory substrate.

During recent years there has been also a growing interest in research of the respiration-linked transport of ions by isolated mitochondria and, particularly, of Ca^{2+} (37, 39, 88). It is not surprising that different results have been produced because there are so many related effects of the rapid accumulation of Ca^{2+} by mitochondria, such as: stimulated respiration, shift of internal and external pH, shift of the redox state, delocalisation of respiratory components and uptake of other ions.

There are two possibilities of measuring mitochondrial Ca^{2+} transport: isotopically, by means of quenching techniques or by using a Ca^{2+} ion-selective electrode. This later

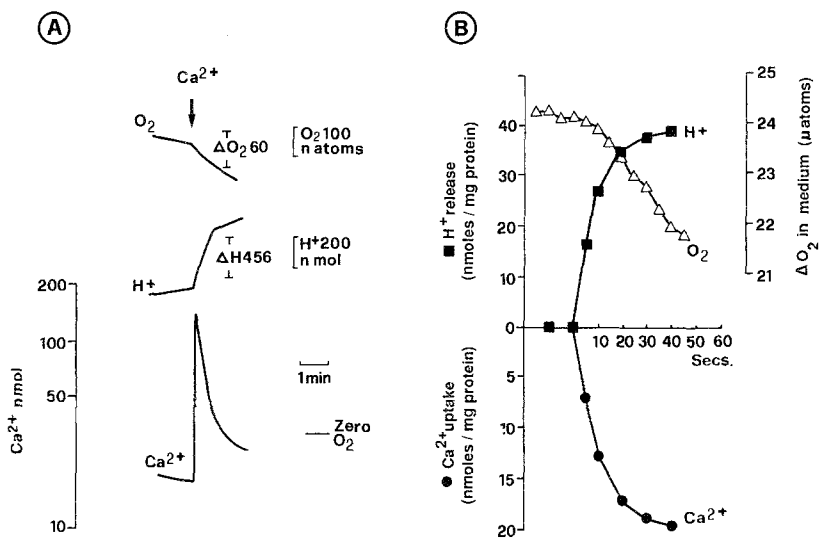

Fig. 2. Mitochondrial calcium transport. Experiments were carried out in a water jacketed perspex chamber (vol. 2.5 ml) maintained at 25 °C containing a Clark type oxygen electrode, a glass pH electrode and a Ca^{2+} electrode inserted through the air-tight fittings. A common reference was used for the Ca^{2+} and pH electrodes and their outputs connected to the inputs of high input impedance operational amplifiers. The input of the amplifiers together with that of O_2 eletrode were recorded on a Watanable pen recorder. Additions to the reaction medium were made through a small hole in the top of the cuvette. The Ca^{2+} and pH electrodes were calibrated by adding known amounts of $CaCl_2$ and HCl. Mitochondria (approximately 2 mg protein) were added to the reaction chamber and allowed to equilibrate. The reaction medium consisted of 120 mM KCl, 3 mM KH_2PO_4, 2 mM Hepes, pH 7.2

A: Simultaneous measurement of oxygen consumption, Ca^{2+} uptake, and proton expulsion by mitochondria from non perfused, freshly excised rabbit heart prepared as described in Figure 1. The addition of 420 nmol Ca^{2+} to mitochondria respiring with glutamate causes a "respiratory jump" with consumption of 60 natoms of O_2. The accumulation of the added Ca^{2+} is associated with the expulsion of 456 nmol H^+.

B: H^+-Ca^{2+} stoichiometry in the absence of a permeant anion. The reaction medium contained no added phosphate and NEM was added to inhibit the transport of endogenous phosphate. Under these conditions, a stoichiometry of $2H^+$ released per Ca^{2+} accumulated is observed.

technique allows simultaneous measurement of cardiac mitochondrial oxygen consumption, Ca^{2+} accumulation and proton movements, as is shown in Figure 2.

Mitochondria may participate directly in the process of Ca^{2+} homeostasis by means of separate influx and efflux pathways located within the inner mitochondrial membrane (37, 39). Since the Ca^{2+} affinity of the uniporter for cytosolic Ca^{2+} accumulation at submicromolar concentration and the rate of Ca^{2+} egress are extremely low, a direct beat to beat participation of mitochondria to intracellular Ca^{2+} movements is considered unlikely (6). On the contrary, their total capacity for Ca^{2+} accumulation is, at least, several times larger than sarcoplasmatic reticulum, suggesting a key mitochondrial role for maintainance of Ca^{2+} homeostasis under pathological conditions, when cytosolic Ca^{2+} concentration increases substantially.

Recently, another important physiological role of mitochondrial Ca^{2+} transport has been suggested. There is evidence in the mitochondrial matrix of Ca^{2+} transient (51, 71, 86). In single resting adult rat myocyte at low stimulation frequency (< 1 Hz), mitochondrial Ca^{2+} content is lower than cytosolic. When the cells are stimulated with β-adrenergic substances and paced at physiological frequency (4 Hz), Ca^{2+} concentration in the mitochondria exceeds the cytosolic content, reaching values of 0.6 μM (61). This range of Ca^{2+} increase stimulates the activity of three enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase. It follows that both flux in the Krebs cycle and NADH formation are significantly enhanced. The Ca^{2+}-induced increase in NADH redox potential might increase the rate of ATP synthesis and it might explain the mechanism of action of compounds able to improve the efficiency of the heart (37, 49, 54). This unique possibility for Ca^{2+} to stimulate contraction as well as metabolic supply occurs within the physiological ranges of cytosolic and mitochondrial Ca^{2+} concentration.

Thus, it is now clear that mitochondria can use the $\Delta\mu H$ for both ATP production and Ca^{2+} transport and at physiological concentration, mitochondrial Ca^{2+} transient plays an important regulatory role on ATP synthesis.

Different is the role of abnormal mitochondrial Ca^{2+} accumulation (26). It is recognized that cytosolic and mitochondrial Ca^{2+} overload is dangerous for the myocyte because it increases the mitochondrial permeability to solutes and competes with ATP production.

Mitochondrial permeability to solutes

There are several conditions that cause a Ca^{2+}-dependent increase in mitochondrial permeability to ions and solutes with molecular weights up to 1.200 Daltons (37). As result there is uncoupling of oxidative phosphorylation and matrix swelling. Two main mechanisms have been proposed to explain the permeability transition of the inner mitochondrial membrane: 1) opening of an unselective pore (45, 47), or 2) induction of permeability defects in the membrane lipid phase, due to accumulation of phospholipids following activation of phospholipase A2 (70, 76).

Besides an increase of mitochondrial matrix Ca^{2+} between 1–10 μM, permeability transition requires a wide variety of factors of very different nature, generically named "inducers" (37). This explains the success of the phospholipase A_2 hypothesis which, being based on a relatively aspecific target, i.e. the membrane itself, accommodates the large number of aspecific inducers which facilitate the transition. It has been reported that cyclosporin A but not phospholipase A_2 inhibits with high affinity the permeability transition (4, 33). In addition, the mitochondrial megachannel, a high-conductance channel identified by patch clamp studies of rat liver mitoplast, is inhibited by cyclosporin A and coincides with the transition pore (3, 69, 82, 83). The role of the permeability transition pore is not known. Clearly, pore opening leads to dissipation of the $D\mu H$ with a potential threat to cell viability.

There are different methodologies to study the mitochondrial permeability pore and transition. Measurement of volume changes have been used to this purpose. As the pore opens, it admits components of considerable size which are driven into the matrix space in a colloidosmotic process. Nephelometric or turbidimetric techniques are used to monitor mitochondrial swelling. This approach is easy, can be conducted in a cuvette using an ordinary spectrophotometer or fluorimeter, allows continuous monitoring of the swelling process but reflects the behaviour of the whole sample of mitochondria.

Alternatively, electromicroscopy and kinetic studies monitoring the flux of tracers with high time resolution can be used (11, 48). These techniques have demonstrated that the permeability transition is an all or nothing event: the pores of each mitochondrion open in a coordinated fashion and swelling takes place in hundreds of milliseconds.

It is also possible to monitor the permeabilization pore by means of application of patch-clamp techniques to mitochondria. This is certainly the most powerful and direct means of studying the pore, although there are several problems (38, 55, 68, 79). The researches on the permeability transition are complicated by the necessity of using a model capable of accommodating a variety of inducing or repressing agents. It is known, that Ca^{2+}, P_i, long chain acyl CoA and oxiradicals induce the permeability transition, thus supporting a role for this process during ischaemia and reperfusion damage, when all these substances rise. Of particular interest are also the effects of compounds known to interact with the adenine nucleotide exchanger such as atractylate and carboxyatractylate, which promote permeabilization (53, 59), or bongkrekate and ADP or ATP, which tends to prevent or delay the process.

An extensive list of inducing and repressing agents can be found in Gunter and Pfeiffer, 1990 (37).

Mitochondria and proton movements: an insight into the relationship between ATP synthesis and calcium transport

Ca^{2+} is transported into the mitochondrial matrix in response to the membrane potential component of the respiration-generated proton motive force. The closely related driving force of both ATP synthesis and Ca^{2+} transport makes the relation between these two processes of interest both under normal and pathological condition, such as post-ischaemic reperfusion. It has been suggested that mitochondria from reperfused tissue utilize restored oxygen for Ca^{2+} transport and not for ATP production (26, 27, 28, 31, 32, 87).

The basic premise of this hypothesis is that mitochondrial Ca^{2+} transport takes preference over ATP synthesis, a topic which we have studied by direct measurement of the two processes (28, 31), or indirectly, by simultaneous measurement of oxygen consumption and of proton and Ca^{2+} movements by using various ion-selective electrodes (88).

A typical tracing of the former technique is reported in Figure 3, which shows O_2, H^+ and Ca^{2+} concentration tracings obtained simultaneously. In Figure 3A, the addition of ADP (475 nmol) to cardiac mitochondria respiring with glutamate produced the well-documented stimulation of oxygen consumption which is maintained until all the added ADP is phosphorylated, at which time the rate of oxygen consumption slows to State 4 respiration, producing an ADP/O ratio of 3.0. During ATP synthesis there is a net uptake from the medium of 451 nmol H^+ and a small, but significant, decrease in the quantity of contaminant Ca^{2+} in the reaction medium (a decrease of Ca^{2+} concentration in the medium means a transport of Ca^{2+} into the mitochondria). It is difficult to establish if this represents a true mitochondrial Ca^{2+} accumulation, as it may be also due to a chelation of Ca^{2+} by the newly synthesized ATP.

In Figure 3B the addition of Ca^{2+} (420 nmol) also produced an increased rate of respiration or respiratory jump. However, oxygen consumption during Ca^{2+} accumulation is

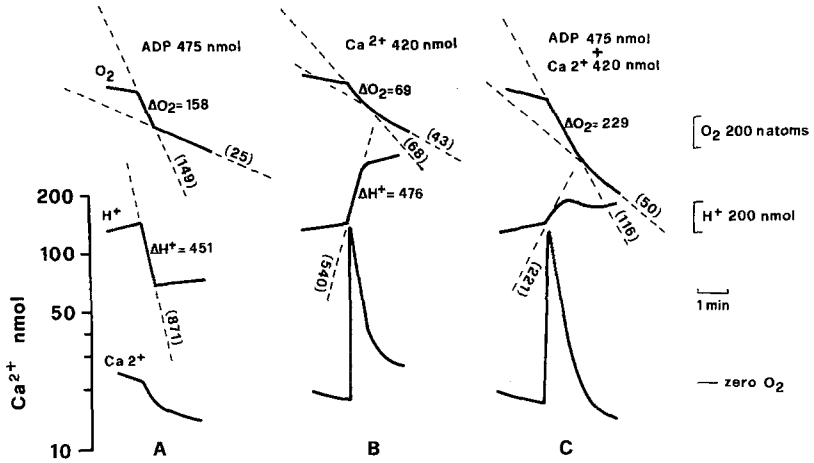

Fig. 3. Simultaneous measurement of mitochondrial respiration and H^+ and Ca^{2+} movements. Experiments were carried out as indicated in Figure 2. Respiration was initiated by adding 3 mM K glutamate. Respiration and ion movements were then monitored following the addition of ADP or Ca^{2+} or a combination of the two.
ΔO_2 values represent natoms oxygen consumed during a respiratory jump; ΔH^+ values represent nmoles H^+ absorbed or ejected. Numbers in parenthesis represent rates; natoms oxygen/mg protein/min and nmoles H^+/mg protein/min, respectively.
Additions were as follows: (A) 475 nmol ADP; (B) 420 nmol Ca^{2+}; (C) 475 nmol ADP plus 420 nmol Ca^{2+}.

considerably slower than during phosphorylation of ADP to ATP. A total of 69 natoms of oxygen are consumed during the transport of 420 nmol of Ca^{2+}, while 158 natoms of oxygen are consumed for phosphorylating 475 nmol of ADP. The Ca^{2+}/O_2 activation ratio is 6.1 or 2.04 per site for a three site substrate such as glutamate. A net proton extrusion is observed during Ca^{2+} accumulation with a total of 476 nmol H^+ released into the medium during the transport of the added of Ca^{2+}. In these experiments, it is not possible to determine the initial rate of Ca^{2+} accumulation, as this rate is masked by the rising phase of the Ca^{2+} electrode response following Ca^{2+} addition. However, it is possible to determine the total Ca^{2+} accumulation. As can be seen in Figure 3 (B), the Ca^{2+} content of the reaction medium returns to approximately the level seen before the Ca^{2+} addition. This indicates that virtually all the added 420 nmol of Ca^{2+} are accumulated with a H/Ca^{2+} ratio of 1.1.

In Figure 3 (C) are shown the effects of simultaneous addition of 475 nmol ADP and 420 nmol Ca^{2+}. As with the addition of either ADP or Ca^{2+} alone, this simultaneous addition produced an increased rate of respiration, the rate of oxygen consumption being intermediate between the rates achieved during ATP synthesis and Ca^{2+} transport. Total oxygen consumption during this respiration jump is 229 natoms. Following the addition of ADP and Ca^{2+}, there is an initial increase in the rate of proton extrusion. This phase is then followed by a net proton absorption and, finally, a return to the base rate of proton extrusion seen prior to the addition of ADP plus Ca^{2+}. The majority of Ca^{2+} added to the system is accumulated during the proton release phase but Ca^{2+} transport is still occurring during the second net proton absorption phase. Total oxygen consumption is approximately equal to the sum of the oxygen consumption with ADP or Ca^{2+} given separately.

These experiments indicate that during ATP production there is an absorption of H^+ from the medium into the mitochondria. The potential is dissipated and energy for ATP synthesis

is provided. On the contrary, during Ca^{2+} transport, the membrane potential decreases as the positively charged ions enter the mitochondrion. This causes permeability transition, further stimulation of respiration and concomitant H^+ ejection with an outward movement of H^+ from the mitochondria into the medium. Addition of ADP to a mitochondrial suspension results in an absorption of protons by the mitochondria, while the addition of Ca^{2+} results in an extrusion of protons. Thus, when both ADP and Ca^{2+} are added together, the consequent movements of protons represent the net changes resulting from H^+ movements in opposite directions. It is evident that Ca^{2+} transport takes precedence over ATP synthesis as first oxygen consumption is coupled to a net H^+ extrusion which is linked with Ca^{2+} accumulation. Only in the later phase, when the ion is completely accumulated with the mitochondrial matrix, oxygen consumption is coupled with H^+ absorption, suggesting ATP synthesis.

It should be recalled here that these experiments were performed by a single injection of Ca^{2+} in nmolar concentration to mitochondria from freshly excised rabbit heart. The situation is quite different, as it will be explained later, when considering events occurring, in vivo, during post-ischaemic reperfusion.

Further insight into the relationship between mitochondrial Ca^{2+} transport and ATP production can be obtained by measuring directly the rate of these two events in the presence or absence of the most important and physiological regulatory agents of the two processes: ADP and Mg^{2+} (26, 31).

Figure 4A shows the relationship between the initial rate of Ca^{2+} accumulation by mitochondria isolated from freshly excised, non-perfused rabbit hearts and the external free

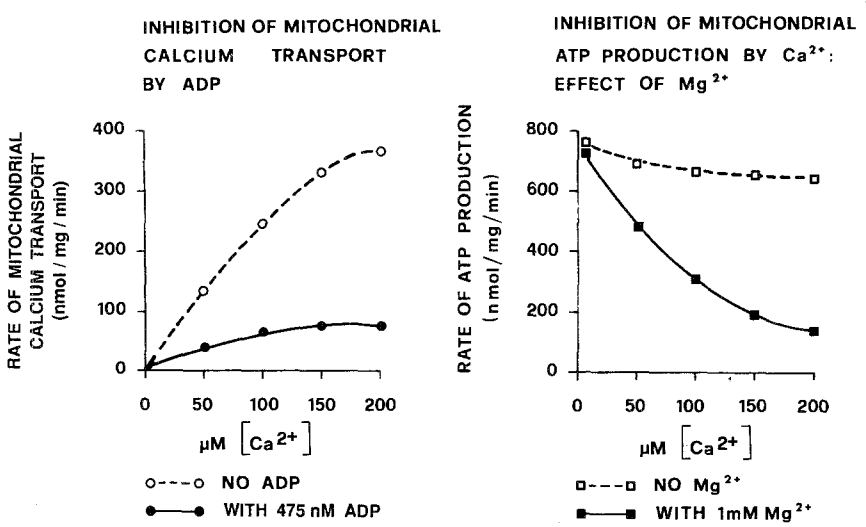

Fig. 4. Effected of ADP and Mg^{2+} on rate of mitochondrial calcium transport and ATP production. Experiments were carried out as indicated in Figure 1.

A: Inhibition of mitochondrial Ca^{2+} transport by ADP. Ca^{2+} transport was initated by adding either 45 Ca^{2+} or 45 Ca^{2+} plus ADP (475 nM) to 2.5 mg of protein mitochondrial respiring with glutamate (3 mM) as substrate.

B: Effect of Mg^{2+} on the Ca^{2+} induced inhibition of ATP synthesis by isolated cardiac mitochondria. ATP formation was initiated by the addition of either ADP (475 nmol) or ADP together with varying amounts of Ca^{2+}. When present, 1 mM Mg^{2+} was included in the reaction medium prior to the additon of mitochondria.

Ca^{2+} concentration. When ADP (475 nmol) and Ca^{2+} are added simultaneously, the initial rates of Ca^{2+} accumulation are reduced. Inhibition occurs over the entire ranges of external Ca^{2+} concentrations used.

Figure 4 B shows that the initial rate of ATP production is also influenced by the simultaneous addition of Ca^{2+} together with ADP. ATP synthesis was monitored in the absence of added Ca^{2+}, that is with a buffered free Ca^{2+} of approximately 10^{-9} M, and in the presence of concentrations up to 200 μM.

The rate of ATP synthesis is lowered in the presence of 12 μM Ca^{2+} and the degree of inhibition increases as the Ca^{2+} concentration increases. Inclusion of 1 mM Mg^{2+} in the reaction medium causes a small decrease in the initial rate of ATP synthesis seen in the absence of added Ca^{2+}. However, in the presence of this concentration of Mg^{2+} the inhibitory effect of Ca^{2+} on ATP synthesis is reduced. Only at Ca^{2+} concentrations of 150 μM and above there is an obvious decrease in the initial rate of ATP synthesis.

Thus, on one hand, oxidative phosphorylation decreases the rate of respiration-supported Ca^{2+} transport, while, on the other hand, the inward movement of Ca^{2+} decreases the rate of ATP production. These two observations lead, once again, to the conclusion that ATP production and Ca^{2+} transport compete for respiratory energy a schematic representation of the two processes is reported in Figure 5.

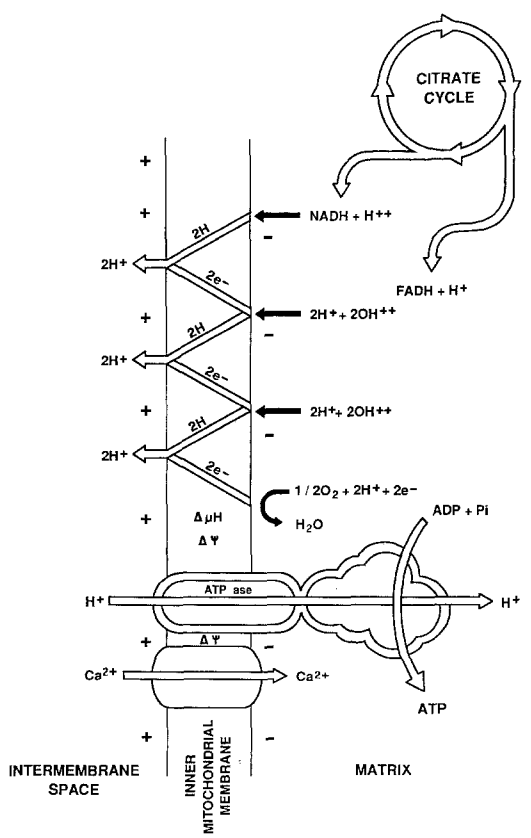

Fig. 5. Schematic representation of ATP production and Ca^{2+} transport across the inner mitochondrial membrane.

Mitochondria and myocardial ischaemia

Myocardial ischaemia is a condition which exists when oxygen supply is not sufficient to meet the rate of mitochondrial oxidation. As consequence, changes of metabolism and function occur and lack of adequate wash-out determine abnormal accumulation of ions and metabolites.

The effects of a total arrest of flow (global ischaemia) on contractile performance of isolated or in situ animal's or human's hearts have been extensively described and recently reviewed (29, 58, 89). In brief, immediately after the onset of ischaemia, developed pressure declines and diastolic pressure increases. If coronary flow is not restored, diastolic pressure further increases, full contracture develops and tissue necrosis begins, leading to infarction. Recovery of metabolism and function is possible only if coronary flow is partially or completely reestablished (reperfusion). Reperfusion can occur spontaneously, i.e.: development of coronary flow or can be induced by several interventions such as thrombolysis, percutaneous intracoronary angioplasty or coronary arteries by pass surgery (29). Usually these interventions are beneficial when started before or during the early stages of acute ischaemia (36,85). Equally, in the laboratory, reperfusion is beneficial when started before or during the early stages of ischaemic contracture. If it is delayed until the contracture is well established, cell damage is worsened and recovery is minimum or absent, leading to the condition named reperfusion damage, although the real existence of this entity is questioned (46).

At mitochondrial level, the onset of ischaemia is determined by an insufficient availability of O_2 for oxidations. As a consequence of the reduced or absent oxidative phosphorylation, intracellular acidosis develops and intracellular creatine phosphate content is rapidly depleted with a concomitant rise in intracellular phosphate, both factors stimulating glycolysis and lactate production. The accumulation of lactate and the hydrolysis of ATP further decreases intracellular pH. Both reduced pH and increased P_i, are the molecular factors responsible for the immediate "down regulation" of contractility, leading to the akinesia of the ischaemic zone (9, 18).

If the oxygen restriction is maintained, mitochondria themselves become targets for ischemic damage, decreasing the possibility of recovery for both metabolism and function. A number of mitochondrial alterations have been described as a consequence of either ischemia or post-ischemic reperfusion. Mitochondria extracted from ischaemic hearts show reduced function, decreased membrane potential (14), and a decreased function of NADH dehydrogenase (41, 72). NMR studies show that in isolated ischaemic hearts oxidative phosphorylation might be still active but oxygen consumption does not correlate with performance, consistent with mitochondrial uncoupling. F_1 F_0 ATPase and the adenine nucleotide translocase are inhibited (41, 72). Figure 6 shows data from our laboratory on the changes induced by ischaemia on mitochondrial function (26). Clearly, there is a reduction in mitochondrial yield, suggesting that some of these organelles when isolated after prolonged ischaemia are structurally altered. Their function, however, shows small changes even after severe ischaemia, suggesting that a residual, function is present even in mitochondria extracted from severely damaged tissues (31, 32). Figure 7 shows further data on superoxide dismutase activity and oxyradical production from the same mitochondria isolated after different period of ischaemia (22, 24, 25). Increasing the duration of ischaemia results in a reduction of mitochondrial superoxide dismutase activity with a concomitant increase of oxyradical production which elicit specific damage at the level of respiratory chain components and could be responsible for the deterioration in function.

The effects of restriction in oxygen availability can be characterized also at the single cell level. Adult cardiac myocytes show a characteristic elongated morphology (rod shape), a

clear sarcomere pattern and are quiescent in the presence of >1 mM extracellular Ca^{2+} (Ca^{2+} tolerant). Under conditions of low intracellular energization, rod shaped myocytes can either change into square forms, maintaining the sarcomeric striature (rigor state) or

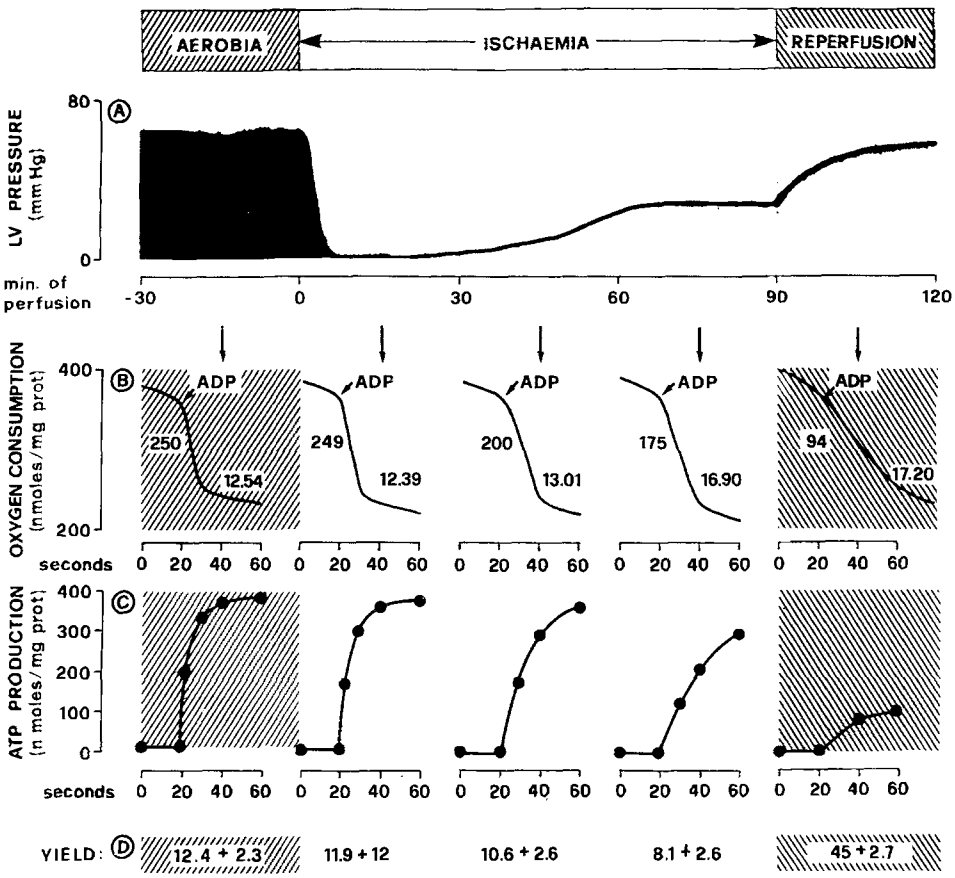

Fig. 6. Effects of 90 min of ischaemia followed by 30 min of reperfusion on mitochondrial function. Paced, isolated and perfused rabbit hearts were used for these experiments. Under control and reperfusion conditions the hearts were perfused with Krebs-Henseleit buffer at a mean coronary flow of 25 ml/min. Ischaemia was induced by reducing coronary flow to 1 ml/min.

A: Typical example of a left ventricular pressure tracing from a whole heart subjected to ischaemia and reperfusion.

B and C: Typical examples of isolated mitochondrial tracing for oxygen consumption and ATP production. The mitochondrial were isolated from hearts which have been perfused under aerobic (for 30 min), ischaemic (for 30, 60 and 90 min) and reperfusion (for 30 min) conditions. The numerical values reported in the oxygen consumption tracing represents rates (natoms oxygen/mg protein/min) consumed by the isolated mitochondria during state III and IV of respiration. Glutamate was used as respiratory substrate.

D: Numerical values (expressed as mean $\pm$ SE of at least six separate experiments) of the yield of mitochondria isolated after each relevant perfusion condition. The left ventricular pressure and mitochondrial oxygen consumption traces are representative of a typical experiment. The other parameters are mean values $\pm$ SE of at least six experiments. Mitochondria were isolated as described in Figure 1 (2).

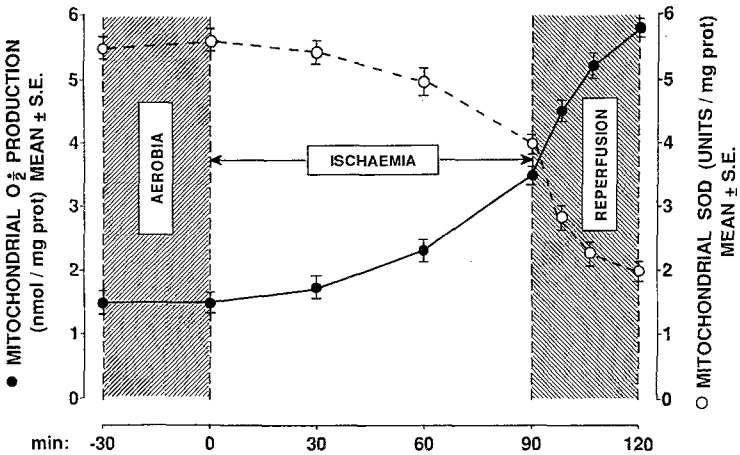

Fig. 7. Effects of 90 min of ischaemia followed by 30 min of reperfusion on the mitochondrial superoxide dismutase activity and O_2 production. Experiments were performed as described in Figure 6. Superoxide dismutase activity and O_2 production were determined as previously described (63, 64).

assume the round dysfunctional form in which the typical sarcomeric striature is no longer distinguishable (hypercontracture). From the functional point of view, rigor is still a reversible condition, while hypercontracture is not. Rod-shaped myocytes exposed to glucose-free anoxia ($pO_2 < 1$ torr) rapidly assume the square aspect (80). All cells exhibit this transition which is probably concomitant with ATP depletion (44). If anoxia is prolonged after the rigor development, the morphology does not show any further change.

If reoxygenation takes place within 5 min after the onset of rigor, all cells partially relengthen retaining a clear sarcomere pattern and the ability to switch in response to electrical stimulation. When cells are kept anoxic for long periods following rigor contracture, recovery is less frequent at reoxygenation and part of the cells hypercontract into rounded dysfunctional forms (80).

Paradoxically, during graded mild hypoxia ($pO_2 > 1$ torr) in the presence of a residual mitochondria oxidative phosphorylation capacity half of the cell undergo hypercontracture after a phase of spontaneous mechanical activity due to intracellular Ca^{2+} oscillation and significant rise in diastolic Ca^{2+} (78). Hypercontracture is not reversed by reoxygenation. Mitochondrial uncoupling blocks this series of events and allows relengthening during reoxygenation.

Thus we can conclude that, usually, mitochondria are quite resistent to ischaemic damage, as their function is retained even after prolonged periods of ischaemia. The presence of residual phosphorylation capacity during ischaemia in isolated myocytes however, is associated with irreversible damage and mitochondrial uncouplers exhibit cardiac protection. Thus mitochondria function during ischaemia might have a paradoxic meaning.

The paradox of ischaemic mitochondria

This term arise from the finding that: 1) intact and normal functioning mitochondria are essential for the recovery of mechanical function during post ischaemic reperfusion; 2) the inhibition of respiratory chain or the addition of uncouplers of oxidative phosphorylation are able to limit the extent of enzyme release in different models of myocardial damage such as post-ischaemic reperfusion, Ca^{2+} paradox (15, 35). These findings, obtained in perfused hearts and isolated myocytes, suggest that the restoration of ATP production by mitochon-

drial oxidative phosphorylation is essential for cell recovery, but, at the same time, it can also contribute to those processes which produce cell necrosis.

This paradox can be explained considering that a residual intracellular content of ATP due to mitochondrial phosphorylation activity impairs sarcoplasmic reticulum function, and causes intracellular Ca^{2+} oscillations and spontaneous contractile waves (5). These events are abolished by ATP depletion which occurs when glycogen stores are depleted and oxidative phosphorylation is completely abolished. The deleterious combination of residual ATP and increase in intracellular Ca^{2+} affects the contraction also at the myofilament level as in myocyte the maintenance of the rod-shape morphology is correlated with ATP concentrations in the bathing solution (2). When permeabilized myocytes are exposed to solutions containing no ATP, they shorten into square form with retained sarcomere pattern and relengthen as soon as ATP in the surrounding medium is increased to millimolar level (65). On the contrary, when exposed to low, (but non-zero) levels of ATP (4–20 μM) myocytes, undergo progressive irreversible hypercontracture. These changes are modulated by Ca^{2+}, which increases the ATP requirements for the maintenance or the recovery of the elongated morphology. These observations provide a rationale for the paradoxical association of mitochondrial function with opposite events such as the recovery of contraction or irreversible damage which occurs during post-ischaemic reperfusion. Two major possibilities can be envisaged: 1) under conditions of mitochondrial inhibition or uncoupling, ATP is virtually absent and cells are kept "frozen" in a rigor state. Reperfusion is accompanied by adequate recovery of mitochondrial function. The restoration of ATP content allows cell elongation or tissue relaxation and intracellular Ca^{2+} rapidly returns to control levels; 2) when mitochondrial function is impaired, but not completely abolished, low levels of ATP impair Ca^{2+} homeostasis and enhance the formation of rigor bands which, in absence of sufficient energy for the relaxation process, produces hypercontracture.

Mitochondria and calcium homeostasis during ischaemia and reperfusion

Intracellular Ca^{2+} rises in the ischaemic zone, although timing of the early rise is still controversial (57, 75). In the isolated papillary muscle cessation of mechanical activity is paradoxically associated with an increased amplitude of Ca^{2+} transients which is probably determined by acidosis (57). Then, the stimulated Ca^{2+} transients gradually decline and are replaced by irregular Ca^{2+} oscillations followed by a large and prolonged increase in intracellular Ca^{2+}. This finding has been interpreted as a large Ca^{2+} release from sarcoplasmatic reticulum which could no longer maintain its Ca^{2+} uptake capacity in absence of ATP.

The mechanism for Ca^{2+} release from the sarcoplasmic reticulum are not completely known. There is evidence of a Ca^{2+} induced release of Ca^{2+} (16). An increased cytosolic concentration of ADP and phosphate also favour Ca^{2+} release from the sarcoplasmic reticulum (43). Optimal pH for the ATP-dependent calcium pump of cardiac sarcoplasmic reticulum is above 7.5 (74). Thus, the ischaemic episode, lowering intracellular pH, should decrease the activity of this enzyme.

The data reported in Figure 8 show that during ischaemia total tissue Ca^{2+} does not change while mitochondrial Ca^{2+} increases, suggesting a redistribution of intracellular Ca^{2+} rather than a net massive accumulation which occurs during reperfusion after prolonged ischaemia. The route by which Ca^{2+} enters the cytoplasm is controversial, but it appears that Ca^{2+} channels are not involved (19). Uncontrolled Ca^{2+} entry could occur through leaks or membrane defects caused by the accumulation of toxic metabolites (lysophospholipids, oxyradicals) and/or by mechanical factors (21, 23). Alternatively, Ca^{2+} overload could result from the impairment of specific pathways utilized for Ca^{2+} uptake and extrusion (84). One possible mechanism is Ca^{2+} entry through the Na^{+}/Ca^{2+} exchanger, which after a period of ischaemia is far from its normal equilibrium due to a rise in the intracellular Na^{+} concentra-

tion. Another suggested mechanism is inhibition of the Na^+/H^+ exchanger by acidosis during ischaemia, followed by reactivation on reperfusion, causing an efflux of H^+ and an influx of Na^+, which, in turn, stimulates Ca^{2+} entry on the Na^+/Ca^{2+} exchanger (81). Whatever the route of entry, the rise in intracellular Ca^{2+} is accompanied by an increase in mitochondrial Ca^{2+} which impairs oxidative phosphorylation (Fig. 8).

Thus, the relationship between mitochondrial calcium transport and ATP synthesis previously described may be a key factor for cell survival during post ischaemic reperfusion (20, 30, 63).

The studies of mitochondria isolated from hearts reperfused after 90 min of severe ischaemia illustrated in Figures 6, 7 and 8 produced the following information: 1) their yield fell, suggesting that these organelles are structurally altered; 2) the production of oxygen free radicals is increased; 3) the isolated mitochondria contained large quantities of Ca^{2+}; 4) the oxidative phosphorylation capability of these organelles is severely disrupted.

What factors could be responsible for the significant alterations in mitochondrial function associated with reperfusion?

As we have pointed out, reperfusion leads to a marked increase in total tissue Ca^{2+} content, when the ADP content is elevated (52). The reintroduction of oxygen to the cell should, therefore, find at least a proportion of the mitochondria in a functional condition and supplied with wide quantities of substrate for their two major roles in the cell, oxidative phosphorylation and Ca^{2+} regulation.

The reported data in Figures 3 and 4 suggest that provided with both ADP and Ca^{2+}, cardiac mitochondria will preferentially accumulate Ca^{2+}, prior to phosphorylating ADP.

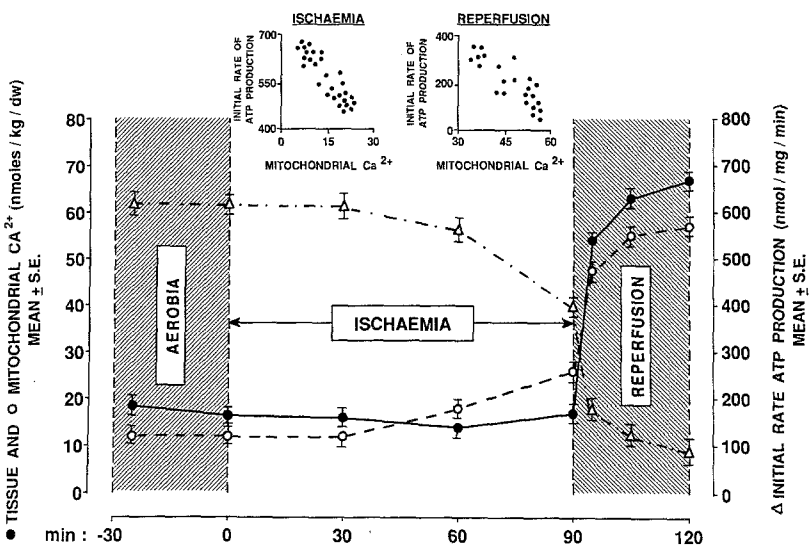

Fig. 8. Effects of 90 min of ischaemia followed by 30 min of reperfusion on the relationship between mitochondrial Ca^{2+} and initial rate of ATP production. Experiments were performed as described in Figure 6. Tissue and mitochondrial Ca^{2+} was measured by atomic absorption as previously described (63). Mitochondria used for the determination of their Ca^{2+} content and ATP production were extracted in a medium containing ruthenium red and lacking EDTA, so they would be prevented from accumulating Ca^{2+} during the extraction procedure. The data reported are mean ± SE of at least six separate experiments. Single data obtained after 90 min of ischaemia and after 30 min of reperfusion are shown in the two top panels.

The reason for such a preference is difficult to conceive. Both reactions are driven by the respiration generated proton gradient but occur via different components of the mitochondrial inner membrane. Opening of the membrane transition pore has been recently proposed as pathogenetic factor of myocardial damage during post ischaemic reperfusion (12, 13, 73). It should be pointed out, however that at the time of reperfusion several changes occur within the cytosol and the mitochondria itself which can either promote or reduce the possibility of pore opening.

First, there is an increase in cytosolic content of P_i. It is likely that during ischaemia P_i equilibrates within the intracellular compartments so that upon reperfusion mitochondria matrix P_i should be higher than in normoxic control and facilitate pore opening. Second, long chain acyl-CoA content increases 2–3 fold during ischaemia as β oxidation flux is reduced (50). This accumulation occurs within the matrix space, since $>90\%$ of cellular CoA is compartimentalized inside mitochondria and induce pore opening. Third, another important promotor of pore activation is the increase in oxygen free radicals production at the movement of reperfusion (17, 21, 23). Finally, even the increased content of Ca^{2+} in the mitochondria may contribute to opening of the pore, particularly in the presence of reduced or collapsed $\Delta\psi$ thus giving rise to a vicious cycle.

Ischaemia and reperfusion also cause changes which are expected to inhibit pore opening. First a reduction in pH is expected to reduce the probability of pore opening (1). As in the case of P_i, there is no information concerning matrix pH in situ. Due to the collapse of $\Delta\psi$ and ΔpH, an acidotic condition is likely to occur under ischaemia, but it is difficult to predict the behaviour of mitochondria pH during reperfusion. Second, free cytosolic and, possibly, intramitochondrial ADP increase with inhibiting effect on pore opening. Finally, it has been recently shown by NMR that, despite the ischaemic and reperfused heart leaks Mg^{2+} (26), the intracellular concentration of the ion increases during ischaemia (56, 62, 77), a process which is most likely related to ATP hydrolysis. Mg^{2+} is expected to inhibit pore opening.

Thus a role for the membrane transition pore will depend on the equilibrium between these and, probably, many other components. If the pore plays an important role in reperfusion damage, its inhibition with cyclosporin A should result in less damage. Unfortunately the effects of cyclosporin A are complex and mediated by at least three different intracellular receptors: the cyclophilins (7, 10, 34, 40, 42). The cyclosporin-cyclophilin complex alters gene transcription (8, 66). It follows that cyclosporin A will cause plelotropic effect at the cellular level due to its multiple interactions within the cyclophilins and others as yet uncharacterized systems, including Ca^{2+} fluxes (64, 67).

Whatever the molecular mechanism involved, based on direct measurements of ATP synthesis, Ca^{2+} transport and net proton movements, we have concluded that a competition exists between ATP synthesis and Ca^{2+} transport, the second process taking precedence over the first. In addition, the relative activities of these reactions may be influenced by a number of factors including the substrate for respiration and concentration of Mg^{2+}, which is a competitive inhibitor of mitochondrial Ca^{2+} transport (Fig. 4).

We can, therefore, summarize that reperfusion of the severely ischaemic myocardium provides oxygen to reactivate mitochondrial respiration, but also produces a large increase of intracellular Ca^{2+}. Under these conditions it is suggested that: 1) the transport of Ca^{2+} into the mitochondria would be stimulated to maximal rates, in an attempt to buffer the large influx of Ca^{2+} across the sarcolemma; 2) as a consequence, the equilibrium between ATP synthesis and Ca^{2+} influx would be dramatically shifted towards Ca^{2+} influx; 3) Ca^{2+} accumulation would be further favored or inhibited by the observed changes of Mg^{2+} from the cell and by the increased production of oxygen free radicals at mitochondrial level.

Inevitably this sequence of events lead to mitochondrial Ca^{2+} overload with subsequent mitochondrial structural damage and, as observed, the loss of the ability to synthesis ATP

even after extraction and resuspension in a Ca^{2+} free medium. Under these conditions we would expect no recovery of tissue ATP or creatine phosphate and no return of normal muscle function.

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