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THE EFFECTS OF CORONARY REPERFUSION ON SARCOLEMMAL Na^+,K^+-ATPase ACTIVITY IN DOGS

(Na^+,K^+-ATPase/regional myocardial blood flow/reperfusion)

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After the ligation of coronary artery for 1 hour (1-hr group), 2 hours (2-hr group) and 4 hours (4-hr group), we investigated myocardial viability through the alterations in sarcolemmal function with measurements of regional myocardial blood flow (RMBF) and sarcolemmal Na^+,K^+-ATPase (ATPase) activity, comparing these groups with a permanently occluded group (permanent group). RMBF was measured in the ischemic region using hydrogen gas generated by electrolysis. Sarcolemmal membrane debris was prepared from ischemic, marginal and non-ischemic regions of the myocardium 7 days after reperfusion. Seven days after reperfusion, RMBF in 1-hr group recovered to control value. Reduction of RMBF in 2-hr group was significantly less than that of RMBF in parmanent group. There was no significant difference in RMBF between 4-hr group and permanent group. In the ischemic region, reduction of ATPase activity in 1-hr 2-hr group was significantly less than that of enzyme

activity in permanent group. There was no difference in enzyme activity between 4-hr agoup and permanent group. In the marginal region, there was no difference in ATPase activity between the reperfusion group and the permanent group. These results show that in 4 hours or more after coronary artery occlusion, little salvage of the myocardium can be induced by acute reperfusion.

With the advent of surgical and nonsurgical revascularization of ischemic myocardium in man, coronary artery reperfusion has become an important therapeutic intervention. Many investigators have reported the effects of reperfusion on ischemic myocardium after various periods of total coronary occlusion, but the value of this intervention remains controversial. Na^+, K^+-ATPase is one of the most important enzymes to maintain the vital reactions by hydrolysis of the terminal high-energy phosphate of ATP and active transport of Na^+ and K^+ across the sarcolemma. Therefore it seems that the decrease of Na^+,K^+-ATPase activity after myocardial ischemia expresses the irreversibility of myocardial cells. In this study we investigated the effects of reperfusion after 1 hour, 2 hours, and 4 hours of coronary artery occlusion on Na^+,K^+-ATPase activity of cell membrance 7 days after reperfusion and regional myocardial blood flow at an early period and after 7 days of reperfusion.

MATERIALS AND METHODS

Forty two mongrel dogs (body weight, 7-15kg) of both sexes were anesthetized with sodium pentobarbital (25-30 mg/kg, iv) and ventilated artificially with room air delivered by a Harvard respirator via a cuffed endotracheal tube. A thoracotomy was performed in the third or fourth left intercostal space and the heart was suspended in a pericardial cradle. A catheter was inserted into the left ventricular cavity through an apical stab wound for monitoring pressure. A segment of the left anterior descending coronary artery (LAD) just distal to the first diagonal branch was gently dissected away from the epicardium and a silk suture was placed at it. The dogs were divided into five

groups according to coronary occlusion time: none (sham group, n=4), 1 hour (1-hr group, n=7), 2 hours (2-hr group, n=13), 4 hours (4-hr group, n=11) and permanent (permanent group, n=7).

Blood for lactate balance was drawn before coronary occlusion, 10 minutes after occlusion and 10, 20, 40 and 60 minutes after reperfusion from coronary great vein and left ventricular cavity. Lactate levels were determined by Lactate-UV-Test(1). Lactate balances were calculated according to the formula:

$$\text{Lactate balance} = \frac{\text{arterio-venous difference}}{\text{arterial value}} \times 100 \quad (\%)$$

Regional myocardial blood flow (RMBF) was measured by using hydrogen gas generated by electrolysis (RBF 1, Biomedical Science)(2). The polarographic current was recorded on a recorder TI 102 (Tokai Irika). A wire-type platinum iridium electrode, 0.08 mm in diameter, was introduced into the heart muscle and fixed at subendocardium in LAD areas (ischemic areas). The RMBF was calculated by the hydrogen clearance method. The hydrogen washout curve was plotted on semilogarithmic paper to obtain the half time (T 1/2) from the middle monoexponential part of the curve. If the curve displayed non-exponential characteristics in its middle region, the data were excluded. RMBF was calculated as follows:

$$\text{RMBF} = \frac{0.693 \times 60 \times 100}{\text{T } 1/2 \text{ (second)}} \text{ (ml/minute/100 g)}$$

The RMBF was determined before coronary ligation (control), immediately, 20, 40, 60 minutes after ligation, before reperfusion and immediately, 20, 40, 60 minutes after coronary reperfusion. The chest was closed in layers and air was eliminated by suction drainage of the chest cavity. On the seventh day, RMBF was measured again. Immediately after the end of the measurements, the dogs were killed with a KCl injection. The heart was removed and pieces of transmural myocardium were sampled from the ischemic (between LAD and second diagonal branch), marginal (between first and second diagonal branch) and non-ischemic areas (indicated by hatched areas in Fig. 1) for the preparation of sarcolemma. The weights of the samples were in the range of 0.4 to 1.5 g.

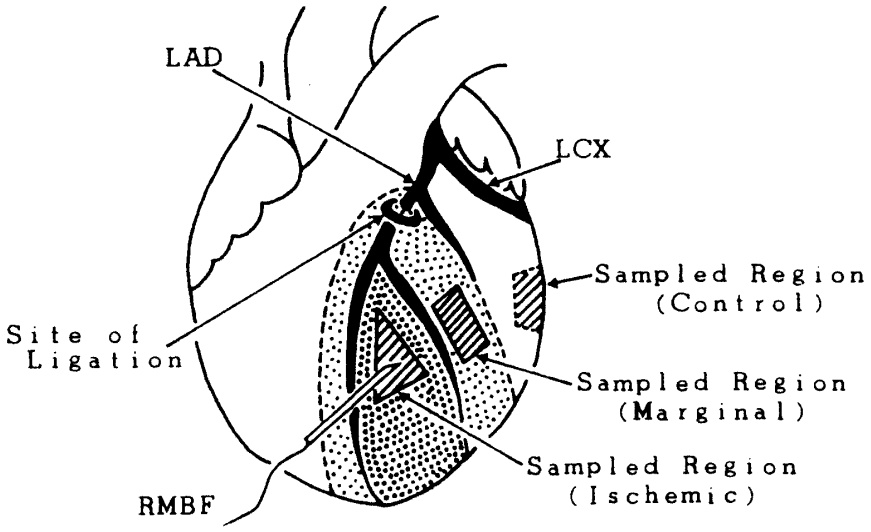

Fig.1 Schematic diagram of main coronary arteries in the canine heart. The site of ligation is indicated by an arrow immediately distal to the first diagonal branch. Platinum-iridium electrode was inserted into the endocardium and regional myocardial blood flow was measured. Sarcolemma was sampled from the hatched ischemic, marginal and non-ischemic regions. Abbreviations: LAD = left anterior descending coronary artery, LCX=left circumflex coronary artery.

Alstyne's method (3). The tissues of the heart were rapidly immersed in ice-cold 0.9% saline. The tissues were weighed and minced with scissors. All of the following procedures were carried out at 2-4°C. The mince was suspended in 4-5 volumes of a medium containing 10 mM $NaHCO_3$ and 5 mM NaN_3, pH 7.0 (medium A5), and homogenized for 15 seconds (Polytron PT-20). The homogenate was centrifuged at 8700 X g for 20 minutes to yield supernatant-1, which was discarded, and pellet-1. The latter was suspended in the same volume of medium A, homogenized in a large glass homogenizing vessel with one pass of a motor-driven Teflon pestle and centrifuged at 8700 X g for 20 minutes. This yielded supernatant-2, which was discarded, and pellet-2 which was dispersed in 6 volumes of 10 mM Tris-HCl, pH 7.4 (medium B) and subjected to four passes of a moter-driven Teflon pestle as above. Centrifugation at 8700 X g for 20 minutes yielded pellet-3, which was discarded, and supernatant-3. The latter was centrifuged at 35000 X g for 20 minutes to obtain supernatant-4, which was discarded, and pellet-4. This pellet was re-suspended in a small glass homogenizer and subjected to five passes of a hand driven Teflon pestle in medium B. The preparation was

stored at -20°C until use. For determination of total ATPase activity (4), incubation tubes containing 0.75 ml of substrate solution were placed in a water bath at 37°C and the reaction was started by adding 0.25 ml of enzyme preparation, providing final concentrations of 3 mM Mg^{++}, 100 mM Na^{+}, 20 mM K^{+}, and 100 mM imidazole-HCl buffer (pH7.2). Incubation was stopped in 60 minutes by adding 1.0 ml of ice-cold 10% trichloroacetic acid. After centrifugation at 1700 X g for 10 minutes 1.0 ml of supernatant was assayed for inorganic phosphate. Mg^{++}-ATPase activity was assayed in the presence of 1.0 mM ouabain and Na^{+}, K^{+}-ATPase activity was calculated as the difference between total ATPase and Mg^{++}-ATPase activities. Inorganic phosphate was determined according to the method described by Fiske and Subbarow (5). Protein determination was carried out using the method of Lowry et al. (6) and Na^{+}, K^{+}-ATPase activity was expressed as micromoles Pi per milligram of protein per hour. The yield of cardiac membranes was 1.0±0.4 mg/g wet weight in ischemic areas, 1.0±0.4 mg/g in marginal areas and 1.1±0.5 mg/g in non-ischemic areas.

Postmortem coronary arteriograms were made to the delineate the occluded bed or risk region (7,8). After the ligation of LAD at the same point, the coronary arteries (LAD, left circumflex artery and right coronary artery) were cannulated separately at their origins and injected simultaneously with a barium sulphate-gelatin mass. The hearts were fixed in formalin for several days and thereafter cut into 5 mm thick rings which were x-rayed. The region of perfusion of the occluded coronary artery was determined from the angiograms of the slices, aided by comparison with the anteroposterior and lateral arteriogram of the entire heart.

Data were analyzed with a repeated-measurements analysis of variance procedure. When overall significance was found with the analysis of variance, Dunkan's multiple range test was used to delineate which paired comparisons were significantly different. All data are expressed as the mean±SD. The level of significance was $p<0.05$.

RESULTS

Regional myocardial blood flow (RMBF)

Table 1 shows RMBF in the ischemic region. There were no significant changes in RMBF in sham group during experiments.

Table 1 Regional myocardial blood flow (ml/minute/100 g) in ischemic region. Values are expressed in mean ± SD.

		after occlusion							after reperfusion				
	control	0min	20min	40min	60min	80min	100min	120min	220min	0min	20min	40min	60min	7days
sham n=4	85±1		84±4	84±4	86†	84±4	84±4	85±5	85±5					88±5
(%)	(100)		(99±2)	(102±4)	(104)	(102±3)	(101±3)	(99±2)	(100±1)					(103±3)
1-hr n=7	95±3	14±11**	26±25**	25±27**						43±30**	56±24**	61±18**	67±12*	93±13
(%)	(100)	(14±11)	(27±26)	(27±29)						(46±34)	(59±27)	(64±20)	(72±17)	(97±6)
2-hr n=13	95±10	20±12**	17±18**	15±19**	22±22**	24±31**	23±31**			59±32**	57±37**	61±37**	67±33**	68±36**
(%)	(100)	(25±20)	(18±19)	(16±19)	(23±22)	(24±30)	(23±31)			(62±32)	(58±37)	(62±37)	(69±32)	(63±39)
4-hr n=9	77±30	21±22**	18±19**	22±25**	18±19**	18±19**	24±26**	18±24**	24±24**	32±35**	40±40**	35±36**	34±35**	30±24**
(%)	(100)	(27±23)	(26±27)	(30±30)	(22±18)	(21±18)	(24±25)	(24±23)	(25±22)	(33±36)	(42±39)	(36±36)	(36±37)	(33±30)
permanent n=7	95±19	32±22**	31±14**	21±23**	18±20**									13±10**
(%)	(100)	(34±24)	(14±35)	(23±26)	(20±30)									(14±10)

* $p<0.05$ compared with control.
** $p<0.01$ compared with control.
† this data is obtained from one experiment.
( ) in percentage of control value.

1-hr group: In the center of the ischemic region, just after occlusion, RMBF was 14±11% of control value and this reduction was significant statistically ($p<0.01$). At the end of 40 minutes after occlusion and before reflow, regional flow was 27±29% of control value. Just after reperfusion, flow was 46±34% of control value. RMBF remained depressed 60 minutes after reperfusion. After 7 days of reperfusion there was no significant difference in regional flow compared to control myocardial blood flow.

2-hr group: In the center of the ischemic region regional flow was significantly reduced after occlusion to 25±20% of control value ($p<0.01$). This flow remained depressed during ligation of LAD. Just after reperfusion, regional blood flow was 62±32% of control value ($p<0.05$). After 60 minutes this reduction persisted. On the seventh day, RMBF flow was 63±39% of control value ($p<0.01$).

4-hr group: RMBF just after occlusion was 27±23% of control value ($p<0.01$). At the end of the occlusion period, 220 minutes after occlusion, RMBF was 25±22% of control value ($p< 0.01$). This reduction in flow at the end of 4 hour occlusion was similar to the reduction in flow observed in 1-hr and 2-hr groups. Just after reperfusion, RMBF remained depressed. Seven days after reperfusion, regional flow was 33±30% of control value ($p<0.01$).

Permanent group: RMBF just after occlusion was reduced to 34±24% of control value ($p<0.01$). After 60 minutes RMBF was 20±23% of control value. Seven days after occlusion, RMBF was 14±10% of control value.

Seven days after reperfusion, reduction of RMBF in sham group, 1-hr group and 2-hr group was significantly less than that of RMBF in permanent group. However, there was no significant difference in reduction of RMBF between 4-hr group and permanent group.

Lactate balance

Table 2 shows lactate balance in 1-hr group, 2-hr group and 4-hr group. No significant lactate derangement was found in sham experiments.

1-hr group: Mean lactate balance in the control period was 26±110%, meaning extraction. It was -129±157% 10 minutes after occlusion, meaning lactate production. It was -40±112%, lactate production, 10 minutes after reperfusion.

Table 2 Lactate balance {(arterio-venous/arterial) × 100} (%) in 1-hr, 2-hr and 4-hr group.

		after occlusion	after reperfusion			
	control	10min	10min	20min	40min	60min
1-hr n=8	26 ±110	-129* ±157	-40* ±112	9 ±19	-3 ±40	11 ±13
2-hr n=11	52 ±63	-295** ±272	-52** ±92	-13* ±47	-13* ±44	-10* ±35
4-hr n=11	21 ±22	-179** ±137	-13* ±47	-11* ±29	-10* ±30	-1* ±14

Values are expressed in mean ± SD.
* $p<0.05$ compared with control.
** $p<0.01$ compared with control.

But 20 minutes after reperfusion, lactate balance was 9±19% and lactate metabolism was returning to lactate extraction.

2-hr group: Mean lactate balance in the control period was 52±63%, meaning extraction. It was -295±272% 10 minutes after occlusion, meaning lactate production. Ten and sixty minutes after reperfusion it was -52±92% and -10±35%, respectively. Lactate metabolism did not return to lactate extraction after 60 minutes of reperfusion.

4-hr group: Mean lactate balance in the control period was 21±22%, meaning extraction. It was -179±137% 10 minutes after occlusion, meaning lactate production. Ten and sixty minutes after reperfusion, lactate balance was -13±47% and -1±14%, respectively. Lactate production persisted at 60 minutes after reperfusion.

With regard to the area at risk there was no difference in the percentage of area at risk/LV weight among each groups (49±19% for the 1-hr group, 39±13% for the 2-hr group, 34±9% for the 4-hr group and 46±19% for the permanent group, respectively).

Na^+,K^+-ATPase activity

Fig. 2 shows the results of Na^+,K^+-ATPase activity from ischemic, marginal and non-ischemic areas.

1-hr group: In ischemic and marginal myocardium, Na^+, K^+-ATPase activity was at the same level of those in the

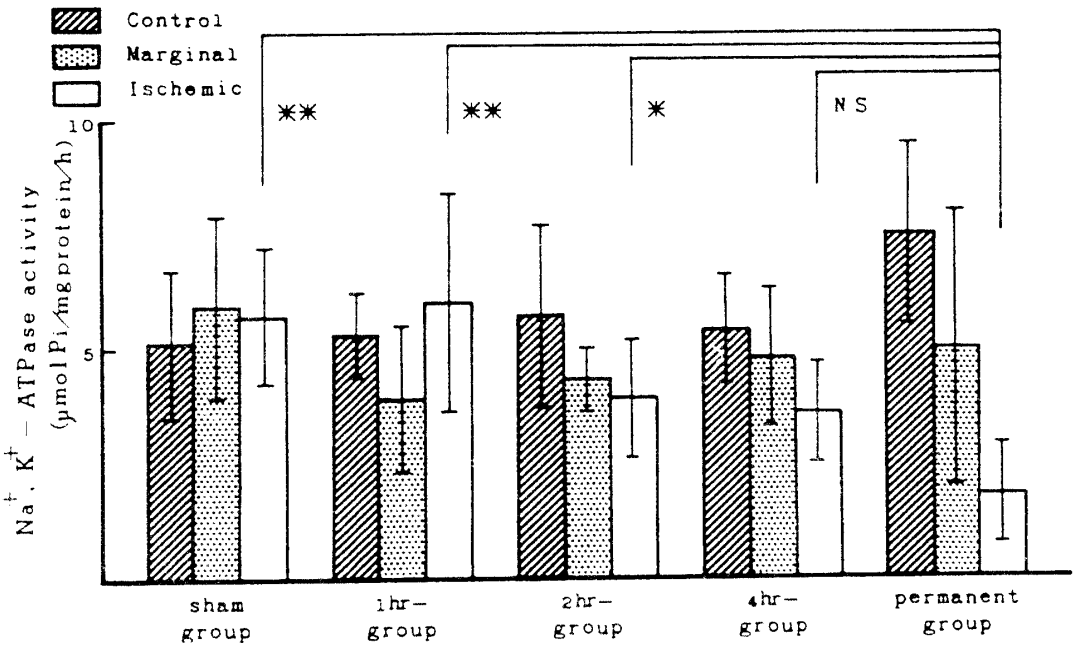

Fig.2 Na^+,K^+-ATPase activity in sarcolemma from non-ischemic, marginal and ischemic regions in each group. Bars represent mean values ± 1SD.
* significant difference ($p<0.05$)
** significant difference ($p<0.01$)

non-ischemic myocardium.

2-hr group: In ischemic myocardium, Na^+,K^+-ATPase activity reduced to 71±32% of non-ischemic activity, and this reduction was not significant. In marginal myocardium, enzyme activity was at the same level as that in the non-ischemic myocardium.

4-hr group: In ischemic myocardium, Na^+,K^+-ATPase activity was reduced to 70±20% of non-ischemic enzyme activity. In marginal myocardium, enzyme activity was at the same level of those in non-ischemic myocardium.

Permanent group: In ischemic myocardium, Na^+,K^+-ATPase activity was significantly reduced to 20±14% of non-ischemic myocardium. In marginal myocardium, Na^+,K^+-ATPase activity was significantly reduced to 59±35% of non-ischemic myocardium.

In marginal region, no difference in Na^+,K^+-ATPase activity was observed among the five groups. There was no difference in Na^+,K^+-ATPase activity between non-ischemic region and marginal region in reperfusion groups, but there was a significant difference in enzyme activity between non-ischemic region and

marginal region in permanent group($p<0.05$). In ischemic myocardium, Na^+,K^+-ATPase activity of permanent group was significantly less than those of reperfusion groups except for the 4-hr group. There was no significant difference in Na^+,K^+-ATPase between permanent group and 4-hr group. In non-ischemic myocardium of permanent group, Na^+,K^+-ATPase activity increased as compared to sham group, but this augmentation was not significant. There were no significant differences among ischemic, marginal and non-ischemic areas in sham group.

DISCUSSION

The maintenance of membrance function and integrity is essential for proper cellular function. The cardiac sarcolemma maintains the proper distribution of electrolytes between the intracellular and extracellular spaces by an active transport mechanism. Sarcolemmal Na^+,K^+-ATPase is known to be one of the most important enzymes for maintaining the vital reactions by hydrolysis of the terminal high-energy phosphate of ATP, almost all of which is produced in the mitochondria under aerobic metabolism. It regulates the intracellular and extracellular Na^+ and K^+ concentration by active transport mechanism and these monovalent cations are very important for regulation of cellular volumes and contractility of the myocardium.

The sudden onset of myocardial ischemia induced by occlusion of the coronary artery is associated with metabolic, functional and ultrastructural alterations. The loss of ATPase activity in the ischemic state would be responsible for causing not only functional damage but also irreversible anatomic changes in the involved myocardial cell. On the other hand, RMBF is one of the most important factors in restoring the myocardial cells. In reported studies, RMBF was measured by microsphere or hydrogen inhalation. Microsphere method cannot be repeated in the same individual and hydrogen inhalation method requires more time than local hydrogen generation method by electrolysis, which we used in this study. This method has several outstanding advantages(9,10). One of these advantages is its applicability to repeated measurements of RMBF in tissues without hydrogen inhalation. When several electrodes are used, RMBF at several sites can be measured simultaneously, and the measurements can be

obtained as absolute values (11). The apparatus and procedure are extremely simple. Therefore we think that this method is useful for RMBF measurements, particularly in the light of the fact that compared to other methods a relatively large number of measurements can be made.

In our study, during myocardial ischemia RMBF was significantly reduced and lactate was produced in all groups of coronary ligation. After reperfusion lactate production was reduced in all reperfusion groups and restoration of lactate balance was observed in the 1-hr group. Lactate balance was not restored within 60 minutes after reperfusion in the 2-hr and 4-hr groups. These results indicated that aerobic metabolism recovered from ischemic state within 60 minutes after coronary reperfusion in the 1-hr group.

On cardiac Na^+,K^+-ATPase activity in ischemic state, Schwartz et al. (12) reported that one day after coronary ligation, tissue taken from ischemic area showed no enzyme inhibition but 7 days after ligation, ischemic tissue had consistently less enzyme activity than control tissue in the preparation of sarcolemmal fraction by treatment. On the other hand, Beller et al. (13) reported a slight reduction in Na^+,K^+-ATPase activity after 2 hours of myocardial ischemia and marked decrease in it after 6 hours without collateral circulation. However, in these studies measurements of Na^+,K^+-ATPase activity were performed at an early stage (several hours) after reperfusion. Repair of tissue may require several days or a few weeks. During these periods, it could be determined whether myocardial cells would survive or not. The decreased Na^+,K^+-ATPase activity could be directly involved with cell death that follows. From this point of view, we measured ATPase activity 7 days after reperfusion. Our results showed that Na^+,K^+-ATPase activity of the ischemic area tended to reduce in 2-hr and 4-hr groups as compared to that of non-ischemic areas, and that the difference in enzyme activity in ischemic area between 4-hr group and permanent group was not significant. In marginal region, ATPase activity was not significantly reduced in 1-hr, 2-hr and 4-hr groups when compared to non-ischemic enzyme activity from the same heart, but in permanent group enzyme activity was significantly reduced. These results show that in dogs reperfusion 2 hours after coronary occlusion can produce substantial salvage of severe ischemic myocardium, but

reperfusion after 4 hours of coronary occlusion cannot. Our results differ from those of Schwartz et al. (12). We think that this may be due to the difference in samples used. We cannot help using smaller size of myocardial specimens at the center of the each region for the more accuracy and specificity. Their samples may contain the sample of our "marginal region" because of the larger size of myocardium. Our results also differ from those of Beller et al. (13). In their experiments, collateral circulation was ligated but not in our experiments. On the other hand, regions of moderate ischemia are potentially salvageable for up to 4 hours in dogs. RMBF indicate also the same results. In short, RMBF was restored with reperfusion after 1 hour of coronary occlusion but RMBF was not recovered after 2 hors and 4 hours of occlusion. Reduction of RMBF in 2-hr group was less than that of RMBF in 4-hr group. Although there was no significant difference in RMBF on the seventh day between 4-hr group and permanent group, reduction of RMBF in 2-hr group was significantly less than that of RMBF in permanent group. However we did not find a good correlation between the regional blood flow and ATPase activity 7 days after reperfusion. This discrepancy seems to indicate that RMBF after reperfusion reflects the ineffective redistribution of flow. Therefore restoration of RMBF does not always imply the recovery of myocardial viability. But we concerned that the normal function of sarcolemma is necessary to the integrity of myocardial cells, the decreased Na^+,K^+-ATPase activity always implys the dysfunction of myocardium and cell damage. We think the decreased ATPase activity is directly involved with cell death and ATPase activity expresses the viability of myocardial cell rather than RMBF. Maroko et al. (14), Ginks et al. (15) and Costantini et al. (16) have reported almost the same results as ours by other methods.

In summary, coronary artery reperfusion after 1 hour and 2 hours of coronary artery occlusion results in substantial salvage of RMBF and ATPase activity in the ischemic myocardial region. After 4 hours of coronary artery occlusion, little salvage of RMBF and ATPase activity occurs with reperfusion in the most severely ischemic region. However the marginal region could be salvaged by coronary artery reperfusion after 4 hours of coronary artery occlusion. Since the amout of potentially salvageable myocardium progressively decreases as the period of ischemia are

prolonged, time is the most critical variable involved in delaying or preventing ischemic myocardial cell death. These results may help clarify the time constraints during which coronary revascularization by thrombolysis or percutaneous transluminal coronary revascularization may be expected to result in significant salvage of ischemic myocardium in man.

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