Mechanism(s) of Altered Mitochondrial Calcium Transport in Acutely Ischemic Canine Hearts

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SUMMARY Studies were undertaken to determine the mechanisms leading to altered mitochondrial function in ischemic myocardium. A new procedure has been developed to routinely isolate 60-70% of the total mitochondrial protein from heart tissue. After 1 hour of ischemia, mitochondria exhibit decreases of more than 50% in phosphorylating respiration for both NADH- and succinate-linked substrates compared to controls. However, no significant decreases in the efficiency of mitochondrial ATP synthesis (ADP:O) or ATPase activity are observed. Rates of substrate-driven Ca²⁺ uptake exhibit decreases greater than that seen with phosphorylating respiration with incomplete uptake and premature release of Ca²⁺. Spectrophotometric measurements in ischemic heart reveal rapid oxidation or loss of mitochondrial NADH with marked "swelling" of the inner membrane compartment; both changes parallel the loss of Ca²⁺. Significant losses in intramitochondrial adenine nucleotides also are found. Mitochondrial retention of accumulated Ca²⁺ can be restored by addition of small amounts of exogenous adenine nucleotides (ATP or ADP) with concomitant attenuation of both NADH oxidation and "swelling." The data indicate that, following 1 hour of ischemia, the efficiency of mitochondrial ATP production is still relatively intact whereas both electron transport chain activity and calcium transport are severely compromised. These decreases appear to be related to selective membrane damage in the mitochondrial inner membrane.


IT IS generally agreed that approximately 1 hour following severe ischemic insult, the myocardial cell has reached the point of irreversible injury (Jennings and Ganote, 1974). A variety of mechanisms have been invoked to explain the onset of irreversible cell death in the ischemic myocardium. No clear mechanism(s) have yet been established to explain the rapid dysfunction and death of the myocardial cell following ischemia (Jennings and Ganote, 1974; Hillis and Braunwald, 1977). The mitochondrial fraction of the heart cell constitutes approximately 35% of the membrane protein (Page and McCallister, 1973) and is the principal source of ATP in the normally functioning heart (Neely and Morgan, 1974). It is clear from previous studies that the energy-linked functions of mitochondria are rapidly compromised following the onset of ischemia (Jennings and Ganote, 1976; Lochner et al., 1975; Sobel, 1974; Wood et al., 1979). However, it is not clear whether these changes represent a primary factor in the onset of irreversible myocardial cell death (Jennings and Ganote, 1976; Sobel, 1974). The lack of a close correlation between energy metabolism and loss of contractile function during ischemia (Sobel, 1974; Williamson et al., 1976) has led to speculation on the role of Ca²⁺ in altered myocardial performance (Williamson et al., 1976). The beat-to-beat regulation of intracellular Ca²⁺ in the myocardial cell is a complex process (Dhalla et al., 1977). Alterations in the control of this Ca²⁺ could rapidly affect myocardial contractility. Ca²⁺ transport in heart mitochondria is not a well characterized process (Scarpa, 1979). This is true particularly with regard to regulation of the retention and release phases of the cation. However, it is apparent that the energy requirements and mechanisms involving mitochondrial Ca²⁺ retention and release are different than those associated with active accumulation (Lehninger et al., 1978; Scarpa, 1979).

Various reports have demonstrated rapid depletion of intracellular adenine nucleotides following ischemic insult (Gudbjarnason et al., 1970; Neely et al., 1973; Jennings et al., 1978; Wollenberger and Krause, 1968). These results appear to correlate with the reduced energy-producing capacity of the myocardial cell mitochondria following ischemia. However, myocardial contractile function is more rapidly affected than can be explained by the temporal decreases in intracellular high energy phosphates (Neely et al., 1973; Sobel, 1974; Wollenberger and Krause, 1968). Recently, it has been shown that increased levels of lipid metabolites during ischemia, including acylCoA esters and lysophospholipids, may contribute to inhibition of cellular membrane transport processes, as well as actual membrane damage (Ahumada et al., 1979; Owens et al., 1979; Shrago et al., 1976). The loss of membrane integrity has been suggested as one of the primary events associated with myocardial ischemia (Jen-
nings and Ganote, 1974; Sobel, 1974). However, the specificity of this membrane damage is not established.

A consistent problem in comparative studies involving cardiac pathologies has been the criticism of obtaining "representative" populations of mitochondria from experimental tissue compared to control. A technique for isolating putative, separate populations of subsarcolemmal and interfibrillar mitochondria from heart has recently been published (Palmer et al., 1977). The overall mitochondrial yield reported by these investigators was 70% of total mitochondrial protein found in cardiac tissue. Preliminary studies in our laboratory, using the specificity of this membrane damage is not established (Palmer et al., 1977). The overall mitochondrial yield reported by these investigators was 70% of total mitochondrial protein found in cardiac tissue. Preliminary studies in our laboratory, using the above-mentioned method in control and ischemic hearts, revealed parallel decreases in mitochondrial functions due to ischemic insult regardless of the initially higher activities in one population over the other (Sordahl, 1978). We have developed a method to obtain a single, "clean," representative population of heart mitochondria, employing a combination of previously described techniques. The results indicate that a 60-70% recovery of mitochondria from heart can be obtained routinely by this procedure.

In this report, the integrative role between the energy-producing capacity of mitochondria and energy-linked calcium transport have been studied. The period of 1 hour post-ischemia was chosen since it represents the "point of irreversibility" in myocardial cell damage. The intent is to magnify those changes in mitochondrial energy-linked functions that are clearly altered and can be correlated with other parameters of ischemic myocardial dysfunction.

Methods

Twenty-five mongrel dogs weighing 15-20 kg were anesthetized with sodium pentobarbital (30 mg/kg, iv). Positive pressure ventilation was maintained by endotracheal intubation and a Harvard respirator (model 607). A left thoracotomy was performed through the 4th intercostal space. The pericardium was incised and left circumflex coronary artery was dissected at the distal margin of the left atrial appendage. A ligature was placed around the artery at this point and tied. This procedure results in an ischemic area centered in the subendocardial region of the posterior papillary muscle of the left ventricle (Jennings and Ganote, 1974). At the end of 1 hour, the heart is removed and the tissue immediately chilled to 4°C. The mitochondria from ischemic tissue were isolated from the subendocardial region of the posterior papillary muscle. In all the experiments reported here, the anterior left ventricular wall of each heart served as a control to the ischemic side used in these experiments.

Mitochondria are isolated using modifications of previously published procedures (Palmer et al., 1977; Sordahl et al., 1971). Briefly, heart tissue (1-2 g) is minced into very small pieces with scissors. One-gram portions of tissue are put into individual size C glass homogenizers (A.H. Thomas Co.), and 20 ml of 0.25 m sucrose, 5 m Tris-HCl, 10 mM EGTA* medium, pH 7.2, are added (5% wt/vol homogenate). The tissue is homogenized with a Polytron PT-20 tissue processor (Brinkmann Instruments) at a setting of 4.6 for 4 seconds. The homogenate is then centrifuged at 27,000 g for 10 minutes. The supernatant, which contains no cytochrome c oxidase activity, is discarded. The single-gram pellets are combined and 1 mg Nagarse (Enzyme Development Corp.) per gram starting tissue is added (Nagarse solution is made up by adding 10 mg Nagarse enzyme to 10 ml of the sucrose-Tris-EGTA medium plus 100 μl 1.0 mM Tris base). The suspension is thoroughly mixed with a vortex mixer and allowed to incubate at 4°C for 8 minutes. It has been determined that optimally functional and intact mitochondria are obtained with this length of incubation time. After incubation, a solution of 0.18 mM KC1, 5 mM Tris-HCl, 10 mM EGTA, 0.5% bovine serum albumin (Fraction V, Sigma) at pH 7.2 is added to obtain a 5% wt/vol homogenate. This suspension is homogenized by making one pass with a motor-driven Teflon pestle and then centrifuged at 500 g for 5 minutes. The supernatant is poured through cheesecloth and centrifuged at 12,000 g for 10 minutes. The mitochondria are "washed" once and resuspended in 0.18 mM KC1, 5 mM Tris-HCl, 0.5% bovine serum albumin medium, pH 7.2, at a final concentration of approximately 30 mg/ml. Protein determinations are made by a biuret method (Jacobs et al., 1966).

Cytochrome oxidase activity was determined in the total homogenate and subsequent fractions with an established spectrophotometric procedure (Wharton and Tzagoloff, 1967). Mitochondrial respiratory activity was measured polarographically by a previously published technique (Sordahl et al., 1971). Mitochondrial ATPase activity was determined using a linked-enzyme assay monitoring NADH oxidation at 340 nm (Albers and Koval, 1962; Schwartz et al., 1969). The ATPase activity was inhibited essentially 100% by addition of oligomycin. After oligomycin addition, a small amount of residual activity remained which was completely inhibited by rotenone. This activity represented oxidation of NADH by the mitochondria in the assay system and was subtracted from the initial rates to obtain accurate ATPase values. Adenine nucleotides were determined from perchloric acid extracts of the mitochondrial suspensions by a previously published spectrofluorometric method (Estabrook et al., 1967). The Ca** and Mg** contents of mitochondria were assayed by atomic absorption

* Abbreviations: EGTA, ethylene bis(oxyethylene nitrilo tetraacetic acid); Fi, inorganic orthophosphate.
spectrophotometry from 0.5 N HCl extracts of the protein. For Ca2+ determinations, 1% LaCl3 was included in the acid extracts to prevent phosphate interference.

Mitochondrial respiration during Ca2+ uptake was measured by polarographic means, and substrate-supported Ca2+ uptake was measured by a previously described dual-beam spectrophotometric method at the wavelength pair 541-504 nm (Asimakis and Sordahl, 1977; Scarpa, 1972). Ca2+ transport was also routinely assayed using 45Ca2+. Millipore filtration and liquid scintillation techniques to assure that artifacts (e.g., non-specific absorbance changes) in the dual-beam spectrophotometric procedure were not occurring. The basic assay medium for all mitochondrial experiments consisted of: 0.25 M sucrose, 1 mM Tris-HCl, 75 mM KCl, pH 7.2, 1.7 mM Pi, 1.7 mM substrate, and 1 mg/ml mitochondrial protein. Ca2+ was added at a final concentration of 150 μM in all assays. Temperature for all assays was 30°C. Dual-beam spectrophotometric calcium transport assays were conducted with the inclusion of 50 μM murexide (ammonium purpurate), succinate as substrate, and 1 μg rotenone/mg mitochondrial protein. Changes in the absorbance (A) of mitochondria ("swelling-contraction") were conducted at 30°C in a Aminco DW-2 spectrophotometer in the split-beam mode and operating with λ1 at 520 nm (Sordahl and Asimakis, 1978). The instrument was optically attenuated to a zero baseline with full scale changes from 0 to 0.1 A. The incubation conditions for the swelling-contraction experiments were identical to those for the calcium transport studies with the omission of murexide in the media. It has been established clearly that these measurements reflect conformational changes in the mitochondrial inner membrane compartment related to functional state (Stoner and Sirak, 1969; Wrighlesworth and Packer, 1968). Ca2+ was added to initiate the reaction and the changes in absorbance were followed with time. Changes in oxidation-reduction steady states of intramitochondrial pyridine nucleotides were measured by dual-beam spectrophotometry at the wavelength pair 340-374 nm (Chance, 1972).

Statistical analyses used Student's paired t-test. The data were analyzed with a Wang 2200 series statistical program (Wang Instrument Laboratories, 1973). All values were expressed as the mean ± SE.

Results

Table 1 shows averaged values obtained for the percent yield of mitochondria obtained from control and ischemic heart tissue based on cytochrome oxidase (mitochondrial marker enzyme) and protein recovery. The range of values for total homogenate cytochrome oxidase specific activities was 0.192 to 0.256 and 0.185 to 0.243 for control and ischemic preparations, respectively. Control mitochondrial cytochrome oxidase specific activities ranged from 0.870 to 1.1 and ischemic activities from 0.80 to 0.95. The range of values for yield of mitochondrial protein was 57.4 to 66% for both control and ischemic preparations. Generally a 60% or better recovery of the total mitochondrial population in both control and ischemic heart tissue was obtained. Electron micrographs of the isolated mitochondrial suspensions revealed intact mitochondria that were free of other subcellular materials (data not shown).

A greater than 50% reduction in mitochondrial respiratory activity is observed after 1 hour of ischemia with both NADH- and succinate-linked substrates (Fig. 1A; controls, filled bars; ischemic, unfilled bars). However, no reduction in the efficiency of ATP synthesis (ADP:O) is observed in ischemic heart mitochondria (Fig. 1B; unfilled bars) compared to controls (Fig. 1B; filled bars). Measurements of mitochondrial ATPase activity in control (Fig. 2; solid circles) and ischemic (Fig. 2; unfilled circles) preparations reveal that no significant differences exist, although the ischemic heart mitochondria are somewhat lower in activity. The ATPase data (Fig. 2) are consistent with the observation that the efficiency of energy coupling has

| Table 1 | Values for Recovery of Mitochondria from Control and Ischemic Heart Tissue |
|---------|------------------|-----------------|-----------------|------------------|
| Fraction | Cytochrome oxidase | Mitochondrial protein |
|          | S.A.†             | T.A. †           | % Yield         | mg/g wet wt. †   | % Yield         |
| Control (n = 15) |                     |                  |                 |                  |
| Total homogenate | 0.232             | 64,032           | 100             | 43.4             | 100             |
| Nuclear pellet | 0.085             | 23,460           | 36.6            |                  |                  |
| Post-supernatant | 0                 | 0                | 0               |                  |                  |
| Mitochondria | 0.983             | 39,615           | 61.8            | 27.5             | 63.4            |
| Ischemic (n = 14) |                     |                  |                 |                  |
| Total homogenate | 0.218             | 63,348           | 100             | 47.2             | 100             |
| Nuclear pellet | 0.099             | 24,999           | 40.1            |                  |                  |
| Post-supernatant | 0                 | 0                | 0               |                  |                  |
| Mitochondria | 0.880             | 37,312           | 59.8            | 28.3             | 59.9            |

* S.A. = Specific activity in: μmol cytochrome c oxidized/min per mg.
† T.A. = Total activity as: Units specific activity × total protein.
‡ mg/g wet wt. = (mg total protein/g heart tissue) × (S.A. total homogenate/S.A. Mitochondria).
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been maintained in ischemic heart mitochondria after 1 hour (Fig. IB).

Determinations of intramitochondrial adenine nucleotides from control (Fig. 3; filled bars) and ischemic heart mitochondria (Fig. 3; unfilled bars) indicate significant reductions in ATP, ADP, and AMP, as well as total adenine nucleotides.

Measurements of substrate-supported Ca\(^{2+}\) uptake by control (Fig. 4A; top solid trace) and ischemic heart mitochondria (Fig. 4B; top solid trace) reveal a marked decrease in the rate of uptake after 1 hour of ischemia. The numbers to the right of the trace are rates of Ca\(^{2+}\) uptake in nmoles/min per mg mitochondrial protein. Ischemic heart mitochondria also exhibit a rapid, premature release of the accumulated Ca\(^{2+}\) (Fig. 4B; top solid trace, upward deflection) compared to controls which do not release the Ca\(^{2+}\) until the system is depleted of oxygen (Fig. 4A; top solid trace, upward deflection). Simultaneous measurements of respiratory activity (Fig. 4, A and B; dashed lines) during active Ca\(^{2+}\) uptake by control and ischemic heart mitochondria indicate that the premature Ca\(^{2+}\) release is not due to uncoupling of mitochondrial respiration (Fig. 4B; dashed line). Independent measurements of the reduced steady state of mitochondrial NADH (Fig. 4, A and B; middle traces) and “swelling” of the mitochondrial inner membrane compartment (Fig. 4, A and B; bottom traces) reveal rapid decreases in NADH during the Ca\(^{2+}\) release phase from ischemic heart mitochondria (Fig. 4B; middle trace, downward deflection). This rapid decrease in the NADH steady state is paralleled by a rapid “swelling” of the mitochondrial inner membrane compartment (Fig. 4B; bottom trace, downward deflection). Addition of exogenous ATP or ADP (1–5 nmol/mg) to ischemic heart mitochondria, in the presence or absence of oligomycin, results in complete uptake and retention of Ca\(^{2+}\) (Fig. 4C; top solid trace) until the system is anaerobic (Fig. 4C; top dashed trace), like that of controls (Fig. 4A; top traces). The addition of exogenous adenine nucleotide also attenuates the rapid decrease in mitochondrial NADH (Fig. 4C; middle trace) and the rapid swelling (Fig. 4C; bottom trace) of the inner membrane compartment observed in the ischemic preparations (Fig. 4B; middle and bottom traces).

Determinations of the Ca\(^{2+}\) and Mg\(^{2+}\) content in control and ischemic heart mitochondria revealed

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**FIGURE 1** Oxidative phosphorylation in heart mitochondria following 1 hour of ischemia. A: Respiratory rates during active phosphorylation (State 3). B: Efficiency (ADP:O) of oxidative phosphorylation. Controls, filled bars; Ischemic, unfilled bars.

**FIGURE 2** Mitochondrial ATPase activity in control (filled circles) and ischemic (unfilled circles) heart mitochondria. No significant differences exist for the range of Mg\(^{2+}\) concentrations used at 0.8, 1.2, 1.6, and 2.0 mM. The P values are 0.28, 0.35, 0.45, and 0.64, respectively. ATPase activity is expressed as: µmol ATP split/mg per hr.

**FIGURE 3** Concentration of intramitochondrial adenine nucleotides from control (filled bars) and ischemic (unfilled bars) heart mitochondria.
Figure 4: Representative spectrophotometric tracings of mitochondrial calcium uptake (upper traces, solid lines) and respiratory rates measured polarographically (upper traces, dashed lines). Middle traces are the measurement of NADH reduced steady states during calcium uptake. An upward deflection indicates reduction and downward oxidation. Bottom traces are the measurement of mitochondrial "swelling" during calcium accumulation, retention, and release. Upward deflection are contraction and downward deflection the "swelling" of the mitochondrial inner membrane compartment. Numbers to the right of the upper solid traces are the rates of calcium uptake in: nmol/min per mg protein. Numbers to the right of the dashed upper traces are the rates of oxygen consumption in: natoms O₂ consumed/min per mg protein. The arrows indicate the addition of calcium (150 μM). ADP was added at a concentration of 5 nmol/mg in these experiments (C).

Discussion

A new method of isolating heart mitochondria has been developed that results in "clean," consistent yields of 60% or more of the total mitochondrial population in heart tissue (Table 1). The yields of mitochondria based on cytochrome oxidase recoveries agree quite well with the estimates of recovery based on total mitochondrial protein. Our data indicate a total of 45-50 mg mitochondrial protein/g wet weight of tissue, which is in close agreement with other recent determinations (Idell-Wenger et al., 1978). No significant differences in total mitochondrial recovery from control and ischemic tissue were observed, and this also is consistent with other investigators’ results (Idell-Wenger et al., 1978). This isolation procedure appears to offer an approach to obtaining a large representative population of mitochondria from both control and ischemic tissue for comparative studies.

The data in Figure 1A indicate that the mitochondrial respiratory chain is more susceptible to ischemic damage than the phosphorylation or energy-coupling mechanism (Figs. 1B and 2). The cytochrome oxidase specific activities of control and ischemic heart mitochondrial preparations exhibited no significant differences (e.g., 0.8-1.2 μmoles cytochrome c oxidized/min per mg). These data suggest that the decreased respiratory activity (Fig. 1) is not due to loss or damage of this particular coenzyme. Addition of exogenous cytochrome c partially restored the respiratory rates of ischemic heart mitochondria toward controls (10-15% increase), but was not effective in completely restoring activity (data not shown). Mitochondrial uncouplers were routinely added during the polarographic assays to ascertain if higher respiratory rates could be obtained in uncoupled ischemic heart mitochondria. The results were negative, suggesting that inhibition of the adenine translocase system in these mitochondria was not rate limiting or inhibited (Shrago et al., 1976).

The decreased rates of substrate-supported Ca²⁺ uptake by ischemic heart mitochondria (Fig. 4B)
are consistent with the observed decreased rates of respiration (Fig. 1A) and the established relationship between electron transport chain activity and Ca\(^{2+}\) transport (Heaton and Nicholls, 1976). A number of studies indicate that the various phases of mitochondrial Ca\(^{2+}\) uptake, retention, and release have differing energy requirements (Harris, 1979; Lehninger et al., 1978; Scarpa, 1979). It has been suggested (Lehninger et al., 1978) that mitochondrial Ca\(^{2+}\) retention is favored when the internal pyridine nucleotides are in a relatively reduced steady state. It is clear from Figure 4B that a rapid decrease in mitochondrial NADH accompanies the efflux of Ca\(^{2+}\). However, the decreasing levels of NADH are also associated with rapid inner membrane swelling during Ca\(^{2+}\) release (Fig. 4B; bottom trace). Recently Harris (1979) has suggested that adenine nucleotide binding to mitochondrial inner membranes functions to decrease Ca\(^{2+}\) leakage by blocking cation loss through specific pathways. In previous studies (Sordahl and Asimakis, 1978) in blocking cation loss through specific pathways. In this laboratory, we have shown that normal heart mitochondria treated with inorganic pyrophosphate exhibit a partial depletion of adenine nucleotides and an inability to retain accumulated Ca\(^{2+}\) that is similar to the results obtained with ischemic heart mitochondria (Figs. 3 and 4B). Further, exogenous addition of small amounts of adenine nucleotides could reestablish Ca\(^{2+}\) retention in pyrophosphate-treated normal heart mitochondria (Sordahl and Asimakis, 1978); this is similar to the effects of adenine nucleotides reported here for ischemic heart mitochondria (Fig. 4C).

It is clear that rapid depletion of cellular high-energy phosphates accompanies myocardial ischemia (Gudbjarnason et al., 1970; Jennings et al., 1978; Wollenberger and Krause, 1968). Recent studies also suggest accumulating lipid metabolites may result in membrane damage associated with ischemia (Ahumada et al., 1979; Owens et al., 1979; Shrago et al., 1976). It appears that the energy requirements for Ca\(^{2+}\) uptake place a great demand on the mitochondrial respiratory chain as reflected in the initial rates of respiration (Fig. 4; upper dashed traces) during active accumulation. The slower rates of respiration following Ca\(^{2+}\) uptake in ischemic heart mitochondria (Fig. 4B; upper dashed trace) would suggest the "energy poise" of the system is insufficient to maintain the internal Ca\(^{2+}\) load. The rapid oxidation (or loss) of NADH (Fig. 4B; middle trace) is a manifestation of this decreased "energy poise." The rapid decrease in NADH associated with Ca\(^{2+}\) loss would appear to be consistent with the hypothesis that a higher reduced steady state of this coenzyme favors Ca\(^{2+}\) retention (Lehninger et al., 1978). However, marked inner membrane swelling (Fig. 4B; lower trace) also is associated with Ca\(^{2+}\) loss. The role of adenine nucleotides in maintaining internal mitochondrial membrane stability in the face of a Ca\(^{2+}\) load (Fig. 4C) appears crucial to the retention of Ca\(^{2+}\) (Harris, 1979). Further work is currently in progress to determine the primacy of one process over the other. The data here suggest a selective membrane damage has occurred in ischemic heart mitochondria which results in greater damage to the inner membrane and energy-transducing system involved with Ca\(^{2+}\) transport than that associated with ATP synthesis.

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