ORIGINAL CONTRIBUTIONS

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Mitochondrial function after global cardiac ischemia and reperfusion: Influences of organelle isolation protocols*)

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Summary

Dog hearts were made globally ischemic for 1 hr at normothermia, at 28 °C, or at normothermia after perfusion with a hyperkalemic cardioplegia solution. After 1 hr of reperfusion mitochondria were isolated from each heart using three protocols involving: processing (homogenization and centrifugation) exclusively in KCl, Tris-EDTA plus albumin (KEA) solution; homogenizing in KEA but washing mitochondria in EDTA-depleted media (KA); or processing exclusively in EDTA-free medium.

Postreperfusion contractile measurements indicated that only hypothermic hearts had function not significantly different from nonischemic controls, but significantly better than that of normothermic, nontreated ischemic hearts. Mitochondrial studies indicated no differences between ischemic-hypothermic, potassium-arrested or nonischemic groups. Mitochondrial function paralleled contractile function only in the severely damaged hearts made ischemic at 37 °C without perfusion with cardioplegic solution. These comparisons were not dependent upon whether mitochondrial function was assessed in terms of respiratory rates, respiratory control (R.C.) or ADP : O ratios, or the oxidative phosphorylation rate (O.P.R.; State 3 oxygen consumption rate X ADP : O ratio).

Adding EDTA to organelles isolated by gradually removing EDTA after homogenization in KEA had differing effects. In the ischemic nontreated group, adding EDTA increased State 3 rate, decreased State 4 rate, and increased both R.C. ratios and the O.P.R. Adding EDTA to mitochondria from nonischemic hearts or hearts which were hypothermic or potassium-arrested decreased State 4 rates, increasing the R.C. ratio, but did not affect State 3 rates or the O.P.R. Initial

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homogenization of tissue in EDTA-free medium gave poorly-functioning mitochondria in all groups, and readministration of EDTA was without benefit.

We concluded that hypothermia was superior to normothermic cardioplegia in terms of contractile function, but not based on mitochondrial function. This suggests that in some hearts subjected to ischemic damage, defects of ATP utilization, rather than of ATP synthesis, may predominate.

We also concluded that for studies of cardiac mitochondrial function, EDTA or a similar chelator must be present in homogenizing media to provide meaningful data, but the chelator can be removed and readministered as desired during subsequent processing. However, the qualitative and quantitative effects of EDTA readministration depend upon the severity of ischemic damage to the myocardium from which the organelles are isolated.

Key words: cardioplegia, global ischemia, heart, hypothermia, mitochondria, myocardium, oxidative phosphorylation

Introduction

Many studies have documented alterations of mitochondrial function after cardiac ischemia. Some have provided strong evidence that impaired mitochondrial oxidative phosphorylation is an important biochemical lesion which contributes to contractile dysfunction, particularly after a severe ischemic insult. However, a variety of factors can influence the *in-vitro* behavior of mitochondria and so may affect the validity of conclusions made about possible relationships between mitochondrial and contractile status, or about the mechanisms of action of interventions used to reduce ischemic injury.

Some influential factors relate to inherent biochemical differences of mitochondrial subpopulations in the myocardium (13, 14, 19, 26, 28), and their apparently differing responses to pathological states such as ischemia (14, 28). Density gradient centrifugation techniques (13–15, 19, 28), rather than the widely-used differential centrifugation protocols, are suited best to evaluating these often subtle inherent differences. However, regardless of the centrifugation techniques used to isolate mitochondria, numerous preparative factors relating to tissue homogenization and the composition of solutions used to isolate or assay mitochondria can affect the functional characteristics of the organelles and can conceivably affect data interpretation.

To supplement a study designed to evaluate and compare the efficacy of several intraoperative interventions on cardiac contractile and hemodynamic function of dogs subjected to global cardiac ischemia, we examined the effects of three variations of a widely used mitochondrial isolation protocol (2, 3, 5, 8, 9, 11, 12–17, 20, 22, 25–28, 30, 10) on the oxidative phosphorylating activity of cardiac mitochondria. Since ethylenediamine tetraacetic acid (EDTA) is often used in mitochondrial isolation solutions, and Peng and colleagues (20) recently demonstrated its marked influences on isolated mitochondrial function, we evaluated whether it selectively altered the *in-vitro* behavior of organelles isolated from hearts which had incurred differing degrees of ischemic injury. We also attempted to evaluate which measures of mitochondrial respiration or phosphorylation were particularly suitable reflections of the contractile status of the heart from which the organelles were isolated.

Materials and methods

Surgery

Healthy mongrel dogs of either sex, weighing 20 to 25 kg, were anesthetized with pentobarbital sodium (25 mg/kg, i.v.) and mechanically ventilated with pure oxygen (positive end expiratory pressure was 2 cm H_2O). The chest was opened, and standard procedures were used to place cannulas necessary for making various hemodynamic and cardiac contractile measurements, and for instituting cardiopulmonary bypass (CPB).

Dogs were assigned randomly to one of the following groups:

Nonischemic controls

Dogs were placed on partial CPB for 1 hr at 37 °C, and then taken off pump for an additional hour. The heart was then excised and studied as described below. This group served as a control for the remaining groups.

Ischemic, nontreated

After placing the dogs on CPB we cross-clamped the aorta to produce 1 hr of global ischemia at 37 °C. No intervention was used to lessen ischemic injury. After ischemia the aortic clamp was removed and the heart was rested for 45 min while the dog was on partial bypass. The heart was excised 15 min after discontinuing partial bypass and restoring normal circulation.

Ischemic, hypothermic

Dogs were cooled to an intramyocardial temperature of 28 °C (total body hypothermia) during partial bypass, the aorta was cross-clamped, and the pericardial sac was filled with cool (28 °C) physiological saline solution (Na^+, 152 mEq/l; Ca^{2+}, 4.5; K^+, 5.4; HCO^-_3, 24; Cl^-, 177; and mannitol, 12.5 g/l). After 1 hr of ischemia the heart was reperfused as described above, and then excised.

Ischemic, potassium cardioplegia-pretreated

Immediately after cross-clamping the aorta we perfused the hearts of dogs in this group with physiological saline solution supplemented with KCl to give a K^+ concentration of 25 mEq/l. This produced prompt cardioplegia. The hearts were ischemic for 1 hr at 37 °C. They received a brief perfusion with cardioplegic solution 30 min after the start of ischemia, and again at the end of the ischemic period. Then they were reperfused 45 min while the dog was on partial bypass, and finally the heart was allowed to resume full circulatory support for 15 min before it was excised.

None of the dogs received inotropic drugs after ischemia.

Tissue sampling and analysis

The heart was excised quickly and placed in a basin containing normal saline at 4 °C to cool the tissue promptly. The heart was blotted and weighed. We cut transmural strips (approximately 5 mm thick) of left ventricular free wall and interventricular septum, and placed them in a beaker of either KEA (0.18 M KCl; 10 mM Tris-EDTA; 0.5 % bovine serum albumin, pH 7.40 at 4 °C) or KA solution (identical to KEA, but EDTA was omitted). We collected approximately 50 g of tissue in KEA and 25 g in KA.

Right ventricular free wall tissue was discarded because its mitochondria have intrinsically lower *in vitro* functional capacities than organelles isolated from the left ventricular wall (26). We selected KEA medium as the basic isolation solution because it is widely used and is superior, in several ways (11), to many other media.

Mitochondrial isolation

From a single heart, mitochondria were isolated simultaneously using three protocols described below. All steps were carried out at 4 °C. Tissue strips were trimmed with scissors, epicardial and endocardial tissue was discarded, and the trimmed tissue was placed in fresh KEA or KA. When all tissue was trimmed, the samples were blotted and weighed quickly, and sufficient tissue was reserved to yield 40 g in KEA and 20 g in KA. The tissue was minced finely with scissors, and KEA or KA solution was added to give 4 ml of solution per gram of trimmed tissue wet weight. The samples were divided into aliquots each containing approximately 5 g of minced tissue in 20 ml of solution.

The tissue aliquots were homogenized with three 5-sec bursts of a Tekmar® Tissuemizer Model SDT (the SDT-182E shaft was cooled to 4 °C before use) with the Control Module speed setting at 30. There was a 60 sec rest period between each homogenization of a given aliquot. To avoid contamination of KA solution with EDTA, tissue in KA was homogenized first, and the generator shaft was disassembled and cleaned before homogenizing tissue in KEA. Homogenates in a given medium were pooled, sufficient fresh medium was added to give 20 ml of homogenate per gram of tissue wet weight, and the homogenates were then mixed and divided into aliquots of approximately 200 ml each. The entire process was done in a cold room (4 °C).

The three isolation protocols tested involved only differential centrifugation, as outlined in Figure 1. One protocol (I) involved mitochondrial isolation exclusively in KEA. Another mitochondrial fraction (Protocol II) was obtained from half of the initial KEA homogenate, but the mitochondrial pellet was sequentially rinsed, resuspended with ("washed") and recentrifuged in solutions containing 1.0, 0.1 and 0.0 mM Tris-EDTA in modified KEA medium. Protocol III involved organelle isolation exclusively in EDTA-free medium. All pellets were gently resuspended using Teflon and glass homogenizers.

The final organelle pellets were washed with and resuspended in a small, measured volume of the appropriate medium (KEA or KA). The protein concentration of each suspension was determined using a Biuret method, using bovine serum albumin as a protein standard. The measured protein concentration of organelles resuspended in KEA was corrected to account for the 5 mg of albumin contained in each milliliter of solution. The organelle suspensions were kept on ice and all assays of respiratory activity were completed within 2 hr of recovery. The sequence of assaying the three organelle fractions was varied randomly each day.

Mitochondrial oxidative phosphorylation

We used a Gilson Oxygraph® with a Clark-type oxygen electrode. The basic assay medium contained 0.22 M sucrose, 4 mM K_2HPO_4, and 13.5 mM morpholinopropanesulfonic acid (MOPS) buffer, pH 7.4 at 30 °C. The final mitochondrial protein concentration in the electrode cuvette was 1.04 ± 0.06 mg/ml. Respiratory substrates tested were Tris-glutamate plus Tris-malate (9.4 mM each, final concentration), or Tris-succinate (7.5 mM, plus 1 μg rotenone per mg mitochondrial protein). Some experiments involving organelles resuspended in EDTA-free solution (Protocols II or III, Tables 3 and 4) were conducted without adding EDTA to the cuvette. In other experiments with these mitochondria we added Tris-EDTA to give a final concentration of 1.0 mM. The final Tris-EDTA concentration in the medium used to assay the function of mitochondria isolated and resuspended in KEA was 0.94 mM. Mitochondria, substrate, and EDTA, when added, incubated in the assay medium for 2 minutes, and the initial State 4 respiratory rate (substrate, phosphate present; ADP absent) was recorded. Then 500 nmoles of Tris-ADP were added, and State 3 rates (substrate, phosphate, ADP present) were recorded. We determined the exact concentration of ADP stock solutions spectrophotometrically, using a millimolar

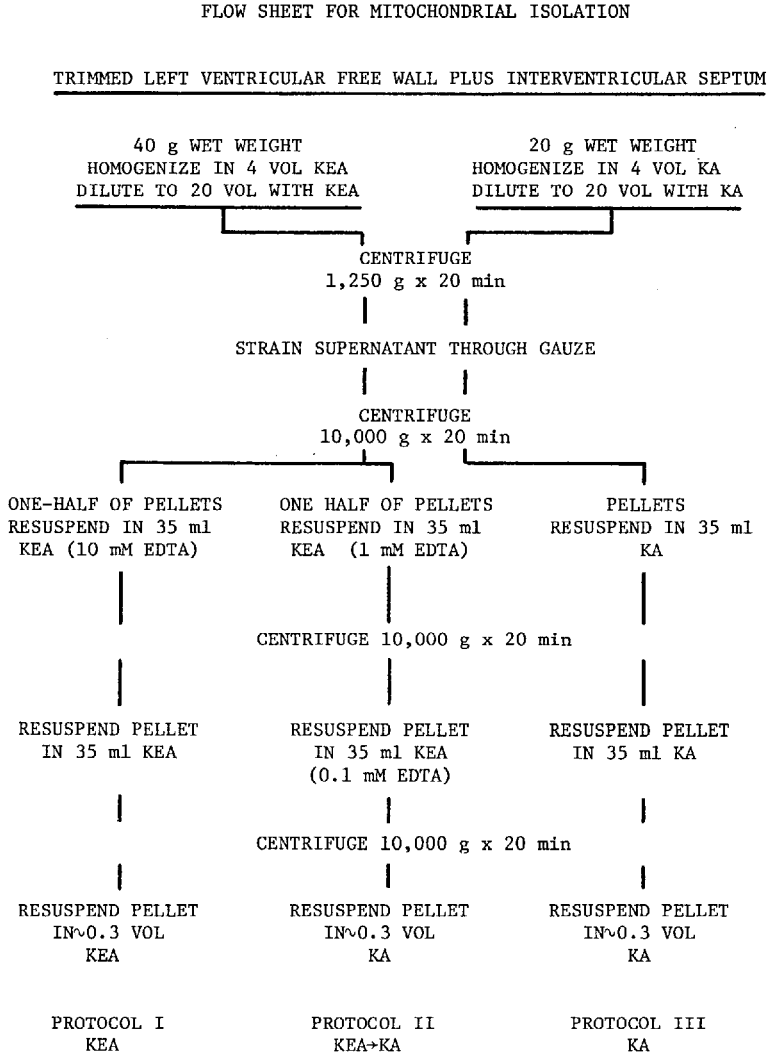

Fig. 1. This flow-chart summarizes the three protocols used to isolate mitochondria. From the left ventricular free wall and interventricular septum of each heart, mitochondria were isolated simultaneously using all three methods.

extinction coefficient of 15.4 at 259 nanometers. For any set of experimental conditions the mitochondria were assayed in triplicate, and when possible ADP was added twice during each run. Data from triplicate assays were averaged, and this value was used to calculate the overall mean values reported for each group in the Results section.

The respiratory control ratio (R.C.R.) was calculated as the quotient of the State 3 rate and the subsequent State 4 rate. Some mitochondria, particularly those isolated in EDTA-free medium from ischemic hearts, failed to return to a basal respiratory rate after the first ADP addition, and for these preparations we calculated a

respiratory control index (R.C.I.) as the quotient of the State 3 rate and the initial State 4 rate. The ADP : O ratio of mitochondria which returned to State 4 respiration after adding ADP was calculated as natoms of oxygen consumed during phosphorylation of the 500 nmoles of added ADP. The oxidative phosphorylation rate (O.P.R.) was calculated as the product of the State 3 respiratory rate and the ADP : O ratio. It estimates the amount of ATP synthesized per milligram of mitochondrial protein per minute (2, 3).

Mitochondrial Ca^{2+} content

The Ca^{2+} content of organelles isolated by Protocol II was assayed using standard atomic absorption spectrometry techniques. We did not assay Ca^{2+} content of organelles isolated by the other protocols.

Statistics

Data are summarized as arithmetic means plus or minus one standard error of the mean (SEM). Where noted in the text, single classification analysis of variance (ANOVA), or paired or nonpaired (group) t-tests were calculated. When ANOVA indicated no significant group differences, individual nonpaired t-tests were not made. In some instances, derived data such as R.C.R. or ADP : O ratios were subjected to logarithmic transformation, and the means plus or minus the 95 % confidence limits were used to make between-group comparisons. However, in these cases, conclusions about group differences were the same as when nontransformed data were subjected to ANOVA or t-tests, and so for simplicity the values of derived data are reported as noted above.

Results

Contractile status

There were no significant between-group differences for any of the various contractile and hemodynamic indicators which we evaluated before ischemia. Amongst these, we selected left ventricular maximum dP/dt and cardiac index as being representative indicators of cardiac status for comparison with mitochondrial data. Figures 2 and 3 summarize postischemic values for these two variables, and indicate that our experimental interventions produced groups of hearts with ischemic injury which qualitatively ranged from mild (ischemic, hypothermic) to severe (ischemic, nontreated).

Organelle yields

The overall yield (mg Biuret protein recovered per gram tissue wet weight) was 3.11 ± 0.12 mg/g [Table 1; ANOVA indicated that sample means in the various groups estimated a common population mean ($P < 0.25$)]. Although these values are low in comparison to some published values (16, 21, 27, 29), the data indicate no between-group differences of yield which might be attributable to ischemia, the interventions studied, or the isolation protocols used. The data suggest that isolating mitochondria exclusively in KA gave a decreased yield when tissue was taken from ischemic hearts subjected to either hypothermia or potassium cardioplegia pretreatment, but these yields were not significantly different from those obtained with other isolation protocols.

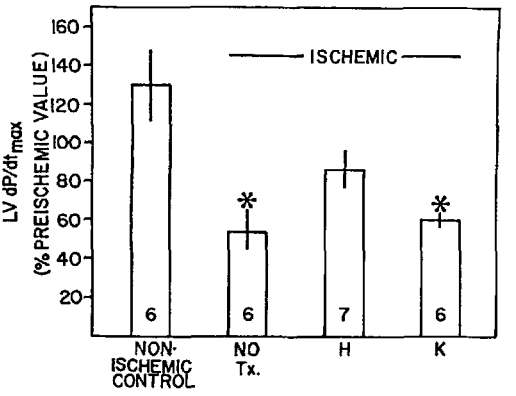

Fig. 2. Postischemic left ventricular dP/dt_{max} was measured after 45 min on partial bypass and 15 min of normal unassisted circulation, immediately before excising the heart. Values are reported as arithmetic means ± 1 S.E.M., expressed as a percent of preischemic values. The pooled preischemic left ventricular dP/dt_{max} was 2744 ± 126 mm Hg · sec^{-1}. An asterisk (*) denotes a statistically significant difference from values measured at a corresponding time in nonischemic control hearts. Of the ischemic-reperfused hearts, only those made hypothermic (H) during ischemia did not incur significant losses of dP/dt, compared to their preischemic values (paired t-tests). (K) denotes normothermic, potassium-arrested hearts.

Mitochondrial Ca^{2+} content

The organelle fractions isolated from nonischemic control hearts using Protocol II contained 3.64 ± 0.43 nmoles Ca^{2+}/mg protein (mean ± S.E.M.). Values for organelles isolated from ischemic hearts which were hypothermic or received cardioplegia solution were 2.47 ± 0.53 and 2.53 ± 0.59 nmoles/mg, respectively (not significantly different from control).

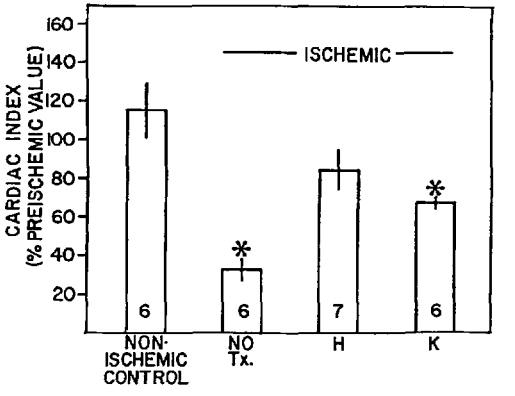

Fig. 3. Postischemic cardiac index was measured at the same time as noted for dP/dt (Figure 2). Pooled preischemic cardiac indexes for all hearts was 3.06 ± 0.10 l · min^{-1} · M^{-2}. As was the case with dP/dt, only hearts made hypothermic during ischemia did not incur significant losses of cardiac index, compared to preischemic values in the same group.

Table 1. Effect of global ischemia, protective interventions, and isolation media on organelle yields[a].

	Isolation protocol[b]		
	I (KEA)	II (KEA → KA)	III (KA)
Group			
Nonischemic control	3.12 ± 0.39	3.03 ± 0.46	2.78 ± 0.46
Ischemic, nontreated	2.90 ± 0.42	2.73 ± 0.45	3.10 ± 0.32
Ischemic, hypothermic	3.78 ± 0.39	3.39 ± 0.34	2.70 ± 0.24
Ischemic, K^+-cardioplegia	4.10 ± 0.57	3.20 ± 0.49	2.41 ± 0.31

[a] Numbers in table represent organelle yields, calculated as mg of protein recovered per gram of trimmed tissue wet weight. Single classification ANOVA indicated that all group means estimated a common population mean ($F_{11,66} = 1.34$). Values are reported as means ± one standard error.

[b] Solution compositions and centrifugation times are noted in text and Figure 1.

Organelles isolated from nontreated ischemic hearts contained 5.95 ± 0.89 nmoles Ca^{2+}/mg ($P < 0.05$).

Mitochondrial oxidative phosphorylation

The data comparing the effects of ischemia, protective interventions, and the influences of isolation media on *in vitro* mitochondrial function, are summarized in Tables 2, 3 and 4.

Table 2 shows data obtained from organelles isolated exclusively in KEA. This protocol resembles best the isolation techniques reported in the majority of published mitochondrial studies, and our data for mitochondria isolated from nonischemic control hearts indicate that regardless of the substrates used, these organelles had *in vitro* function which clearly was equivalent to accepted literature values for comparable experimental conditions, and to values for normal canine left ventricular myocardium (26). Similar results were obtained by initial homogenization in KEA followed by gradual removal of EDTA during isolation, and readministration of the agent in the assay medium (Table 3).

Table 4 shows data from organelles isolated exclusively in EDTA-free medium. For most groups prepared this way, only a small number of preparations demonstrated ADP-stimulated respiration, and the addition of Tris-EDTA to the assay medium at a concentration (1 mM) close to that of organelles assayed after isolation in KEA (protocol I, Table 2) failed to improve mitochondrial function significantly. Adding EDTA also failed to change significantly the number of preparations which showed an increased (State 3) respiratory rate when ADP was added, based on data analysis with Fisher's Exact Test (24). Using the same data analysis we found that the responsiveness of the preparations to ADP, whether in the presence or absence of added EDTA, did not depend upon the substrate used.

Table 2. Respiratory function[a] of mitochondria isolated from nonischemic of ischemic-reperfused hearts using isolation protocol I[b].

			Oxygen consumption (n Atoms O/min/mg protein)				
Group	*N*	*Substrate*[c]	*State 3*	*State 4*	*R.C.R.*	*ADP : O ratio*	*O.P.R.*
Nonischemic	6	G + M	185 ± 15	12 ± 1	15.0 ± 0.8	2.9 ± 0.1	541 ± 48
control		SUC	185 ± 13	39 ± 2	4.8 ± 0.4	1.7 ± 0.1	320 ± 27
Ischemic,	6	G + M	91 ± 4*	9 ± 1	10.0 ± 0.6*	2.9 ± 0.1	277 ± 9*
nontreated		SUC	112 ± 7*	31 ± 4	3.8 ± 0.4*	1.6 ± 0.1	182 ± 12*
Ischemic	7	G + M	165 ± 14	13 ± 1	13.2 ± 0.7	2.7 ± 0.1	542 ± 46
hypothermic		SUC	179 ± 14	40 ± 4	4.5 ± 0.3	1.5 ± 0.1	277 ± 25
Ischemic,	6	G + M	149 ± 9	13 ± 0	11.8 ± 0.7*	2.7 ± 0.1	409 ± 34*
K^+- cardioplegia		SUC	167 ± 8	41 ± 3	4.2 ± 0.2*	1.6 ± 0.1	269 ± 23*

[a] Values are reported as means ± 1 standard error of the mean.

[b] Organelles were isolated exclusively in KEA. Mitochondrial respiration was studied in a medium containing 0.22 M sucrose, 4.4 mM K_2HPO_4, 310 μM Na_2ADP, and mitochondria (1.04 ± 0.06 mg protein/ml), in 13 mM morpholinopropanesulfonic acid (MOPS) buffer (pH 7.40 at 30 °C). Final [EDTA] was 940 μM. Substrates are noted below.

[c] G + M, glutamate plus malate (9.4 mM each, Tris salts); SUC, succinate (7.5 mM, Tris salt), plus rotenone (1 μg/mg mitochondrial protein)

* Indicates a significant difference ($p < 0.05$ based on nonpaired t-tests) from corresponding value in nonischemic control group.

Table 3. Respiratory function[a] of mitochondria isolated from nonischemic or ischemic-reperfused hearts using isolation protocol II[b]: Effects of added EDTA.

		Oxygen consumption (n Atoms O/min/mg protein)				
Group	*Tris-EDTA (mM)*	*State 3*	*State 4*	*R.C.R.*	*ADP : O ratio*	*O.P.R*
Substrates: Glutamate + malate						
Nonischemic	0	241 ± 29	22 ± 1	11.0 ± 1.0	2.9 ± 0.1	694 ± 87
control	1	264 ± 24	16 ± 1#	15.4 ± 1.0#	3.0 ± 0.1	684 ± 73
Ischemic,	0	78 ± 4*	16 ± 1*	5.0 ± 0.4*	2.6 ± 0.1	201 ± 12*
nontreated	1	113 ± 6*#	12 ± 2#	9.5 ± 0.8*#	2.8 ± 0.1#	319 ± 18*#
Ischemic,	0	215 ± 20	25 ± 2	8.8 ± 0.7	2.6 ± 0.1	562 ± 56
hypothermic	1	223 ± 17	16 ± 1#	14.3 ± 0.9#	2.7 ± 0.1	613 ± 58
Ischemic,	0	218 ± 20	29 ± 3*	7.9 ± 1.1	2.6 ± 0.1	569 ± 69
K^+-cardioplegia	1	218 ± 14	17 ± 1#	13.4 ± 0.8#	2.8 ± 0.1#	617 ± 52
Substrate: succinate						
Nonischemic	0	246 ± 22	55 ± 7	4.8 ± 0.6	1.7 ± 0.1	413 ± 47
control	1	236 ± 19	43 ± 3#	5.6 ± 0.5#	1.8 ± 0.1	423 ± 42
Ischemic,	0	114 ± 16*	41 ± 5*	2.8 ± 0.3*	1.4 ± 0.1	158 ± 24*
nontreated	1	128 ± 11*	39 ± 5	3.5 ± 0.3*#	1.6 ± 0.1	203 ± 23*#
Ischemic,	0	234 ± 18	72 ± 6*	3.3 ± 0.3	1.5 ± 0.1	345 ± 35
hypothermic	1	231 ± 19	50 ± 4#	4.7 ± 0.3#	1.6 ± 0.1	371 ± 34
Ischemic,	0	234 ± 12	71 ± 7*	3.5 ± 0.6	1.5 ± 0.1	352 ± 39
K^+-cardioplegia	1	227 ± 11	48 ± 3#	4.8 ± 0.4#	1.7 ± 0.1#	389 ± 31

[a] Values are reported as means ± 1 standard error of the mean. The number of preparations studied is the same as in Table 2.

[b] Isolation involved initial tissue homogenization in KEA, organelle resuspension in KA, and is described in text and Figure 1. Except for [EDTA], assay medium was identical to that noted in Table 2.

* Indicates a significant difference ($P < 0.05$ based on *nonpaired* t-tests) from corresponding value in nonischemic control group.

# Indicates a significant difference ($P < 0.05$ based on *paired* t-tests) from value obtained in absence of added EDTA.

Table 4. Respiratory function[a] of mitochondria isolated from nonischemic or ischemic-reperfused hearts using isolation protocol III[b]: Effects of added EDTA.

Group	Tris-EDTA (mM)	Preparations responding to added ADP	Oxygen consumption (n Atoms O/min/mg protein) State 3	State 4	R.C.R.[c] or R.C.I.[d]	ADP : O ratio	O.P.R.
Substrates: glutamate + malate							
Nonischemic control	0	1 of 6	103	17 ± 4	3.5[c]	2.0	206
	1	4 of 6	47 ± 27	10 ± 2#	4.0 ± 1.2[c]	2.0 ± 0.2	111 ± 74
Ischemic, nontreated	0	0 of 6	N.D.	12 ± 2*	N.D.	N.D.	N.D.
	1	0 of 6	N.D.	10 ± 1	N.D.	N.D.	N.D.
Ischemic, hypothermic (H)	0	3 of 7	96 ± 27	19 ± 3	3.6 ± 0.8[c]	2.0 ± 0.2	207 ± 72
	1	3 of 7	106 ± 24	15 ± 2#	5.6 ± 0.5#	2.4 ± 0.1	260 ± 65
Ischemic, K^+-cardioplegia	0	2 of 6	44 ± 29	23 ± 4	2.2 ± 0.4[c]	2.0 ± 0.1	84 ± 54
	1	4 of 6	49 ± 17	12 ± 2#	4.1 ± 0.6#[c]	2.1 ± 0.2	110 ± 33
Substrate: succinate							
Nonischemic control	0	2 of 6	114 ± 2	73 ± 10	1.8 ± 0.2[d]	N.D.	N.D.
	1	2 of 6	93 ± 4	68 ± 4	1.4 ± 0.1[d]	N.D.	N.D.
Ischemic, nontreated	0	5 of 6	62 ± 12*	68 ± 21	1.3 ± 0.1*[d]	N.D.	N.D.
	1	1 of 6	46	60 ± 9	1.1	N.D.	N.D.
Ischemic, hypothermic	0	1 of 7	163	90 ± 9	2.7[d]	1.3	212
	1	2 of 7	133 ± 17*	84 ± 5*	2.4 ± 0.5*[c]	1.2 ± 0.1	196 ± 66
Ischemic, K^+-cardioplegia	0	1 of 6	98	80 ± 12	1.5[d]	N.D.	N.D.
	1	2 of 6	102 ± 4	70 ± 5	1.8 ± 0.1*[d]	N.D.	N.D.

[a] Values are reported as means ± 1 S.E.M.
[b] Organelles were isolated exclusively in KA. Unless noted above, the assay medium contained no EDTA.
[c] Value reported is a Respiratory Control Ratio (R.C.R.) as defined in Methods.
[d] Value reported is a Respiratory Control Index (R.C.I.) as defined in Methods.
N.D. Not determined: because of the poor function of the preparation these values could not be measured or calculated.
* Indicates a significant difference ($P < 0.05$ based on nonpaired t-tests) from corresponding value in nonischemic control group.
# Indicates a significant difference ($P < 0.05$) from value obtained in absence of added EDTA.

Tables 2 and 3 show that with the exception of the ADP : O ratio, most indicators of mitochondrial function were significantly depressed when the organelles were isolated from hearts made ischemic for 1 hr at 37 °C with no protective intervention before or during ischemia. Nevertheless, evaluating data for mitochondria of nontreated or treated ischemic hearts showed subtle differences of function within or between groups. These differences depended upon the isolation protocol, incubation medium composition (substrate; presence or absence of EDTA), and upon the particular measured or derived variable considered indicative of organelle status. This dependency can be seen in mitochondria isolated from hearts perfused with a potassium cardioplegia solution. When mitochondria were isolated exclusively in KEA (Table 2), in the presence of glutamate plus malate, both R.C.R. and O.P.R. were significantly depressed, although the slight decreases of respiratory rates and the ADP : O ratio, which are used to calculate R.C.R. or O.P.R., were not statistically different from values measured in mitochondria isolated from nonischemic control hearts. In contrast, when mitochondria from potassium-arrested ischemic hearts were isolated by a protocol involving initial homogenization in KEA, with final resuspension in an EDTA-free medium (Table 3), neither R.C.R. nor the O.P.R. differed significantly from values in the nonischemic control group, regardless of whether EDTA was added to the assay medium.

Comparing data in Table 2 with those in Table 3 revealed that the isolation protocol had little effect on the overall poor function of mitochondria isolated from nontreated ischemic hearts. However, examining data for mitochondria in other groups, including the nonischemic control, shows the effects of maintaining EDTA throughout the isolation, its removal during isolation, and the readdition of EDTA to some of the preparations.

Removing EDTA during isolation and omitting it from the assay medium consistently and significantly increased both State 3 and State 4 respiratory rates ($P < 0.05$ based on paired t-tests), whether mitochondria were respiring in the presence of glutamate plus malate, or of succinate. In relative terms, the increase of State 4 respiratory rates was greater than that of State 3, and since the R.C.R. is the quotient of these two rates, removing EDTA decreased this ratio. EDTA removal had no significant effect on ADP : O ratios of mitochondria from hearts other than the ischemic-nontreated ones, and so calculated O.P.R. values were generally increased compared to values obtained with organelles isolated exclusively in KEA (Table 2).

When tissue was homogenized in KEA but EDTA was gradually removed during mitochondrial isolation, the readministration of EDTA to the isolated organelles generally decreased State 4 respiratory rates significantly, but did not alter State 3 rates significantly. Thus, the net effect of adding 1 mM EDTA was to increase calculated R.C.R. values to a level not significantly different from those obtained when KEA was used throughout, and the final EDTA concentration in the assay medium was 0.94 mM. This EDTA-dependent effect on respiratory rates and R.C.R. did not apply to mitochondria isolated from ischemic-nontreated hearts.

Removing EDTA during isolation significantly decreased the ADP : O ratios of mitochondria obtained from ischemic-nontreated hearts, com-

pared to values obtained when KEA was used throughout isolation. For the ischemic-nontreated group, and for the group which received cardioplegic solution before ischemia, we found that adding 1 mM EDTA to mitochondria obtained by Protocol II (Figure 1) significantly increased calculated ADP : O ratios compared to values measured in the absence of EDTA (Table 3). Numerically this increase was slight, but it was consistent and statistically significant based on paired t-tests, and did not depend upon the substrate used.

For a given set of experimental conditions we found no instance in which calculated values of R.C.R., but not of O.P.R., were significantly different from control; the converse was also true. However, there were many instances in which ADP : O ratios and respiratory rates of mitochondria from ischemic hearts were not different from control, despite the fact that R.C.R. and O.P.R. were significantly decreased.

Discussion

Our contractile and hemodynamic data indicated that modest hypothermia or potassium-induced arrest at 37 °C lessened cardiac damage produced by 1 hr of global ischemia. The protection afforded by hypothermia was greater than that provided by hyperkalemic normothermic arrest, as only hearts in the former group consistently had functional indices which were not significantly different from those measured in nonischemic controls. The greater protection occurred although hearts in the hypothermic group continued to contract for several minutes after aortic cross-clamping, while the perfusion of a hyperkalemic solution, even at normothermia, caused prompt asystole. Other indicators of cardiac function (left ventricular stroke work index, minute work, etc.) supported the conclusion that hypothermia was generally more efficacious than normothermic cardioplegia.

Our current data, and other data from our laboratory (1) and elsewhere (9), suggest that a direct relationship between contractile and mitochondrial function does not exist necessarily or invariably, although this apparent disparity may be affected by preparative factors or species-related differences (4, 6) in the tolerance of cardiac mitochondria to ischemia. An alternate proposal is that one or more other organelles are more susceptible (sensitive) to ischemic insults. Gillette and colleagues (5) used a model similar to ours and found differential decreases of microsomal (sarcoplasmic reticulum) and mitochondrial function after ischemia. They emphasized a strong association between depressed postischemic contractility and lesions of microsomal calcium (Ca) metabolism. Interestingly, they found that 1 hr of hypothermic ischemic arrest at 26 °C provided considerably less protection than that which we obtained at 28 °C. Nevertheless, the above information collectively supports the proposal that with modest ischemic injury, defects of ATP utilization, rather than of ATP supply, may predominate.

Peng and colleagues (20) reported an alleged Ca-related depression of State 3 respiratory rates, ATP production and ADP : O ratios of mitochondria isolated from regionally-ischemic or ischemic-reperfused porcine hearts. Adding EDTA or EGTA increased ADP-stimulated (State 3) rates

of oxygen consumption and the efficiency of ATP synthesis when the organelles were isolated in EDTA- or EGTA-free media. Neither chelator affected mitochondria isolated from nonischemic and allegedly normal regions of these hearts, and the organelles isolated from these regions had relatively good *in-vitro* function. In contrast, we found that initial tissue homogenization in EDTA-free medium was unsuitable, even when nonischemic hearts were studied. The reason for this apparent discrepancy is not known.

Since mitochondrial ATPase is very sensitive to Mg^{2+}, and Mg-induced ATPase activiation and regeneration of ADP would increase an apparent State 4 respiratory rate after initial ADP addition, it is also possible that Mg may somehow participate in the observed response to the chelators. Peng and colleagues (20) reported that EGTA, a comparatively good chelator of Ca but not of Mg, produced EDTA-like effects, suggesting that the common mechanism involved specific modulation of Ca, rather than of Mg, to "reverse" abnormal oxidative phosphorylation. However, they published only a few ostensibly representative oxygen electrode tracings showing the comparative effects of EDTA as opposed to EGTA, and so it is difficult to state with certainty whether either agent had preferential effects on State 4 rates which might indirectly reflect ATPase activity due to Mg.

In accord with Peng's attribute of Ca-dependency, we found that added EDTA affected basal respiratory rates before ADP was added, as well as after ADP addition, at a time when it is being phosphorylated to ATP, which in turn could be dephosphorylated to regenerate ADP. We also observed that oligomycin, added at concentrations which blocked ADP-stimulated respiration and ATP synthesis, did not alter the effects of EDTA addition on basal respiratory rate. In contrast, however, we did find that in some instances adding 1 mM Tris-EDTA produced greater improvement of R.C.R., primarily by increasing State 3 rates and decreasing State 4 rates further, than did 1 mM Tris-EGTA. These latter observations, although not repeated enough for statistical evaluation, would argue in favor of Mg chelation and perhaps suppression of ATPase activity, as an important mechanism by which these manipulations "improve" mitochondrial function.

Finally, we wished to evaluate which of the commonly-used indicators of mitochondrial respiration and phosphorylation might be selectively influenced by ischemia or the methods which we used. Recently Edoute and colleagues (3) considered whether mitochondrial respiratory rates, R.C.R., O.P.R. or ADP : O ratios were "suitable" indicators of organelle integrity. They showed that under some conditions the respiratory control index and ADP : O ratios adequately reflected organelle status, but in other situations, involving ischemia, the respiratory control indexes and ADP : O ratios of mitochondria isolated from ischemic hearts did not differ from values for normal mitochondria, yet when either State 3 respiratory rates of the O.P.R. values for these two groups were compared, the differences were statistically significant. They argued that the normalized rate of mitochondrial ATP synthesis, estimated as the O.P.R., might be more biologically relevant than a measure of respiratory control for evaluating organelle activity *in situ* and for possibly relating

mitochondrial status to the contractile status of the heart from which they were isolated. However, we found that whenever R.C.R. values of mitochondria from ischemic hearts differed significantly from corresponding values in nonischemic hearts, the O.P.R. also differed. Since some pathological or pharmacological interventions can preferentially alter State 3 or State 4 rates, or both, without affecting the ADP : O ratio, it is clear that the calculated O.P.R., but not necessarily the R.C.R., could change markedly. In view of this, it appears prudent to report as many of the accepted variables as might be needed to interpret the effects of any intervention on mitochondrial function.

Overall, based on the methods we used, our data indicate that unless an ischemic insult is severe, modest losses of contractile function in the early postischemic recovery period are not necessarily accompanied by parallel losses of mitochondrial function. Isolating organelles exclusively in EDTA-free medium provided little meaningful data. However, isolating mitochondria in KEA gave excellent results, but the gradual removal of EDTA also provided a preparation with excellent function and one in which the effects of Ca addition or Ca chelation can be evaluated further. As Matlib (13) and Nagao (15) have shown, the combination of mechanical tissue disruption without proteases, followed by organelle fractionation by differential centrifugation, can provide a suitable method for studying mitochondrial function in normal or ischemic heart.

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References

1. Bush, L. R., M. Shlafer, D. W. Haack, B. R. Lucchesi: Time-dependent changes in canine cardiac mitochondrial function and ultrastructure resulting from coronary occlusion and reperfusion. Basic Res. Cardiol. **75**, 551–571 (1980).
2. Dhalla, N. S., J. C. Yates, S. L. Lee, A. Singh: Functional and subcellular changes in the isolated rat heart perfused with oxidized isoproterenol. J. Molec. Cell. Cardiol. **10**, 31–41 (1978).
3. Edoute, Y., J. C. N. Kotze, A. Lochner: Oxidative phosphorylation rate: An index for evaluation of mitochondrial function in myocardial ischemia. J. Molec. Cell. Cardiol. **11**, 831–833 (1979).
4. Feuvray, D., J. de Leiris, D. J. Hearse, S. M. Humphrey: Ultrastructural basis of species variation in myocardial cell damage. J. Molec. Med. **2**, 201–208 (1977).
5. Gillette, P. C., W. W. Pinsky, R. M. Lewis, E. P. Bornet, J. M. Wood, M. L. Entman, A. Schwartz: Myocardial depression after elective ischemic arrest. J. Thorac. Cardiovasc. Surg. **77**, 608–618 (1979).
6. Jennings, R. B., C. E. Ganote: Ultrastructural changes in acute myocardial ischemia. In: Effects of Acute Ischaemia on Myocardial Function, M. F. Oliver, D. G. Julian and K. W. Donald, eds., London, Churchill-Livingstone, pp. 50–74 (1972).
7. Jennings, R. B., P. B. Herdson, H. M. Sommers: Structural and functional abnormalities in mitochondria isolated from ischemic dog myocardium. Labor. Invest. **20**, 548–577 (1969).
8. Kane, J. J., M. L. Murphy, J. K. Bissett, N. deSoyza, J. E. Doherty, K. D. Straub: Mitochondrial function, oxygen extraction, epicardial S–T segment changes

and tritiated digoxin distribution after reperfusion of ischemic myocardium. Amer. J. Cardiol. **36,** 218–224 (1975).

9. Kay, H., G. Asimakis, G. Grotte, J. Hom, J. Fallon, F. Levine, J. Aprille, G. Austin, M. Buckley: Correlation of myocardial and mitochondrial function following ischemia and reperfusion. Circulation **58** (Suppl. 2), II–253 (1978).
10. Lentz, R. W., C. E. Harrison Jr., J. D. Dewey, D. A. Barnhorst, G. K. Danielson, J. R. Pluth: Functional evaluation of cardiac sarcoplasmic reticulum and mitochondria in human pathologic states. J. Molec. Cell. Cardiol. **10,** 3–30 (1978).
11. Lindenmayer, G. E., L. A. Sordahl, A. Schwartz: Reevaluation of oxidative phosphorylation in cardiac mitochondria from normal animals and animals in heart failure. Circulat. Res. **23,** 439–450 (1968).
12. Lochner, A., L. H. Opie, P. Owen, J. C. N. Kotze, K. Bruyneel, W. Gevers: Oxidative phosphorylation in infarcting baboon and dog myocardium: Effects of mitochondrial isolation and incubation medium. J. Molec. Cell. Cardiol. **7,** 203–217 (1975).
13. Matlib, M. A., D. Wilson, W. Rouslin, G. Kraft, P. Berner, A. Schwartz: On the existence of two populations of mitochondria in a single organ: Respiration, calcium transport and enzyme activities. Biochem. Biophys. Res. Comm. **84,** 482–488 (1978).
14. Murfitt, R. R., J. W. Stiles, W. J. Powell Jr., D. R. Sanadi: Experimental myocardial ischemia. Characteristics of isolated mitochondrial subpopulations. J. Molec. Cell. Cardiol. **10,** 109–123 (1978).
15. Nagao, T., M. A. Matlib, D. Franklin, R. W. Millard, A. Schwartz: Effects of diltiazem, a calcium antagonist, on regional myocardial function and mitochondria after brief coronary occlusion. J. Molec. Cell. Cardiol. **12,** 29–44 (1980).
16. Nayler, W. G., E. Fassold, C. Yepez: Pharmacological protection of mitochondrial function in hypoxic heart muscle: Effect of verapamil, propranolol, and methylprednisolone. Cardiovasc. Res. **12,** 152–161 (1978).
17. O'Connor, F., J. L. Castillo-Olivares, M. Gosalvez, D. Figuera: Effect of anoxic cardiac arrest and induced ventricular fibrillation on myocardial mitochondrial respiration and oxidative phosphorylation. J. Surg. Res. **19,** 325–332 (1975).
18. Page, E., J. Early, L. P. McCallister, C. Boud: Copper content and exchange in mammalian hearts. Circulat. Res. **35,** 67–76 (1974).
19. Palmer, J. W., B. Tandler, C. L. Hoppel: Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. J. Biol. Chem. **252,** 8731–8737 (1977).
20. Peng, C.-F., J. J. Kane, M. L. Murphy, K. D. Straub: Abnormal mitochondrial oxidative phosphorylation of ischemic myocardium reversed by Ca^{2+}-chelating agents. J. Molec. Cell. Cardiol. **9,** 897–908 (1977).
21. Scarpa, A., P. Graziotti: Mechanisms for intracellular calcium regulation in heart. I. Stopped-flow measurements of Ca^{++} uptake by cardiac mitochondria. J. Gen. Physiol. **62,** 756–772 (1973).
22. Schwartz, A., J. M. Wood, J. C. Allen, E. C. Bornet, M. L. Entman, M. A. Goldstein, L. A. Sordahl, M. Suzuki: Biochemical and morphological correlates of cardiac ischemia. Amer. J. Cardiol. **32,** 46–61 (1973).
23. Sharma, G. P., K. G. Varley, S. W. Kim, J. Barwinski, M. Cohen, N. S. Dhalla: Alterations in energy metabolism and ultrastructure upon reperfusion of the ischemic myocardium after coronary occlusion. Amer. J. Cardiol. **36,** 234–243 (1975).
24. Siegel, S.: Nonparametric Statistics for the Behavioral Sciences, New York, McGraw-Hill Book Company, pp. 96–101 (1956).
25. Sink, J. D., G. L. Pellom, W. D. Currie, W. R. Chitwood Jr., R. C. Hill, A. S. Wechsler: Protection of mitochondrial function during ischemia by potassium cardioplegia: Correlation with ischemic contracture. Circulation **60** (Suppl. I), I–158 – I–163 (1979).

26. Sordahl, L. A.: Differences in mitochondrial functions from right and left ventricular myocardium from four mammalian species. Compar. Biochem. Physiol. **54B,** 339–342 (1976).
27. Sordahl, L. A., C. L. Johnson, Z. R. Blailock, A. Schwartz: The mitochondrion. In: Methods in Pharmacology, Vol. 1, A. Schwartz, ed., New York, Appleton-Century-Crofts, pp. 247–286 (1971).
28. Sordahl, L. A., W. B. McCollum, W. G. Wood, A. Schwartz: Mitochondria and sarcoplasmic reticulum function in cardiac hypertrophy and failure. Amer. J. Physiol. **224,** 497–502 (1973).
29. Tomlinson, C. W., N. S. Dhalla: Excitation-contraction coupling in heart. IX. Changes in the intracellular stores of calcium in failing hearts due to lack of substrate and oxygen. Cardiovasc. Res. **7,** 470–476 (1973).
30. Wood, J. M., H. G. Hanley, M. L. Entman, C. J. Hartley, J. A. Swain, U. Busch, C.-H. Chang, R. M. Lewis, W. J. Morgan, A. Schwartz: Biochemical and morphological correlates of acute experimental myocardial ischemia in the dog. IV. Energy mechanisms during very early ischemia. Circulat. Res. **44,** 52–61 (1979).
31. Wrogeman, K., E. G. Nylen: Mitochondrial calcium overloading in cardiomyopathic hamsters. J. Molec. Cell. Cardiol. **10,** 185–195 (1978).

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