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Time-dependent changes in canine cardiac mitochondrial function and ultrastructure resulting from coronary occlusion and reperfusion*)

Zeitabhängige Veränderungen von Funktion und Ultrastruktur der Mitochondrien nach Koronarverschluß und Reperfusion beim Hundeherzen

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With 5 figures and 2 tables

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Summary

Time-dependent changes in mitochondrial function and structure resulting from 1 hr of left circumflex coronary artery occlusion followed by 2 to 24 hrs of reperfusion were examined. These changes were correlated with changes in myocardial ultrastructure, tissue water content, infarct size and mitochondrial calcium content. The heart was removed after different periods of reperfusion, and mitochondria were isolated from ischemic and nonischemic regions of the left ventricle. Tissue samples from ischemic and nonischemic myocardium also were taken for electron microscopy and tissue water content determinations. Infarct size was measured by the nitroblue tetrazolium staining method. Oxygen consumption by mitochondria isolated from ischemic and nonischemic myocardium was measured *in vitro*. Mitochondria from ischemic myocardium showed time-dependent decreases in rates of oxygen consumption and tightness of coupling. Electron microscopy revealed progressive ultrastructural deterioration in ischemic myocardium, including accumulation of calcium deposits within mitochondria, a finding corroborated by elevated concentrations of calcium in mitochondria isolated from the same area. Tissue wet-to-dry weight ratios were increased significantly in ischemic myocardium. A small, but significant, decrease in respiratory function was observed in mitochondria isolated from nonischemic myocardium several hrs after reperfusion; however, nomal respiration was observed 24 hrs after release of occlusion. This latter observation indicates that the nonischemic zone also is affected by regional ischemia. The results obtained indicate that temporary left circumflex artery occlusion and reperfusion result in progressively decreasing mitochondrial function and structure within the ischemic myocardium, and that these changes are accompanied by cellular electrolyte alterations.

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The functional and ultrastructural integrity of cardiac mitochondria decline during periods of ischemia. Furthermore, ischemic periods exceeding 40 minutes have been shown to result in irreversible damage to mitochondria within the center of an ischemic zone, regardless of whether or not reperfusion occurs (4, 7, 8–11, 18). In addition, myocardial ischemia results in a rapid decline in the intracellular quantities of high energy phosphate compounds which are produced primarily by mitochondrial oxidative phosphorylation (1, 5, 12). Since normal myocardial cell function requires strict maintenance of ionic gradients (4, 8) and these, in turn, require ample supplies of ATP, myocardial ischemia initiates a vicious cycle. Consequently, the functional capacity of mitochondria plays a crucial role in determining the fate of myocardial cells rendered temporarily ischemic.

These experiments were performed to examine the temporal changes of mitochondrial function and structure within ischemic and nonischemic myocardium at various times (2, 4, 6 or 24 hrs) after release of left circumflex (LCX) coronary artery occlusion. Data were obtained from *in-vitro* measurements of mitochondrial oxygen consumption rates (QO_2) and from electron microscopy (EM). The changes observed in these parameters were correlated with infarct size, tissue water content, and mitochondrial calcium content. The results show that after a constant (1 hr) period of regional myocardial ischemia, mitochondria within the ischemic zone underwent progressive ultrastructural and functional deterioration as reperfusion ensued. An additional finding was that mitochondria isolated from the nonischemic zone displayed a temporary decline in respiratory function, but appeared to regain normal function by 24 hrs after reperfusion.

Materials and methods

A. *Surgery*

Male mongrel dogs (10–16 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and respired under positive pressure with room air. The chest was entered *via* a left thoracotomy, and the pericardium was opened to expose the left ventricle. The LCX was isolated beneath the left atrial appendage, at a site distal to its atrial branch and proximal to major ventricular branches. The LCX was then partially occluded with a silk ligature tied around a 20 gauge needle, and the needle was removed immediately, leaving a vessel lumen diameter not significantly smaller than normal. Immediately following this, the LCX was totally occluded using silastic tubing in a figure eight snare. This model of temporary LCX occlusion, previously described (15), was employed in order to avoid the fatal arrhythmias and hemorrhagic infarction often encountered in occlusion-release experiments that do not employ stenosis. Although the partial constriction used in this model prevents the reactive hyperemia after release of coronary occlusion, it does not alter significantly coronary flow relative to control values, as indicated by measurements of coronary blood flow and regional myocardial force production (15). As such, the stenosis does not produce ischemia itself. The period of occlusion was 1 hr in all experiments, after which time controlled reperfusion through the stenosis was allowed. Sham-operated animals were treated identically to experimental animals except that a loose ligature was placed around the LCX, but the vessel was not occluded or stenosed. Lead II Ecg and arterial blood pressure were recorded

throughout the operative procedure. Arterial blood samples were drawn regularly, and PO_2, PCO_2, and pH were measured using an Instrumentation Laboratory pH/blood gas analyzer. These parameters were maintained within physiological limits by adjusting the tidal volume and respiratory rate. Dogs studied 24 hrs after ischemia and reperfusion were operated on under aseptic conditions and allowed to recover. The next day their arterial blood pressure and Ecg were recorded for 1 hr in the conscious state and then re-anestetized with pentobarbital (20–25 mg/kg, i.v.). After one hour, to allow for redistribution of the anesthetic, hearts were removed from these animals.

B. *Isolation of mitochondria*

At 2, 4, 6 or 24 hrs after release of the LCX occlusion the heart was excised quickly, immersed in cold (4 °C) saline (1.5 l) and taken to a cold room, where all further preparations were done. The heart was washed several times with cold saline and cut into seven or eight transverse slices from which ischemic-reperfused (hereafter referred to simply as "ischemic") and nonischemic myocardium were separated. Small portions from each region also were taken for determinations of water content and for EM. Transmural portions comprising the entire posterior papillary muscle and approximately 1 cm of tissue on either lateral side were used for "ischemic" myocardium. *Jennings* et al. have shown that this area of the left ventricle experiences ischemia upon occlusion of the LCX (8). Samples used for nonischemic myocardium consisted of transmural pieces spanning from the middle of the anterior papillary muscle to the junction of the anterior free wall and interventricular septum. This tissue selection process was maintained constant throughout all studies.

The tissue pieces were trimmed of fat and connective tissue, blotted, weighed, and minced with scissors in cold (4 °C) KEA medium (KCl, 180 mM; EDTA (Tris-salt), 10 mM; bovine serum albumin, 0.5%; pH 7.4). The mince was homogenized with a Tekmar Tissuemizer (three, five-second bursts at 35,000 rpm). Mitochondria were isolated by differential centrifugation according to the method of *Sordahl* et al. (27). The final mitochondrial pellet was resuspended in KEA and its protein concentration measured by the biuret method.

C. *Measurements of mitochondrial respiration*

The respiratory activity of mitochondrial suspensions was measured at 30 °C, using a Gilson Oxygraph, equipped with a Clark oxygen-sensitive electrode. The composition of the incubation medium in which respiration was measured is given in the legend to table 1. The parameters used to assess mitochondrial oxidative phosphorylation are defined below:

State 3 QO_2	=	rate of oxygen consumption stimulated by ADP in the presence of substrate
State 4 QO_2	=	basal rate of oxygen consumption in the absence of ADP, but with substrate present
Respiratory Control Ratio (RCR)	=	State 3 QO_2/State 4 QO_2; an index of tightness of coupling of oxygen consumption to phosphorylation of ADP
ADP : O Ratio	=	nmoles of ADP consumed (presumably phosphorylated to ATP) per natom of oxygen consumed; an index of the efficiency of oxidative phosphorylation

D. *Electron microscopy (EM)*

Samples of ischemic and nonischemic myocardium were taken from the middle of the posterior and anterior papillary muscles, respectively, immediately after cooling the excised heart. Maximum effort was made to cool and dissect the heart rapidly after excision. Approximately 2 min time elapsed between excision of the

heart and dissection of LV myocardium for mitochondrial studies or fixation for EM. Usually, no more than 45 s elapsed between dissection of LV myocardium and immersion of tissue samples (approximately 0.5 g) into fixative (sodium cacodylate 0.1 M; paraformaldehyde, 2.2%; and glutaraldehyde, 2.5%; pH 7.4). These myocardial samples immediately were cut into pieces measuring about 1mm on a side. The tissue was fixed for 12 hrs at room temperature and then rinsed in a cold solution of 8% sucrose in 0.1 M cadodylate buffer, pH 7.4. The samples were post-fixed in cacodylate-buffered 1% osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Blocks were thin-sectioned, placed on grids, and stained with uranyl acetate, followed by lead citrate. Sections were examined with a Siemens 101 transmission electron microscope.

E. *Tissue wet weight: dry weight determinations*

Samples of ischemic and nonischemic myocardial tissue were blotted, weighed and dried in an oven to constant weight. Tissue water content, expressed as per cent water, was calculated from the ratio of tissue wet and dry weights.

F. *Mitochondrial calcium content*

Portions of the mitochondrial suspensions from several experiments were digested in concentrated nitric acid and then diluted with 2 N NaOH. Calcium concentration was determined by atomic absorption spectrophotometry using a standard curve derived from serial dilutions of a $CaCl_2$ standard solution.

G. Infarct size estimation

Approximately 1 cm thick transverse slices of the heart (myocardium not used for harvesting mitochondria, EM, or tissue water content measurements) were incubated for approximately 30 min in a solution of nitroblue tetrazolium (NBT) 0.5%, and phosphate buffer, 10 mM, pH 7.4 (19). After incubation in the NBT solution, the slices were blotted, dissected free of the right ventricle and stained and non-stained areas were separated and weighed. Infarct size was expressed as per cent of LV irreversibly damaged, based on this staining method.

It should be noted that while frank tissue necrosis, or infarction, requires about 24 hrs to develop, NBT has been shown to delineate areas of irreversibly injured myocardium after as little as 2 hrs following the initial insult (15, 19). Thus, although the pathological changes which characterize infarction are incomplete, this staining method enables one to identify myocardial tissue which ultimately will become necrotic.

H. *Statistical analysis*

The differences between mean values from ischemic and nonischemic regions of the same heart were tested for significance using the Student's paired t-test. Differences between groups (2 hrs vs. 4 hrs, 6 hrs, etc.) were tested for significance using a non-paired Student's t-test. Differences at the 95% confidence level were considered statistically significant.

Results

A. *Hemodynamic changes*

No significant differences in heart rate between experimental and sham groups were observed during reperfusion, although a trend towards increased heart rate appeared in the experimental group. Mean heart rate in the experimental dogs increased progressively from a pre-occlusion

value of 165 ± 4 beats per minute (BPM) to 185 ± 13 bpm at 6 hrs. At 24 hrs all experimental animals displayed arrhythmias characterized by ventricular tachycardia with a mean heart rate of 187 ± 15 bpm, 90% of which were ventricular in origin. The only significant difference in mean arterial blood pressure (MAP) between experimental and sham groups occurred during LCX occlusion and, again at 24 hrs after reperfusion. MAP fell from 102 ± 3 mmHg (pre-occlusion) to 93 ± 3 mmHg during occlusion and remained near this level until between 6 and 24 hrs, during which time MAP dropped to 65 ± 4 mmHg.

B. *Mitochondrial respiration*

The State 3 (ADP-stimulated) QO_2's of mitochondria isolated from ischemic myocardium were significantly less than those of mitochondria obtained from nonischemic myocardium at all four periods after release or occlusion (table 1). In addition, a time-dependent decrease in this parame-

Table 1. Comparison of mitochondrial respiratory parameters[a] and yields between ischemic (I) and nonischemic (NI) myocardium.

Group[b]	n[c]	State 3[d] QO_2	State 4[d] QO_2	RCR	ADP: O[e] Ratio	Mitochondrial[f] Yield
2 hr NI	4	256±12	17±4	21±2	2.6±1.6	2.9±0.2
I		154±14*	13±2	13±2*	2.4±0.2	2.2±0.2
4 hr NI	7	164±16††	11±1	16±1†	2.5±0.1	2.5±0.2
I		88±17*,†	8±1	11±2*,†	2.4±0.1	2.1±0.1
6 hr NI	6	165±12	13±1	14±1	2.8±0.3	2.6±0.2
I		76±12	9±1*	9±1*	2.8±0.3	2.5±0.3
6 hr NI	2	238±6	12±1	21±3	2.9±0.1	3.1±0.1
Sham I		220±11	12±1	19±1	3.0±0.1	3.1±0.4
24 hr NI	4	222±29†	13±1	20±1†	3.3±0.1	2.6±0.3
I		30±9*,†	6±1*	7±3*	3.1±0.3	2.1±0.1
24 hr NI	2	268±16	16±1	17±3	3.2±0.1	2.4±0.2
Sham I		268±25	17±1	17±3	3.1±0.2	2.0±0.2

Values reported are mean ± S.E.M.

[a] Mitochondrial suspensions (1-2 mg protein) were added to a cuvette (total volume = 1.5 ml) containing sucrose, 0.32 M; morpholinopropane sulfonic acid (MOPS) buffer, 15 mM, pH 7.4; and K_2HPO_4, 5 mM at 30 °C. Glutamate und malate (5 mM, final concentration each) were added as substrate. ADP (final concentration = 0.33 mM) was added to the cuvette after a 2 min incubation period, and subsequently after at least 1 min of return to State 4 respiration.

[b] Group = time after reperfusion at which hearts were removed and mitochondria isolated.

[c] n = number of experiments (dogs) per group.

[d] units = natoms oxygen consumed/min/mg protein.

[e] units = nmoles ADP consumed per natom oxygen consumed.

[f] Expressed as mg mitochondrial protein/g wet myocardial tissue.

* = Significant difference between I and NI samples ($p < 0.01$).

† = Significant difference between value for the indicated time (group) and immediately preceding time for respective region in the experimental groups ($p < 0.05$ or †† < 0.01).

ter was obtained from the mitochondria isolated from the ischemic region. State 3 QO_2 decreased from 154 ± 14 natoms oxygen min^{-1} mg $protein^{-1}$ at 2 hrs after release, to 30 ± 9, 24 hrs later, with intermediate values at 4 and 6 hrs.

Similar time-dependent changes in RCR occurred. At all times after reperfusion, mitochondria isolated from ischemic myocardium showed significantly lower RCR values than those of the corresponding nonischemic controls (table 1). Statistically significant decreases in this parameter occurred between 2 and 4 hrs and between 6 and 24 hrs, similar to the time-dependent changes seen in State 3 QO_2.

An interesting decline in both State 3 QO_2 and RCR of mitochondria isolated from nonischemic myocardium was observed at 4 and 6 hrs after reperfusion, with values for both parameters returning to normal at 24 hrs (table 1). The decreases were not as great as those of mitochondria from ischemic myocardium, but they were statistically significant, indicating that metabolic alterations occurred even in ostensibly normal myocardium in the first several hours of reperfusion after acute myocardial ischemia.

No time-dependent changes in State 4 QO_2 were obtained in the ischemic group (table 1). However, the values for this variable were significantly lower for mitochondria isolated from ischemic myocardium at 6 and 24 hrs after reperfusion. The duration of reperfusion after ischemia did not affect the ADP: O ratios of mitochondria isolated from either ischemic or nonischemic myocardium in a significant way.

C. *Electron microscopy*

Electron microscopic examination of ischemic myocardium revealed progressive ultrastructural damage as a result of LCX occlusion and reperfusion. The nonischemic portion of the left ventricle (plates 1a, 2a, 3a) showed little or no morphologic change, as was true for sham-operated dogs (plate 4). Ischemic myocardium sampled 2 hrs after reperfusion (plate 1b) showed moderate myofibrillar and mitochondrial morphologic changes. Contraction bands were evident at this time, and mitochondria were moderately swollen. Mitochondrial matrices were slightly clarified, and changes in cristal organization were apparent at 2 hrs. However, these changes were not uniformly apparent. At 4 hrs of reperfusion there was greater evidence of ultrastructural damage (plate 2b). Mitochondria at this time uniformly displayed increased separation of cristae and swelling of the matrix space. Severe contracture and dilatation of the sarcotubular elements were apparent at this time. Contraction bands were more evident at this time. At 6 hrs after reperfusion there was extensive damage within the ischemic zone (plate 3b). Severe myofibrillar disruption and cytoplasmic swelling were evident, in addition to misalignment of sarcomeres. Mitochondrial integrity at this time was severely altered, as evidenced by massive swelling, separation of cristae, and clarification of the matrix space. Most mitochondria within the ischemic zone contained at least one large amorphous density, believed to be precipitates of calcium phosphate (14, 25). No morphological evidence of damage to nonischemic myocardium was apparent at any time throughout the reperfusion period. The

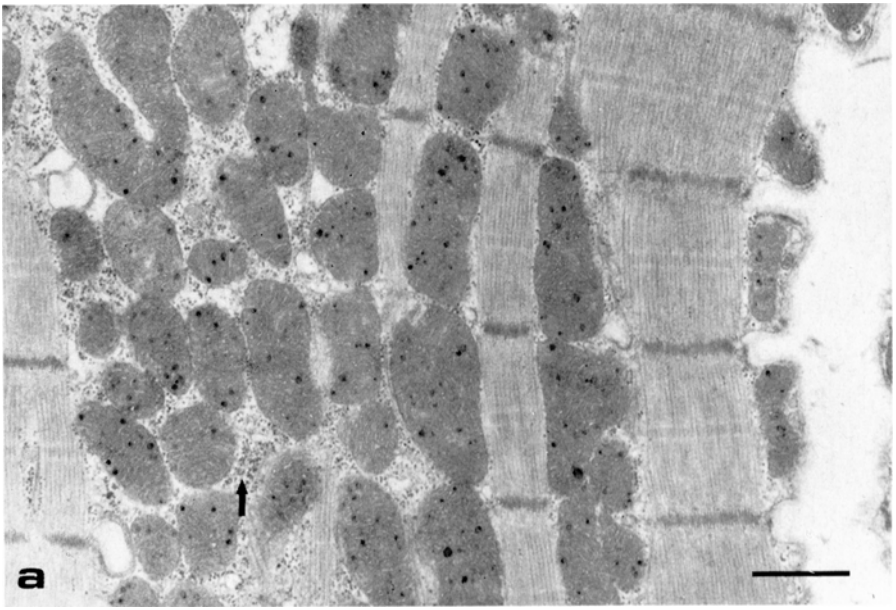

Plate 1a. Electron micrograph of nonischemic left ventricular (LV) myocardium (anterior papillary muscle) fixed at 2 hrs after release of LCX occlusion. Mitochondria appear normal and contain tightly packed cristae, dense matrices, and small dense granules. Glycogen granules (arrow) are abundant, especially in proximity to mitochondria. Overall myofibrillar morphology appears normal. Scale line represents 1 μm in all EM's.

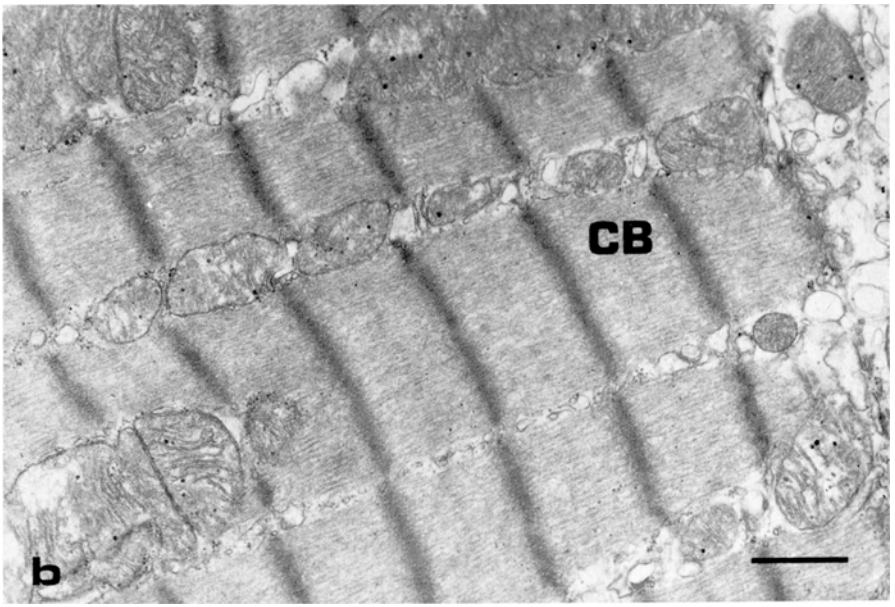

Plate 1b. Electron micrograph of ischemic LV myocardium (posterior papillary muscle) fixed at 2 hrs after reperfusion. Moderate contracture is evidenced by contraction bands (CB). Some mitochondria at this time appear swollen, with cristae separated and matrices slightly clarified. This sample was taken from the same heart shown in plate 1a.

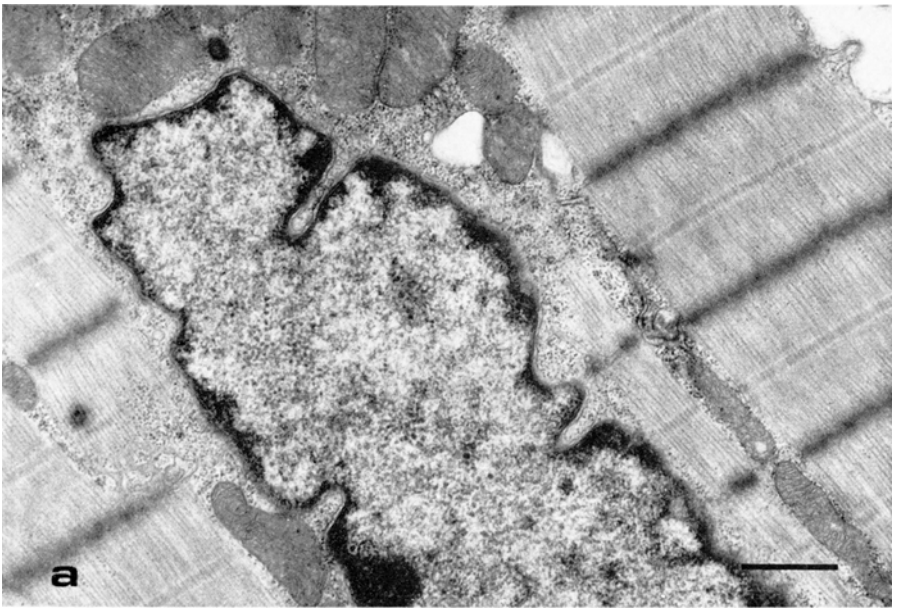

Plate 2a. Electron micrograph of nonischemic LV myocardium fixed 4 hrs after reperfusion. Overall myofibrillar appearance is normal. Glycogen is abundant, sarcomeres well-aligned, and mitochondria are abundant. Mitochondria are morphologically normal, with dense matrices and closely packed cristae. Most mitochondria contain normal small, dense granules. Nuclear chromatin material is diffuse and evenly distributed.

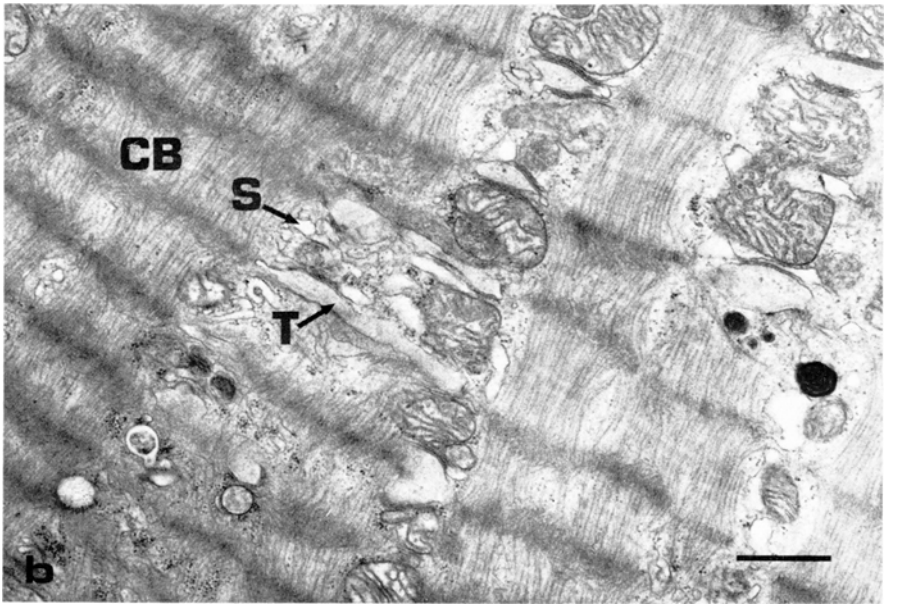

Plate 2b. Electron micrograph of ischemic LV myocardium fixed 4 hrs after release. There is greater evidence of mitochondrial and myofibrillar disruption at this time. Contraction bands (CB) are more evident than at 2 hrs. Mitochondria at this time appear more uniformly disrupted than at 2 hrs; cristae are separated and matrices appear swollen and clarified. Both sarcoplasmic reticulum (S) and t-tubules (T) appear dilated in places. This sample was taken from the same heart as shown in 2a.

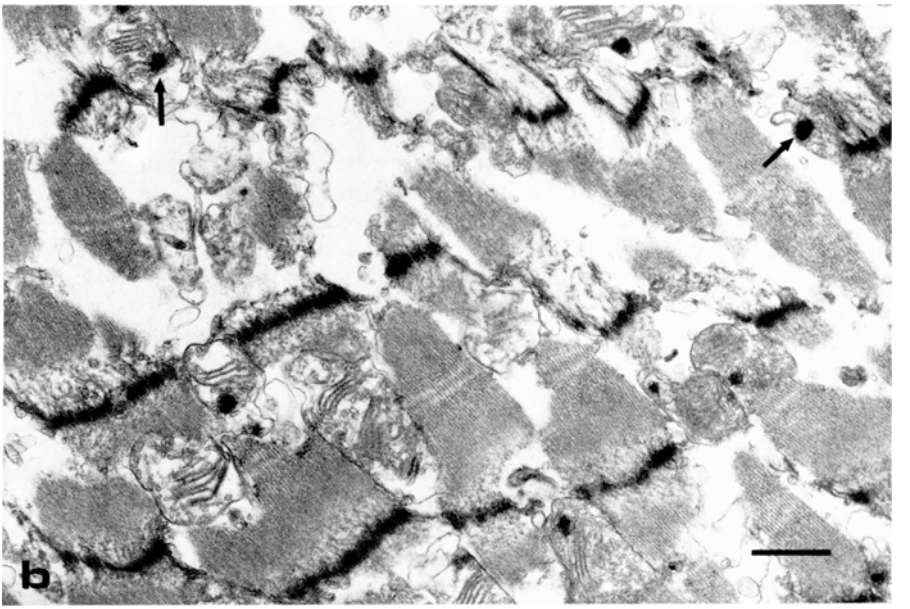

Plate 3a. Electron micrograph of nonischemic LV myocardium fixed 6 hrs after release. Sarcomeres are well-aligned and Z lines are in register. T-tubules appear normal and mitochondria are minimally altered. Glycogen granules are abundant and no myofibrillar alterations are apparent.

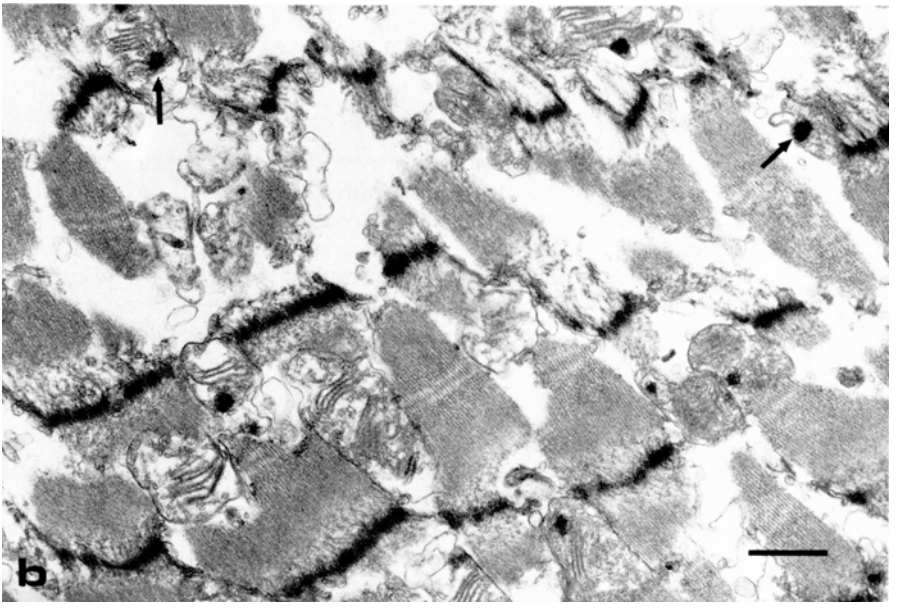

Plate 3b. Electron micrograph of ischemic LV myocardium fixed 6 hrs after release. Extensive ultrastructural damage is evident. The Z lines are misaligned and mitochondria are swollen and show loss of organized cristae and clarified matrices. Most mitochondria contain at least one large dense granule (arrow). There is more extensive myofibrillar swelling than at four hours. This sample was taken from the same heart shown in 3a.

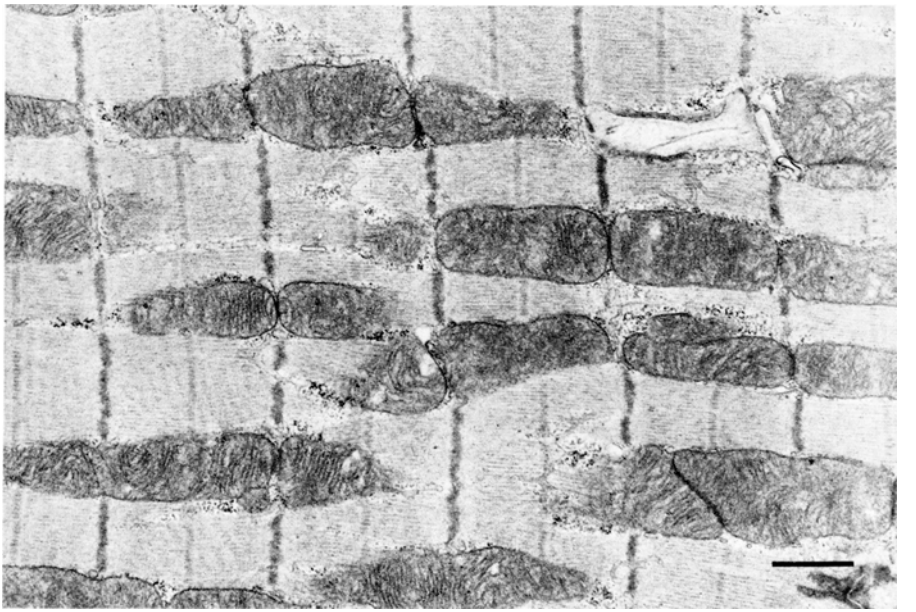

Plate 4. Electron micrograph of myocardium from a sham-operated dog at 6 hours. Tissue was sampled from the posterior papillary muscle, that region rendered ischemic upon LCX occlusion.

myocardium of sham-operated dogs revealed no signs of ultrastructural damage (plate 4).

D. *Tissue water content*

No time-dependent changes in tissue wet: dry weight ratios for either ischemic or nonischemic tissue were obtained (Table 2). At all times, however, this variable was significantly greater in the ischemic tissue, indicating that electrolyte disturbances occurred in this region as a result of ischemia and reperfusion. The water contents of the nonischemic and ischemic myocardium (all time points pooled), expressed as per cent tissue water, were 78.3 ± 0.1 and 80.5 ± 0.2 ($p < 0.001$), respectively.

E. *Mitochondrial calcium content*

The mean calcium concentration of mitochondria isolated from ischemic myocardium (19.3 ± 2.3 nmoles Ca^{2+}/mg protein) was nearly twice that of mitochondria obtained from nonischemic myocardium [10.7 ± 1.9 ($p < 0.05$)] (figure 1). Due to a small number of mitochondrial samples, the values reported represent the composite of all time groups for both ischemic and nonischemic regions, therefore, no time-dependent information was obtained for this variable.

Table 2. Changes in infarct size, tissue wet:dry weight ratios and tissue water content.

Group[a]	n[b]	Infarct Size (% LV)	Wet: Dry Weight Ratio	Tissue Water (%)
2 hr NI	4	15±2	4.69±0.11	78.6±0.5
I			5.02±0.20*	80.1±0.8*
4 hr NI	7	25±3†	4.76±0.28	78.4±1.5
I			5.25±0.26**	80.0±1.1**
6 hr NI	6	25±2	4.56±0.16	77.9±0.7
I			5.15±0.08*	80.6±0.3*
6 hr NI	2	0	4.62±0.17	78.2±0.0
Sham I			4.73±0.15	78.9±0.0
24 hr NI	5	38±5†	4.59±0.05	78.2±0.3
I			5.23±0.15**	80.8±0.5**
24 hr NI	2	0	–	–
Sham I		–	–	

Values reported are mean ± S.E.M.
[a] Group = time after reperfusion [hours (hr)] at which myocardial tissue was taken.
[b] n = number of animals per group.
[c] Infarct size was measured using the nitroblue tetrazolium staining method and is expressed as percentage of left ventricle (LV) which stained positive as irreversibly damaged tissue.
† Significant difference between mean value for the indicated time (group) and immediately preceding time point ($p < 0.05$).
* Significant difference between I and NI myocardium ($p < 0.05$; or $^{**}p < 0.01$).

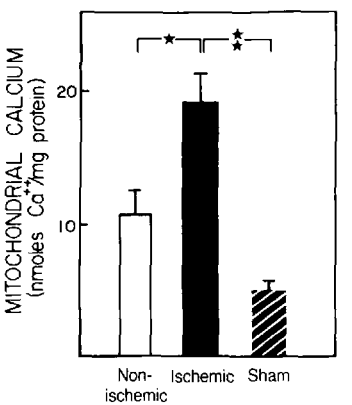

Fig. 1. Mitochondrial calcium content. Mitochondrial calcium concentrations were measured by atomic absorption spectrophotometry in 0.5 $LaCl_3$ diluent. Open bar represents mean of all mitochondrial suspensions isolated from nonischemic myocardium; shaded bar represents ischemic and striped bar represents sham-operated dog groups. ★ = $p < 0.05$; ★★ = $p < 0.01$ based on non-paired t-test.

F. *Infarct size estimation*

Significant increases in infarct size occurred between 2 and 4 hrs and between 6 and 24 hrs after reperfusion. The area of infarcted myocardium consistently expanded in a transmural (endocardial to epicardial) direction, with little or no noticeable extension in the lateral direction. The data are presented in table 2. The temporal changes in infarct size paralleled the progressive declines of mitochondrial structural integrity and respiratory function *in vitro.*

Discussion

The results obtained from this study indicate that time-dependent alterations of mitochondrial structure and function occur as a result of 1 hr of LCX occlusion and controlled reperfusion. Mitochondria isolated from ischemic myocardium showed progressive deterioration of respiratory activity *in vitro,* including declines of State 3 QO_2, State 4 QO_2, and respiratory control ratios. In addition, electron microscopy revealed parallel deterioration of mitochondrial (and overall myofibrillar) ultrastructure during this period of reflow. Thus on the basis of these results it appears that at least the LV myocardium contained in the sampling section underwent additional injury after reperfusion, since changes in mitochondrial ultrastructure and function were greater at 4, 6 and 24 hrs than observed at 2 hrs. Although the possibility of persistent ischemia, despite restoration of LCX coronary blood flow (13) cannot be ruled out, reperfusion injury is largely responsible for the progressive myocardial injury observed in the present study. These results are consistent with previous investigations demonstrating exacerbation of ischemic injury upon reperfusion at single time points after various periods of ischemia (18, 24, 25).

It is noteworthy that the progressive decreases in mitochondrial respiratory function *in vitro* occurred concomitantly with increases in infarct size (tables 1 and 2). This observation relates well to observed functional and ultrastructural changes which occurred in the portion of ischemic LV myocardium sampled in these experiments. It is recalled that a constant, transmural section of LV comprising the posterior papillary muscle was used for "ischemic myocardium" and that samples of ischemic myocardium for electron microscopy were taken from the middle of the posterior papillary muscle. On the basis of the experimental observations and the sampling methods described, it appears that upon reperfusion there were myocardial cells already irreversibly injured due to ischemia and cells in a reversible state of injury (9, 11). The progressive deterioration of mitochondrial structure and function and infarct size probably involved both of these populations of cells. The myocardial cells already irreversibly injured by ischemia probably underwent extensive disruption early in the course of reperfusion (3, 7, 10), while increasing numbers of cells on the borderline between reversible and irreversible injury underwent more extensive injury as reperfusion ensued. This interpretation is consistent with the histological, *in vitro* mitochondrial studies, and infarct size data.

With regard to the sampling process used in these studies, originally, it was desired to sample only tissue known to undergo ischemia as a result of

LCX occlusion for *in vitro* mitochondrial studies. However, preliminary experiments using vital stains excluded this method due to the effects of the stains on mitochondrial respiratory function and to the time-consuming nature of such techniques. Alternatively, a sampling technique in which a constant transmural section, containing the posterior papillary muscle, was decided upon (see Materials and methods). It was realized that sampling only subendocardial tissue would more consistently yield injured myocardial cells. However, without the aid of a staining technique to delineate injured tissue the arbitrary dissection of subendocardial tissue would be more time-consuming and subject to bias and experiment-to-experiment variation. Therefore the transmural sections obtained in the present study probably contained a mixture of dead and viable cells, although all but the most outer (epicardial) layer of cells experienced ischemia during LCX occlusion. At 24 hrs after reperfusion infarcts were, in most cases, transmural within the sampling zone. The time-dependent decline of mitochondrial respiration *in vitro*, coupled with increasing infarct size, and histological observations indicate that both the severity of injury and the percentage of total cells undergoing injury increased during the course of reperfusion.

A major concern relevant to these experiments is the well-known increase in fragility of ischemically injured mitochondria (2, 3, 7, 9, 11, 24). An obvious question regarding the present data is whether only structurally intact mitochondria are isolated and that badly injured mitochondria are absent from the mitochondrial pellet. *Murfitt* et al. (17) demonstrated that subpopulations of mitochondria can be isolated from myocardium by using gentle isolation and density gradient techniques and that ischemia causes shifts in these populations. However, they still reported overall (both populations) decreases in mitochondrial yield (mg protein/g wet tissue) similar in magnitude to those found in this study, which employed standard isolation techniques (27). It is likely that a small portion of severely damaged mitochondria may have been lost during isolation, as reflected by the small decrease in yields compared to nonischemic values, an observation reported previously (10, 17). However, it is believed that most mitochondria within the ischemic myocardium were isolated, based upon several experimental observations. Large time-dependent, or ischemic *vs.* nonischemic, differences in mitochondrial yield were not observed. Also, a very wide range of values of mitochondrial State 3 QO_2 and RCR were obtained for mitochondria isolated from ischemic myocardium (see table 1). Moreover, the badly injured mitochondria (presumed to be irreversibly injured) shown in Plate 3b reveal accumulation of calcium, which was confirmed by actual spectrophotometric measurements of calcium from the same mitochondrial suspensions which were used for respiratory measurements *in vitro*.

In contrast to mitochondrial derangements in the ischemic zone, the observed depressions of State 3 QO_2 and RCR by mitochondria isolated from nonischemic (anterior wall) myocardium were temporary, occurring at 4 and 6 hrs after reperfusion and returning to "normal" at 24 hrs. Furthermore, these functional changes were not accompanied by significant ultrastructural changes. These findings are especially interesting in view of recent observations by *Wood* et al. (29), who reported definite

changes in several biochemical and hemodynamic parameters in both ischemic and nonischemic myocardium after short periods of ischemia (5 and 10 minutes) and reperfusion. Results obtained from the present experiments, as well as those of *Wood* et al. (29), suggest that hemodynamic and biochemical alterations in nonischemic myocardium occur during and after regional ischemia. Using a modification (15) of *Jenning's* LCX occlusion model (8), these are the first experiments to demonstrate that mitochondria within nonischemic myocardium experience apparently reversible changes in respiratory function during the course of reperfusion after 1 hr of total ischemia. The results from the present study provide additional evidence that metabolic and contractile changes occur in nonischemic myocardium as well, after acute regional myocardial infarction (1, 5, 17, 29).

Several possible explanations exist for the interesting temporary decline in respiratory function of mitochondria isolated from nonischemic myocardium. First, it is possible that samples of nonischemic myocardium were contaminated by infarcted tissue. This is unlikely because a constant myocardial sample, far removed from the area at risk of infarction from LCX occlusion (4, 6–10, 14, 24, 25), was used in all experiments. Also, mitochondria isolated from "nonischemic" myocardium 24 hrs after reperfusion would be expected to display even more decreased respiratory function *in vitro*, relative to 6 hrs, if contamination by infarcted tissue was the reason, since the infarct increased in size during this time. Furthermore, the high State 3 respiratory rates and RCR's of mitochondria isolated from myocardium of sham-operated dogs argue against the surgery or anesthesia as contributing factors in the time-dependent decreases in mitochondrial function in either regions of the left ventricle. Secondly, regional myocardial ischemia has been shown to cause a paradoxical systolic bulging in the ischemic portion of ventricle (5, 28), rendering that portion acontractile, impairing ejection and cardiac output. However, by 24 hrs the injured myocardium becomes infarcted and less compliant, and, although akinetic, this infarcted area tends to impair ejection less (5). This temporary phenomenon may explain, at least in part, the decline in mitochondrial function within the nonischemic zone if one supposes that extensive contractile and biochemical demands are imposed upon the uninjured portion of LV. Thirdly, *Gudbjarnason* et al. (1) examined the changes in levels of high energy phosphate compounds in both ischemic and nonischemic myocardium after experimental myocardial ischemia. They found that contractility of ischemic myocardium ceased early after acute coronary occlusion, when ATP levels were comparatively high (80% of normal). In contrast, contractility was maintained or increased in the nonischemic zone where ATP levels were observed to be lower than in the ischemic zone, suggesting that metabolic adjustments occur in nonischemic myocardium in an attempt to compensate for the acontractile portion of left ventricle. It seems likely that whatever metabolic changes occur may be manifested as alterations in mitochondrial respiration, considering the crucial role of the organelle in providing metabolic energy. Also, it is possible that changes in sympathetic nervous activity may play a role in the temporary decline in mitochondrial function in the nonischemic zone (21, 22). Excessive release of

norepinephrine and possibly defects in its neuronal re-uptake (23) would be expected to cause increased mechanical and metabolic demands upon the non-infarcted portion of the left ventricle. Also, catecholamines have been shown to uncouple oxidative phosphorylation (26).

Results from wet and dry weight determinations (table 2) indicate that ischemia and reperfusion result in significant cell swelling and disturbances in electrolyte gradients. *Jennings* et al. (2, 5, 6, 10, 24, 25) have demonstrated that as little as 20 min of reperfusion after 40 min of LCX occlusion produce marked, immediate electrolyte alterations. In those experiments, parallel increases in cell water and $[Na^+]$ and $[Cl^-]$ occurred in the posterior papillary muscle. Our study indicates that disturbances in electrolyte gradients occurred, based upon tissue wet and dry weight determinations.

In summary, these results indicate that progressive, time-dependent alterations in mitochondrial function and structure occur as a result of reperfusion of ischemic myocardium. The decreased respiratory function correlated well with electron microscopic evidence of progressive morphological damage, notably to mitochondria. Changes in tissue water content, which probably reflect alterations of electrolyte gradients, also were observed in ischemic tissue at all times after reperfusion. Another noteworthy finding from these experiments was a temporary decline in respiratory function by mitochondria isolated from ostensibly nonischemic myocardium at 4 and 6 hrs after reperfusion. The exact cause for this is not known with certainty. It may involve an attempt by the uninjured myocardium to compensate for the failing portion of left ventricle.

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Zusammenfassung

Untersucht wurden zeitabhängige Veränderungen in Struktur und Funktion der Mitochondrien, die durch einstündigen Verschluß und 2- bis 24stündige Reperfusion des Ramus circumflexus der linken Koronararterie erzeugt wurden. Diese Veränderungen wurden mit Veränderungen der myokardialen Ultrastruktur, dem Wassergehalt des Gewebes, der Infarktgröße und dem mitochondrialen Calciumgehalt korreliert. Das Herz wurde nach verschiedenen Reperfusionszeiten entnommen und die Mitochondrien aus ischämischen und nichtischämischen Gebieten des linken Ventrikels isoliert. Ebenso wurden Gewebeproben von ischämischem und nichtischämischem Myokard für Elektronenmikroskopie und Bestimmung des Wassergehaltes des Gewebes entnommen. Die Infarktgröße wurde durch die Anfärbung mit Nitroblau-Tetrazolium bestimmt. Der Sauerstoffverbrauch der Mitochondrien aus ischämischem und nichtischämischem Myokard wurde *in vitro* gemessen. Mitochondrien aus ischämischem Myokard zeigten eine zeitabhängige Abnahme des Sauerstoffverbrauchs und seiner Bindung an die Phosphorylierung von ADP. Die Elektronenmikroskopie zeigte eine fortschreitende Zerstörung der Ultrastruktur im ischämischen Myokard, einschließlich einer Zunahme der Calciumablagerungen in Mitochondrien, was mit erhöhten Calciumkonzentrationen in Mitochondrien aus dem gleichen Gebiet übereinstimmte. Im ischämischen Myokard war die Relation Feuchtgewicht/Trockengewicht signifikant erhöht. Eine

geringe, aber signifikante Abnahme der Atmung wurde in Mitochondrien, die nach einigen Stunden Reperfusion aus nichtischämischem Myokard isoliert worden waren, beobachtet; aber nach 24 h Reperfusion fand sich normale Atmung. Letzteres weist darauf hin, daß auch das nichtischämische Gebiet von der regionalen Ischämie betroffen ist. Die Ergebnisse zeigen, daß vorübergehender Verschluß des Ramus circumflexus der linken Koronararterie und Reperfusion zu fortschreitender Zerstörung mitochondrialer Funktion und Struktur führen und daß diese Veränderungen von Änderungen des Electrolytstatus der Zelle begleitet werden.

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