Regional Changes in Mitochondrial Respiration in Acute Myocardial Ischemia

Comparison of the Inner and Outer Heart Muscles

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SUMMARY

Impairment of mitochondrial respiration in acute myocardial ischemia was studied in the inner and outer layers of canine heart muscle by the determination of oxidative phosphorylation and several respiratory enzymatic activities of isolated mitochondria. As early as 15 min after coronary ligation, the respiratory control ratio decreased as the result of a reduction in the oxygen consumption rate in state 3 to 72% of the control ratio in the inner layer. However, in the outer layer, it dropped to 74% after 1 to 2 hours. The oxygen consumption rate in state 4 and the ADP/O ratio were not significantly altered in both cardiac sublayers. In parallel with a decrease in oxygen consumption rate in state 3, Mg^{++} -dependent ATPase and DNP-stimulated ATPase activities of isolated mitochondria reduced significantly in both sublayers, followed by a sequential increase in Mg^{++} -dependent ATPase activity. Succinate dehydrogenase activity increased in ischemia for 3 hours in the inner layer, and for 6 hours in the outer layer, respectively; cytochrome oxidase activity reduced in both sublayers during the same period. Mitochondrial respiration is impaired in acute myocardial ischemia much earlier in the inner layer by a decrease in oxygen consumption rate in state 3, and there is a chronological delay in the development of ischemic mitochondrial changes in the outer myocardium.

Additional Indexing Words:

Acute myocardial ischemia Mitochondrial respiration Polarography Subendocardium Subepicardium Canine heart

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IT is well known that cardiac muscle depends on aerobic metabolism at a rate of more than 95%, which is accomplished solely by mitochondria. Soon after cessation of coronary blood flow, myocardial metabolism shifts from aerobic to anaerobic glycolysis. The cellular energy level falls abruptly, followed by a reduction in cellular pH as a result of decreased ATP, increased lactate, and related changes. The impairment of mitochondrial function in acute myocardial ischemia has long been one of the most interesting problems concerning the critical point of myocardial irreversible injury. For this reason many morphologic and biochemical studies have examined mitochondrial changes in the ischemic myocardium.¹⁾⁻⁶⁾ It is now widely accepted that the subendocardium is injured earlier than the subepicardium in the acute stage of transmural ischemia,⁷⁾ which is observed as a subendocardial infarction in human cases. From this point of view, we divided the ischemic myocardium to the inner and outer sublayers after induction of experimental acute myocardial ischemia in the dog, and examined the respiration level of mitochondria from these tissues by determining the rate of mitochondrial oxidative phosphorylation, respiratory enzymatic activities and ATPase activities.

MATERIALS AND METHODS

All the reagents used were reagent grade. Adenosine diphosphate (ADP), and adenosine triphosphate (ATP) were obtained from Sigma Chemical Co. (purities greater than 99%). 2, 4-dinitrophenol (DNP) was from Wako Pure Chemical Industries.

Surgical procedures: Forty-five adult mongrel dogs, weighing 12 to 25 Kg, were anesthetized with intravenous injection of sodium pentobarbital (Nembutal®, 30 mg/Kg body weight). A left-sided thoracotomy was performed under positive pressure respiration with room air using a Harvard respirator. The anterior descending branch of the left coronary artery was isolated and ligated completely with a silk thread just below the first diagonal branch. The occurrence of transmural myocardial ischemia was confirmed by a continuous ST segment elevation of more than 0.5 mV in the leads near V₃ and V₄. Four to 5 dogs were subjected to each period of ischemia (15, 30 and 60 min, and 2, 3, 4, 6 and 12 hours after coronary ligation) under Nembutal anesthesia. In 5 sham operated dogs, the coronary artery was isolated but not ligated. After the appropriate survival time, the beating heart was immediately excised and washed briefly with an ice-cold solution containing 0.21 M mannitol, 0.07 M sucrose and 0.0001 M ethylenediaminetetraacetate (EDTA) buffered with 0.01 M tris maleate (pH 7.4), and packed in ice. Approximately 4 Gm of heart muscle was removed from the postischemic cy-

notic portion of the anterior left ventricular wall, including the anterior portion of the interventricular septum. The non-ischemic inferior portion of the left ventricle was used as a control. The ischemic myocardium was divided into 2 parts in the middle of heart muscle and they were designated the sub-endocardium and the subepicardium.

Mitochondrial preparation: The preparation of mitochondria was performed by the modified method of Chance and Hagihara,⁸⁾ utilizing crystalline protease (Nagarse, Nagase Co. Ltd.). All the procedures were carried out at 4°C unless otherwise mentioned. Briefly, myocardial tissue was chopped with scissors, minced finely with a stainless steel razor blade, and homogenized gently in 5 volumes of the extraction medium containing 0.21 M mannitol, 0.07 M sucrose, 0.0001 M EDTA, 0.01 M tris-HCl (pH 7.4) and 5 mg/Gm of crystalline protease with a loose teflon-glass homogenizer in 5 strokes. The homogenate was kept in ice for 20 min and spun at $9,000\times G$ for 1 min. After an addition of the solution without protease, the suspension was further homogenized slowly at about 500 rpm with a regular teflon-glass homogenizer. The homogenate was spun at $500\times G$ for 5 min and the supernatant was centrifuged at $9,000\times G$ for 10 min twice. The precipitate was resuspended in a small volume of the medium. Protein concentration was determined by a biuret procedure using bovine serum albumin as a standard.

Measurement of oxidative phosphorylation: Mitochondrial oxidative phosphorylation was measured with an oxygen electrode (Oxygen Consumption Recorder PO-100A, Yanagimoto Mfg. Co. Ltd.), according to the method of Hagihara,⁹⁾ in a reaction medium containing 0.01 M KCl, 0.0001 M EDTA, 0.002 M $MgCl_2$, 0.25 M sucrose and 0.02 M potassium phosphate buffer (pH 7.2) at 25°C. Succinate was used as a substrate and ADP was added to estimate oxidative phosphorylation. Oxygen consumption rates in states 3 and 4, respiratory control ratio and ADP/O ratio were determined from a polarographic record by the method of Chance and Williams.¹⁰⁾

Measurements of ATPase activity: Mitochondrial ATPase activity was examined by the modified method of Pullman et al.¹¹⁾ in a medium containing 0.21 M sucrose, 0.02 M KCl, 0.001 M DNP or 0.03 M $MgCl_2$, 0.02 M tris-maleate (pH 7.2) and 3 mM ATP at 25°C. The amount of phosphate liberated in the reaction was assayed by the method of Fiske and SubbaRow.¹²⁾

Determination of succinate dehydrogenase (SDH) activity: SDH activity was determined by the modified method of Singer et al.¹³⁾ The reaction was carried out in a medium containing 60 mM phosphate buffer (pH 7.6), 1 mM KCN, 72 μM 2, 6-dichlorophenolindophenol (DCIP), 0.1 mM phenazine methosulphate, 0.75 mM $CaCl_2$ and 0.02 M succinate at 25°C. Enzymatic activity was expressed as μ moles DCIP reduced/mg protein/min.

Fig. 1. Oxygen consumption rate in state 3 of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. The reaction mixture contained 0.01 M KCl, 0.0001 M EDTA, 0.002 M $MgCl_2$, 0.25 M sucrose and 0.02 M potassium phosphate buffer (pH 7.2) at 25°C. Values are expressed as mean $\pm$ SD. * $p < 0.01$, ** $p < 0.001$.

Fig. 2. Oxygen consumption rate in state 4 of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. The reaction mixture is described in Fig. 1.

Fig. 3. Respiratory control ratio of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. Values are expressed as mean $\pm$ SD. * $p < 0.01$, *** $p < 0.001$.

Determination of cytochrome oxidase activity: Cytochrome oxidase activity was determined using bovine cytochrome C as a substrate, according to the modified method of Oriei et al.¹⁴⁾ Enzymatic activity was expressed as specific activity/mg protein/sec at 25°C.

Results are expressed as mean $\pm$ SD of number of experiments indicated. Statistical analyses were performed using Student's t-test.

RESULTS

Electron microscopic observations of the mitochondrial fraction revealed that contamination from other organelles was very low in mitochondrial fractions from the both non-ischemic and ischemic heart muscle (not illustrated). The respiratory functions of mitochondria from the non-ischemic heart muscles were not statistically different in each stage of ischemia, and also not different from those of the sham-operated dogs. Therefore the data in the non-ischemic myocardia are summarized as the control.

Fig. 1 shows the oxygen consumption rate in state 3. $213.8 \pm 25.3 \text{ m}\mu\text{Atoms O/mg protein/min}$ was exhibited in the non-ischemic myocardium. In the inner layer, 15 min after coronary ligation, the oxygen consumption rate decreased significantly to 63% of the control rate ($p < 0.001$). It con-

Fig. 4. DNP-stimulated ATPase activity of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. The reaction mixture contained 0.21 M sucrose, 0.02 M KCl, 0.001 M 2,4-dinitrophenol, 0.02 M tris-maleate (pH 7.2) and 0.003 M ATP at 25°C. Values are expressed as mean $\pm$ SD. * p < 0.01, ** p < 0.001.

Fig. 5. Mg^{++} -dependent ATPase activity of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. The reaction mixture contained 0.21 M sucrose, 0.02 M KCl, 0.03 M $MgCl_2$, 0.02 M tris-maleate (pH 7.2) and 0.003 M ATP at 25°C. Values are expressed as mean $\pm$ SD. * p < 0.01, ** p < 0.001.

Fig. 6. Succinate dehydrogenase activity of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. The reaction mixture contained 0.06 M phosphate buffer (pH 7.6), 0.001 M KCN, 72 μ M 2,6-dichlorophenol-indophenol, 0.1 mM phenazine methosulphate, 0.75 mM CaCl_2 and 0.02 M succinate at 25°C. Values are expressed as mean $\pm$ SD. * $p < 0.01$.

tinued to decrease during 12 hours of ischemia. In the outer layer, the oxygen consumption rate in state 3 decreased more slowly than in the inner layer, falling to 62% of the control rate at 1 hour after coronary ligation.

The oxygen consumption rate in state 4 is shown in Fig. 2. The control value was 39.2 ± 7.3 m μ Atoms O/mg protein/min. In the ischemic heart muscle, the rate in state 4 was not significantly altered in either sublayer.

The respiratory control ratio (RC), which is the ratio of the rates in state 3 and state 4, decreased significantly to 72% of the control ratio at 15 min after coronary ligation in the inner layer and to 74% at 1 to 3 hours in the outer layer, and the RC decreased monotonically during 12 hours of ischemia (Fig. 3). By contrast, the ADP/O ratio was not changed significantly either in the inner or in the outer layers.

The DNP-stimulated ATPase and Mg^{++} -dependent ATPase activities are shown in Figs. 4 and 5, respectively. The DNP-stimulated ATPase activity was 9.20 ± 2.59 μ moles Pi/mg protein/hour and the Mg^{++} -dependent ATPase activity was 13.47 ± 3.92 μ moles Pi/mg protein/hour in the control myocardium. The ADP-stimulated ATPase activity decreased significantly to 61% of the control activity in the inner layer and to 62% of the control in the outer

Fig. 7. Cytochrome oxidase activity of mitochondria from the ischemic canine heart. Closed circles and open squares indicate the rates in the inner and outer layer, respectively. The reaction mixture contained 0.15 M phosphate buffer (pH 6.0) and 0.015 M cytochrome C at 25°C. Values are expressed as mean $\pm$ SD. * $p < 0.01$.

Fig. 8. Percent activities of parameters of mitochondrial respiration in the ischemic canine heart. The inner layer data of the ischemic myocardium are shown in the right panel and the outer layer data on the left panel.

layer at 15 min after coronary ligation ($p < 0.001$). At 30 min, they were 47% and 55% of the non-ischemic heart muscle controls for the inner and outer layers, respectively. On the other hand, Mg^{++} -dependent ATPase activity was initially reduced to 47% of the control activity in the inner layer and to 57% in the outer layer at 15 min after coronary ligation. However, after 1 to 2 hours the activities rose steadily to reach 153% of the control myocardium value in the inner layer and 149% of the outer layer control at 12 hours.

SDH activity is shown in Fig. 6. The control myocardial activity was 23.41 ± 10.73 μ moles DCIP/mg protein. The activity of the inner layer remained in the control level until at 2 hours after coronary ligation, then increased rapidly after 3 to 4 hours. The increased activity reached 268% of the control value at 3 hours. The activity of the outer layer did not change from 15 min to 4 hours, and increased to 247% at 6 hours. SDH activities kept increasing through 12 hours after coronary ligation both in the inner and outer layers.

Cytochrome oxidase activity is shown in Fig. 7. The control value was 0.238 ± 0.041 /mg protein/sec. As in the case of SDH activity, cytochrome oxidase activity remained at the control level from 15 min to 2 hours after coronary ligation in the inner layer, and from 15 min to 3 hours in the outer layer. The activities of both layers then began to decrease, reaching 65% of control at 3 hours in the inner layer ($p < 0.01$), and 66% at 6 hours in the outer layer ($p < 0.01$), respectively. These results are summarized in Fig. 8 as percent change for each parameter.

DISCUSSION

Mitochondrial structure and function in acute ischemic myocardial injury have received considerable attention because mitochondria are the sole microorganelles conducting aerobic metabolism, which accounts for more than 95% of the energy needs of cardiac muscle.

The utilization of alkaline protease in the preparation procedure, introduced by Chance and Hagihara,⁸⁾ results in a preparation containing both subsarcolemmal and interfibrillar mitochondria without impairment of mitochondrial function.¹⁵⁾ In addition, this method reduces contamination by the digestion with alkaline protease. Electron microscopic observation of the mitochondrial fraction revealed satisfactory purity both in the non-ischemic and ischemic heart muscles.

Since the 1950s, morphologic changes in mitochondria in myocardial ischemia have been reported often in the literature. Jennings et al^{2),3),16)} observed swollen mitochondria with disordered cristae at 15 to 30 min after coro-

nary ligation; the appearance of intramitochondrial dense deposits at 40 min was defined as the first cellular sign of irreversible ischemia. On the biochemical aspect, reductions in oxygen consumption rate and respiratory control ratio were noted over 12 hours after myocardial ischemia.⁶⁾ A reduction in the oxygen consumption rate in state 3 was reported by Schwartz et al¹⁷⁾ in the ischemic myocardium 7 days after coronary ligation, and later at 60 min by Jennings et al.¹⁸⁾ By contrast, Yamagami et al¹⁹⁾ observed a deterioration of the oxygen consumption rate in state 4. In this study, the disturbance of mitochondrial respiration in myocardial ischemia was attributed to a reduction in the oxygen consumption rate in state 3. The inner myocardium was affected much earlier by ischemia than the outer layer (as early as 15 min after coronary ligation). By contrast, Hayashi et al²⁰⁾ found no differences in the respiratory activities between the inner and outer layers at 30 min after occlusion of one of the small branches of the anterior descending artery.

Regarding these metabolic differences between the subendocardium and the subepicardium, Griggs and co-workers have reported that regional blood flow is smaller in the subendocardium,²¹⁾⁻²³⁾ the capillary distribution is lowered to a greater extent^{24),25)} and tissue level of ATP decreased more in the ischemic state in the subendocardium than in the subepicardium.²⁶⁾ The delay in the development of ischemic changes in the outer layer in this study may be a function of these anatomical and physiologic differences in heart muscle.

In our previous reports, a simultaneous reduction in Ca^{++} -stimulated ATPase activity and the Ca^{++} -uptake rate of the sarcoplasmic reticulum were observed in ischemia for 90 to 180 min,²⁷⁾ and a decrease in sarcolemmal $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$ was observed within 60 to 90 min after coronary ligation.²⁸⁾ Mitochondrial ischemic changes developed much earlier than these membrane effects. It should be taken into consideration that impairment of mitochondrial function induces a decrease in the production of ATP, and this lowered ATP level would facilitate the degradation of the membrane system since Jennings et al recognized the close relationship between lowered ATP and the appearance of fine structural changes in acute myocardial ischemia.²⁹⁾

Hagihara has described 3 types of disturbances of oxidative phosphorylation with polarographic patterns, a disturbance of oxidation, a disturbance of phosphorylation and an uncoupling of oxidative phosphorylation.³⁰⁾ The polarographic pattern of ischemic heart mitochondria in the present study, a reduction in respiration rate in state 3 with a normal range of state 4, is a disturbance of phosphorylation. This also supported by changing patterns of mitochondrial ATPase activities, since DNP-stimulated ATPase activity was reduced already in ischemia for 15 min, whereas Mg^{++} -dependent ATPase ac-

tivity increased after an initial reduction in the inner layer.³⁰⁾ It is possible that this increase in Mg^{++} -dependent ATPase activity exhausts the remaining ATP in mitochondria in the ischemic heart muscle and exacerbates the ischemic injury.

The SDH and cytochrome oxidase activities were retained until 3 hours after coronary ligation, even in the inner layer. In the recent study by Rouslin and Millard,³¹⁾ an impairment of mitochondrial phosphorylating respiration was observed as a lesion in the NADH-coenzyme Q reductase segment of the electron transport chain. Reduction in the activities of complex I was also recognized in congestive heart failure.³²⁾ Even though SDH and cytochrome oxidase activities were unchanged, impairment in the electron transport system would have occurred from the initial state of myocardial ischemia. The tight binding of these enzymes to the mitochondrial inner membrane may account for this phenomenon,³³⁾ or latent SDH activity might have been activated by a reduction in inhibitors such as malonate in myocardial ischemia.³⁴⁾

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