Regional metabolism during coronary occlusion, reperfusion, and reocclusion using phosphorus^{31} nuclear magnetic resonance spectroscopy in the intact rabbit

Few studies have examined metabolic consequences of coronary occlusion and reperfusion using phosphorus^{31} nuclear magnetic resonance (^{31}P-NMR) in an intact animal model. Accordingly, we developed a model to study serial changes in myocardial metabolism in the intact open-chest rabbit. Ten animals underwent 20 ± 2 minutes of regional coronary occlusion and 60 ± 10 minutes of reperfusion followed by reocclusion. Cardiac-gated ^{31}P-NMR spectra were obtained with a regional surface coil over the ischemic area during baseline, occlusion, reperfusion, and reocclusion conditions. Phosphocreatine fell with both the initial and second ischemic insults to 65% ± 5% of baseline for the first occlusion ($p < 0.01$) and tended to decrease to 89% ± 8% of baseline for the second occlusion ($p = 0.07$), with normal levels reattained in the intervening period of reperfusion (99% ± 5% of baseline, p = NS). Concordant inverse changes were seen with inorganic phosphates. At occlusion levels of inorganic phosphates were 135% ± 10% of baseline ($p < 0.05$) and 139% ± 10% of baseline at reocclusion ($p < 0.05$). Levels of adenosine triphosphate decreased during occlusion to 78% ± 9% of baseline and were significantly lower than baseline during the second occlusion (75% ± 5% of baseline, $p < 0.01$). The ratio of phosphocreatine to inorganic phosphates, when compared with values at baseline, decreased at occlusion (49.6% ± 4.7% of baseline, $p < 0.01$) and at reocclusion (64.7% ± 4.9% of baseline, $p < 0.01$), with a normal ratio reattained in the intervening period of reperfusion (93.3% ± 3.1% of baseline, p = NS). We conclude that reperfusion restores levels of phosphocreatine and adenosine triphosphate while returning levels of inorganic phosphates to baseline. Deleterious changes in high-energy phosphate metabolism are not potentiated by reocclusion in this model. ^{31}P-NMR spectroscopy holds promise as a technique to noninvasively monitor intracellular biochemical processes serially during various interventions in the intact animal model. (Am Heart J 1989;117:53.)

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Phosphorus^{31} nuclear magnetic resonance spectroscopy (^{31}P-NMR) is a unique method for examining metabolic events in a noninvasive, non-tissue destructive manner. With this technique, one can measure the relative intracellular concentrations of the high-energy phosphate molecules, phosphocreatine (PCr), and adenosine triphosphate (ATP), along with the inorganic phosphate (Pi) molecules released during the hydrolysis required for the energy-consuming processes of the cell. When myocardial cells are rendered ischemic, anaerobic glycolysis cannot meet the energy requirements of the ischemic myocardium, and as a result levels of PCr and ATP decrease, with concurrent reciprocal increases in the inorganic phosphate products of high-energy phosphate degradation. When ischemia is reversed, these changes in high-energy phosphates revert to normal.[1]

The clinical relevance of the metabolic effects of

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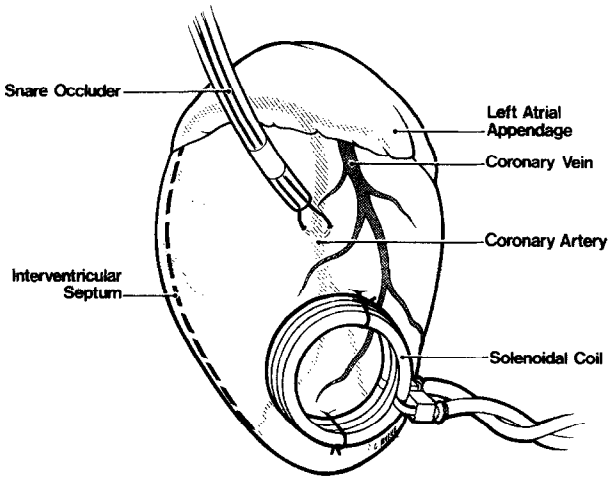

Fig. 1. Instrumentation used in the model. A suture is placed about the subepicardial marginal branch of the left circumflex coronary artery and brought out through a plastic snare. The radiofrequency solenoidal coil is anchored distal to the suture over the region of myocardium to be rendered ischemic.

ischemia and reperfusion has assumed increased importance in recent years with the availability of interventions to reverse coronary occlusion in the setting of acute myocardial infarction. Moreover, it has become apparent that successful recanalization of occluded coronary arteries may only be temporary in a proportion of patients.[2] The metabolic consequences of such reocclusions has not been extensively studied,[3] and there have been no previous studies of reocclusion after reperfusion with ^{31}P-NMR. Accordingly, we developed an intact rabbit model[4] to investigate the high-energy phosphate metabolic consequences of myocardial ischemia and reperfusion using ^{31}P-NMR spectroscopy. Furthermore, the metabolic effects of a second coronary artery occlusion after successful reperfusion were examined.

EXPERIMENTAL METHODS

Animal Model (Fig. 1). Ten healthy New Zealand white rabbits (3.0 ± 0.25 kg) were anesthetized initially with xylazine (10 mg/kg) intramuscularly followed by ketamine (40 mg/kg) also via intramuscular injection. An internal jugular vein was isolated and cannulated with a polyethylene tube for venous access. Anesthesia was maintained with frequent small intravenous boluses of ketamine (1 to 2 mg) through this central venous line. The trachea was then isolated, and animals were intubated via tracheostomy. Rabbits were then ventilated via a Harvard rodent ventilator on a 95% oxygen and 5% carbon dioxide mixture. The strength of the magnetic field required that this respirator remain a remote distance from the animal. Accordingly, two approximately 5 m lengths of tubes were attached to the inspiratory and expiratory ports of the ventilator. These tubes were then each connected to a one-way valve and joined at a T-piece from which a short (6-inch) length was attached to the endotracheal tube. This set-up, along with the alternating valve action of the Harvard respirator, minimized respiratory dead space. Arterial blood gasses were sampled in a series of pilot experiments and revealed pH and P_{CO_2} consistently in the normal range with oxygen saturation always above 95%, thereby assuring adequate function of the respirator apparatus.

The animals then underwent a left lateral thoracotomy through the fifth intercostal space. The pericardium was excised, allowing visualization of the lateral surface of the left ventricle. The large marginal branch of the left circumflex coronary artery, which runs subepicardially in the rabbit,[5] was identified. A 6-0 suture was then placed through the myocardium underneath this artery slightly distal to the atrioventricular groove. The suture was then brought through a polyethylene tube, which served as a reversible snare occluder. The same solenoidal radiofrequency coil as will be described, was used in all experiments. It was placed on the surface of the myocardium distal to the suture snare. A brief (5- to 10-second) occlusion was performed to better identify the ischemic area of myocardium and aid in proper placement of the coil previously described. ECG leads were then placed on the animal's extremities, and rabbits were wrapped in an insulated covering before being placed in the spectrometer.

Reliability of the reversible snare occluder was validated in several ways: (1) A brief (5- to 10-second) occlusion as previously described was done in each animal with the development of cyanotic, dyskinetic myocardium, along with blanching of the coronary artery distal to the suture. (2) In a series of pilot experiments, radioactive microspheres were injected into the left atrium before, during, and after occlusion using an identical suture snare. These studies revealed a near-total ablation of subendocardial blood flow in the myocardium distal to the occlusion that normalized with release of the suture snare. (3) Several of the experimental animals ($N = 6$) remained occluded for 3 hours on completion of the experimental protocol as will be described. After this prolonged occlusion, these animals were killed, and the hearts were excised. The hearts were sliced and stained with the dehydrogenase substrate–specific triphenyl tetrazolium chloride. Infarct in the myocardium distal to the coronary artery occlusion was demonstrated in each animal so studied. (4) Typical ST segment elevation was seen relative to baseline tracings on the ECG monitor. (5) Typical changes in ^{31}P-NMR spectroscopy as previously reported in the literature were seen consistently during appropriate experimental conditions.[6-10]

NMR spectroscopy. Experiments were performed using a General Electric/Nicolet CSI-II nuclear magnetic resonance spectrometer with a 22 cm bore, operating at 2.0 Tesla. The surface radiofrequency coil used was constructed of 0.8 mm copper wire and configured to four

turns with a diameter of 13 mm. The axial length of 6 mm assured near-optimal circuit Q, and the four-turn coil design resulted in concentration of the B1-field along the coil's axis, thus minimizing signal contamination from nonischemic tissue. A balanced tuning circuit[11] with grounding of the unbalanced side inside the magnet bore reduced capacitive losses and eliminated spurious resonances of the coaxial circuit formed by the bore and the radiofrequency cable. Insulation of the entire coil with silicone rubber prevented corrosion. The short T2 proton resonance from this insulating material was well separated from the water proton signal and posed no problem during shimming, when the receiver gain was set to the level of tissue proton signal. Sensitivity profiles of this coil from phantom studies revealed maximum sensitivity at the coil surface, with 80% of the signal recovered from within 6 mm of axial distance. This coil, while within the rabbit, after being centered in the magnetic field, was tuned with the external tuning circuit to 34.62 MHz, the phosphorus resonant frequency at this field strength.

ECG gating was used to reduce motion-related line broadening. ECG signals from the animals were fed into a standard monitor fitted with a custom-made R-wave trigger output, assuring signal acquisition during the same period of the cardiac cycle. Local magnetic field homogeneity was optimized by shimming on the proton signal with a pulse width of 30 μs, yielding water line widths between 0.3 and 0.5 ppm. Gated ^{31}P spectra were acquired over a period of 10 ± 1 minutes by averaging 256 transients with a 2-second interpulse delay. Also, one spectrum was acquired for seven of the 10 animals with an 8-second interpulse delay before any intervention for calculation of partial saturation factors. Spectra were transformed with a line broadening of 10 Hz. The chemical shift for phosphocreatine was defined as zero parts per minute by convention.[12]

Experimental protocol. After acquisition of baseline spectra, including the spectra with an 8-second interpulse delay as will be described, the suture snare was tightened to produce coronary artery occlusion without disturbing the rabbit's position within the NMR unit. Two more spectra were then acquired consecutively during the 20 ± 2 minute ischemic period. This duration of occlusion was chosen for two reasons. First, the lateral borders of ischemia are established within 15 minutes of occlusion; second, although there will be some degree of damage to myocardium, some muscle will not be irreversibly damaged, with the greater proportion of this viable tissue more subepicardial and, thus closer to the surface coil.[13] On completion of the occlusion period, the suture snare was then released, allowing myocardial reperfusion. Serial spectra were then acquired for the next 60 ± 10 minutes (the period of reperfusion), and the results were averaged. Finally, a second period of occlusion was produced by again tightening the same suture snare. Two more spectra were acquired during this period, after which animals were killed. After death, animals were carefully inspected to ensure that the coil remained anchored in its original position and had not moved during the protocol. Heart rates were recorded at each time point as previously described.

Data analysis. The areas under the five phosphate spectral peaks were calculated with the aid of a Lorenzian curve-fitting program supplied with the CSI computer. A two-step procedure was used with the peak positions for α- and β-ATP, and the linewidths for all peaks were fixed during the final peak fit. Data area was expressed as percentage change from baseline for each respective peak. The ratio of PCr to Pi was also calculated at each time point. The chemical shift of the Pi peak relative to PCr was measured at occlusion and reocclusion as an estimate of intracellular pH.[14] All data are presented as mean ± SEM. Comparisons within groups were made using a two-way analysis of variance. When significant F statistics were obtained, paired t tests were used to distinguish which time periods differed significantly. Because multiple comparisons were performed, the Bonferroni inequality adjustment was used to modify the acceptable α-level.[15]

RESULTS

Ten animals were used in this protocol. Two animals died as a result of ventricular arrythmia during reperfusion. Results for these two animals are included only for baseline and occlusion measurements.

There was no significant increase or decrease in heart rate during any intervention. Because our method of data analysis normalized the area under each respective peak to its value at baseline, the need to correct for partial saturation of each peak was obviated. However, the partial saturation ratios were calculated during baseline conditions by comparison to spectra acquired with a repetition time sufficiently long to allow for complete relaxation. The only peak that revealed significant partial saturation was PCr with a ratio of 1.35.

Given the heterogeneity of the Pi peak in the intact animal, accurate measurement of chemical shift for determination of intracellular pH was difficult during nonischemic periods. However, with an ischemic insult levels of Pi rose to an extent that allowed the ability to distinguish this compound from the 2,3-diphosphoglycerate of blood and other phospho-mono and di-esters that also resonate at this frequency. Our experiment revealed no significant change in the chemical shift and hence intracellular pH when values at occlusion were compared with values at reocclusion.

The results of the ^{31}P-NMR measurements of high-energy phosphates are summarized in Table I and Fig. 2. Pi increased significantly during occlusion as a result of continued hydrolysis, with decreased replenishment of PCr and ATP during the ischemic insult. The levels of Pi promptly

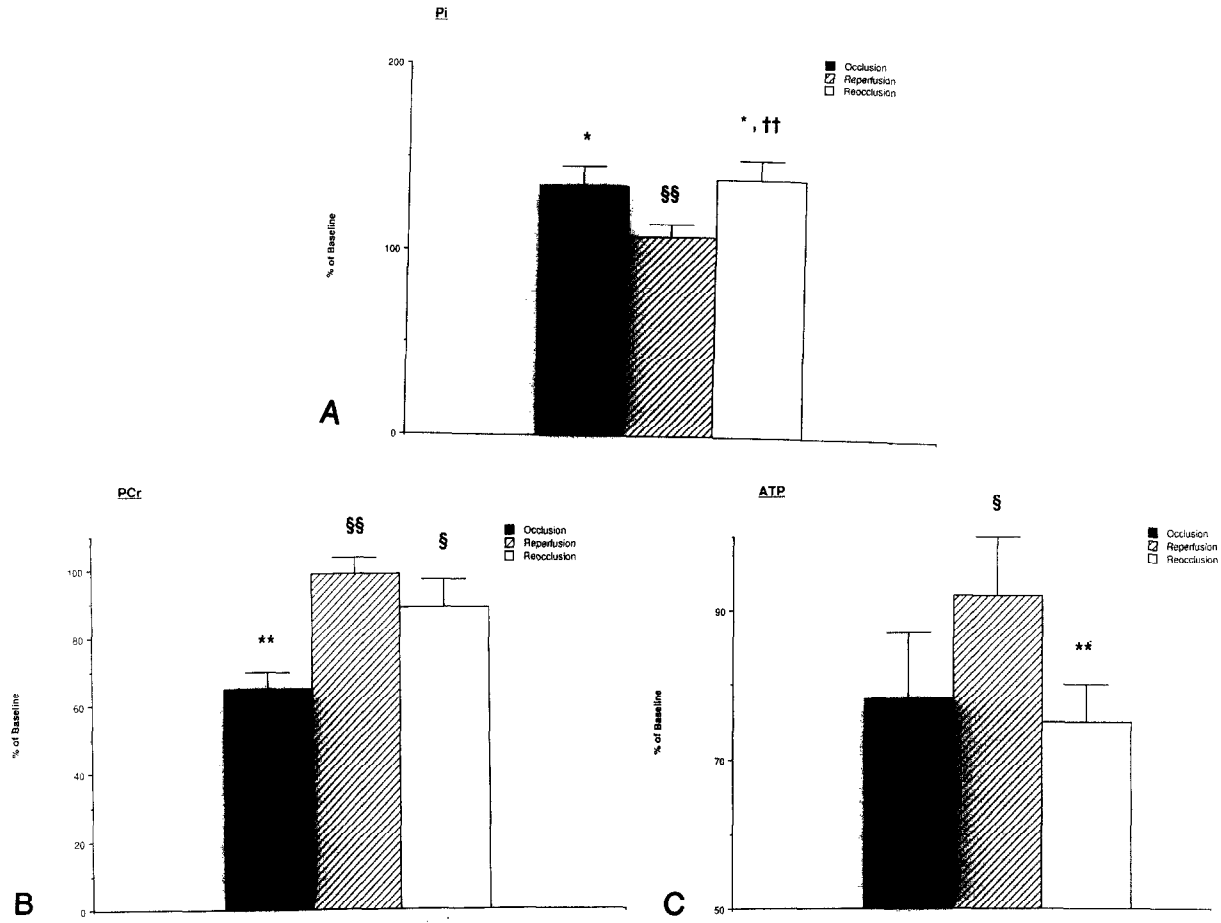

Fig. 2. Results of ^{31}P-NMR measurements of high-energy phosphates. **A** demonstrates the increase in inorganic phosphates *(Pi)* during occlusion. The levels of Pi promptly returned to baseline with reperfusion. With a second occlusion Pi again increased significantly compared with baseline and reperfusion levels. **B** shows changes in the levels of phoshocreatine *(PCr)* compared with baseline. Significant decrease at occlusion is seen compared with baseline. With reperfusion levels returned to baseline values. With a second occlusion the levels of PCr again dropped relative to baseline but not as much as during the first occlusion. **C** shows the amounts of adenosine triphosphate *(ATP)* measured at the β-phosphate resonance, which is unique for ATP. Levels of ATP decreased at occlusion relative to baseline, although not quite reaching statistical significance. With reperfusion the amount of ATP increased to near baseline levels and was significantly more than at occlusion. With reocclusion, the amount of ATP decreased significantly compared with baseline. $*p < 0.05$ compared with baseline; $**p < 0.01$ compared with baseline; $\S p < 0.05$ compared with the first occlusion; $\S\S p < 0.01$ compared with the first occlusion; $\dagger\dagger p < 0.01$ compared with reperfusion.

returned to baseline with reperfusion. During the second occlusion, Pi again increased significantly compared with baseline and reperfusion levels; furthermore, the rise in Pi was nearly identical during the second occlusion period compared with levels measured at the first ischemic insult (135% ± 10% vs 139% ± 10%, respectively, p = NS). Levels of PCr decreased significantly with occlusion to 65% ± 5% of levels during baseline ($p < 0.01$). With reperfusion this decrease promptly returned to baseline values. During the second ischemic insult, the amount of PCr again fell to 89% ± 8% of baseline. This decrease was significantly less than the magnitude noted during the first occlusion period ($p < 0.05$). The β-resonance of ATP was used to measure levels of ATP, since it is the only phosphate found at this frequency, thus representing a relatively pure ATP signal. Levels of β-ATP decreased with occlusion, although not quite reaching statistical significance when compared with baseline. With reperfusion the amount of β-ATP increased to near baseline levels and was significantly more than at occlusion ($p < 0.05$). With reocclusion, the amount of β-ATP again decreased to 75% ± 5% of baseline ($p < 0.01$).

The ratio of levels of PCr to Pi was also calculated

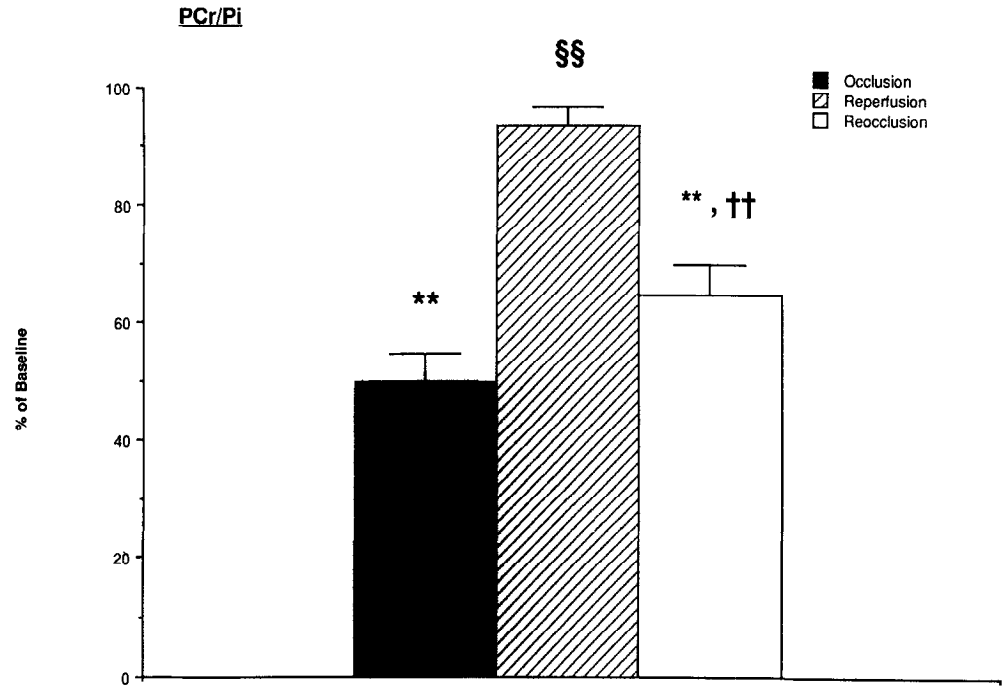

Fig. 3. The ratio of phosphocreatine *(PCr)* to inorganic phosphates *(Pi)* expressed as percentage of baseline. This ratio decreased significantly at occlusion and promptly normalized during reperfusion. During the second occlusion period, this ratio again decreased significantly compared with baseline and reperfusion; however, the decrease did not differ from that noted during the first occlusion. $**p < 0.01$ compared with baseline; $§§p < 0.01$ compared with the first occlusion; $††p < 0.01$ compared with reperfusion.

at each time, point (Fig. 3). This ratio decreased significantly with occlusion and promptly normalized with reperfusion. With a second ischemic insult, the ratio again decreased compared with both baseline and reperfusion; however, there was no significant difference during the second occlusion when compared with the first.

DISCUSSION

Our data demonstrate that coronary occlusión in the intact animal model produces a decrease in PCr and β-ATP, with a concomitant rise in the level of inorganic phosphates. On reperfusion, PCr, Pi, and β-ATP immediately reverted to baseline conditions. The resynthesis of PCr by oxidative metabolism is in agreement with the observations of others[1, 6, 16, 17] and probably relates to preservation of the inner mitochondrial membrane during reperfusion.[18] After reocclusion, PCr again tended to decrease with an increase in Pi, but no potentiation of these metabolic abnormalities was noted.

Our observations are consistent with those of Reimer et al.,[3] who, using serial biopsies and standard biochemical assays, noted rapid restoration of adenylate charge with reperfusion. However, in contrast to our findings, they noted only a slight repletion of ATP after reperfusion. This apparent discrepancy may be related to the fact that their tissue sampling was subenodocardial, whereas in our study the sampling was transmural and somewhat weighted toward the epicardium because of the position of the surface coil. Thus the relatively prominent repletion of ATP noted in our study may be caused by sampling of less profoundly ischemic tissue. In accordance with our findings, Reimer et al.[3] found that repeated brief periods of regional ischemia resulted in no further depletion of ATP. They concluded that intermittent reperfusion pre-

Table I. High-energy phosphate metabolites at three time points; all values are mean percentages of values at baseline ± SEM

	Occlusion	*Reperfusion*	*Reocclusion*
Pi	135 ± 10*	108 ± 7†	139 ± 10*,‡
PCr	65 ± 5§	99 ± 5†	89 ± 8‖
β-ATP	78 ± 9	92 ± 8‖	75 ± 5§

*$p < 0.05$ compared with baseline.
†$p < 0.01$ compared with occlusion.
‡$p < 0.01$ compared with reperfusion.
§$p < 0.01$ compared with baseline.
‖$p < 0.05$ compared with occlusion.

vented cumulative metabolic defects perhaps by restoring the capacity for high-energy phosphate production or washing out of deleterious metabolites.

Most previous studies of myocardial metabolism during ischemia that used ^{31}P-NMR have been performed in isolated heart models. Although considerable knowledge has been gained from these in vitro studies of cardiac metabolic and physiologic functions, the ultimate description of these processes must be in the regulation and integration in the intact, living animal. There are many advantages to examination of the intact heart. Since the heart is maintained by the animal, the in vivo model does not have problems related to viability and instability compared with the buffer-perfused in vitro system. In addition, the in vivo model is more physiologic with adequate oxygen, blood pressure, osmolarity, temperature, electrolytes, and nutritional substrate provided by the animal. To this end, we and others have used the intact animal model[7,8] in conjunction with ^{31}P-NMR to examine high-energy phosphates and intracellular pH in vivo.

Few ^{31}P-NMR studies of the heart have been reported in the intact animal model. Grove et al.[7] studied global myocardial ischemia produced by hypoxia in intact rats. They noted a decrease in PCr and ATP with a concurrent rise in Pi analogous to our findings. In addition, relatively few studies have specifically examined regional ischemia in either an intact animal model[4,8] or the isolated heart.[9] Malloy et al.[10] studied the effects of treatment with propranolol on regionally ischemic intact rabbit myocardium. They observed phosphorus NMR changes with coronary artery occlusion similar to our results that were attenuated by propranolol. Recently, Stein et al.[8] reported a study involving open-chest cats that underwent regional myocardial ischemia with a surface coil placed over the region of interest. They noted a rapid drop in levels of PCr with ischemia and concurrent increases in Pi. Levels of ATP also decreased with coronary artery occlusion although more gradually and did not reach significance until 15 minutes after occlusion.

Our results are also in agreement with previous reports of coronary occlusion and reperfusion, which used standard biochemical techniques[1] and NMR studies.[6,16,17] For example, Neurohr et al.[6] used ^{31}P-NMR in an intact, in vivo, globally ischemic model to study high-energy phosphates within the entire myocardium. They also noted prompt decreases in PCr with ischemia with normalization shortly after reoxygenation and analogous, converse changes in Pi. They observed no detectable fall in ATP with a brief anoxic insult. However, an intact animal model of regional myocardial occlusion and reperfusion is limited to only one report.[19] In this study by Guth et al.,[19] similar changes in PCr, ATP, and Pi occurred as in our study, and ATP did not return to baseline levels. In accordance with our results, these studies did not note an overshoot of PCr with reperfusion as previously reported by others using both biochemical[20] and NMR techniques.[17] However, it is possible that such an overshoot could be missed with the level of time resolution inherent in our model.

Since reocclusion after coronary reperfusion continues to be a common clinical problem,[2] our metabolic data on reocclusion have additional clinical significance. It is important to note that our results demonstrate that reocclusion is not associated with a potentiation of high-energy phosphate depletion. In fact, the decrease in PCr was significantly less with the second occlusion compared with the first occlusion. In view of the fact that brief periods of coronary occlusion cause prolonged functional depression,[21] and that a repeated period of brief ischemia does not have a cumulative detrimental effect on left ventricular function,[22] a possible explanation for the relative preservation of high-energy phosphates is that energy use for contractile function is lower before the second occlusion period. This hypothesis is supported by work from Reimer et al.,[3] who noted no cumulative depletion of the adenine nucleotide pool after as many as four 10-minute episodes of coronary artery occlusion. The observation that deleterious changes in high-energy phosphates were not potentiated by repeated ischemic insults may imply that the rate of infarction, if it is ultimately related to the level of high-energy phosphate content, may be similar after reocclusion compared with the initial ischemic insult. Experiments by Schaper et al.[23] report that the rate of infarction correlates with the amount of ATP "overspending" by ischemic myocardium, which is defined by contrasting the rate of fall of tissue ATP during ischemia with the aerobic ATP production made possible by collateral blood flow. However, since the kinetics of infarction are difficult to define during repeated ischemic insults, the precise interrelationships between high-energy phosphate levels and myocardial necrosis may be difficult to establish.

Limitations of this study include the fact that our coil may have collected some signal from partially ischemic and nonischemic myocardium, as well as ischemic tissue, and that there may have been difficulties in separating the measured peaks from overlying resonances. One further methodologic

point deserves comment. At present there is no consistency in spectral analysis of ^{31}P-NMR data by investigators, and this fact may contribute to small variations in results among different reports. Our method of spectral analysis involves normalizing each peak to its respective preintervention baseline. We believe that this method of analysis holds advantages over others, since it obviates the need for correction of peak intensities for partial saturation.

In summary, we have developed an intact rabbit model to study dynamic changes in high-energy phosphate metabolism in the setting of reversible regional myocardial ischemia using ^{31}P-NMR spectroscopy. Our results with coronary artery occlusion and reperfusion are consistent with those previously reported in the literature using biochemical techniques. Significantly, we noted no potentiation of the deleterious changes in high-energy phosphates with a second episode of severe regional myocardial ischemia. In addition to increasing our knowledge regarding basic metabolic mechanisms of normal and abnormal myocardial function, phosphorus NMR spectroscopy may soon have direct clinical application. Thus ^{31}P-NMR spectroscopy should have the potential to offer unique insights into the study of human myocardial ischemia.

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