Prolonged coronary artery occlusion–reperfusion sequences reduce myocardial free radical production: An electron paramagnetic resonance study

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Our purpose was to determine whether prolonged myocardial ischemia attenuates free radical production after early reperfusion. Twenty-two mongrel dogs underwent left anterior descending coronary artery occlusion for 20, 40, or 60 minutes followed by 30 minutes of reperfusion. Electron paramagnetic resonance spectroscopy was used to measure ascorbate free radical in the coronary vein effluent. Ascorbate free radical production during reperfusion was significantly ($p < 0.05$) reduced in the dogs undergoing 60 minutes of coronary artery occlusion compared with the dogs undergoing 40 and 20 minutes of occlusion. We conclude that prolonged myocardial ischemia results in less free radical production on reperfusion than do shorter periods of ischemia followed by reperfusion. (Am Heart J 1996;132:1147-55.)

Myocardial reperfusion after a brief episode of coronary artery occlusion results in prolonged impairment of contractile function that is completely reversible.[1] This phenomenon, referred to as "myocardial stunning,"[2, 3] has been associated with the production of reactive oxygen metabolite species including superoxide radicals, hydrogen peroxide, and hydroxyl radicals.[4] Free radical species are thought to originate from cellular sources in the myocardium and blood as a result of ischemia–reperfusion sequences.

We hypothesized that prolonged coronary occlusion, sufficient to cause myocardial necrosis, would damage the cellular components responsible for free-radical generation. This condition would then result in attenuation of free radical production after reperfusion of the myocardium. To investigate this hypothesis we monitored ascorbate free-radical production, a new marker of free radical generation and total oxidative stress,[5] in open-chest dogs undergoing variable duration coronary artery occlusion–reperfusion sequences.

METHODS

Animal preparation. An open-chest canine model was used for coronary occlusion–reperfusion studies. Mongrel dogs of either sex were fasted overnight and anesthetized with intravenous fentanyl–droperidol 0.13 ml/kg followed by pentobarbital 20 mg/kg. The animal was then endotracheally intubated, and ventilation was begun with a volume-cycled respirator. The arterial blood gas was maintained in the physiologic range by adjusting the tidal volume, respiratory rate, fraction of inspired oxygen content, and serum bicarbonate as necessary. Intravascular access was obtained through the internal jugular veins, femoral arteries, and femoral veins bilaterally. Arterial blood pressure was monitored from the left femoral artery. The electrocardiogram was monitored from limb leads. A left lateral thoracotomy was performed in the sixth intercostal space, and the heart was suspended in a pericardial cradle. The left atrial appendage was retracted back to allow adequate exposure of the heart. The left anterior descending (LAD) coronary artery was isolated proximally, and a snare was placed around the vessel for occlusion. A catheter was advanced from the right internal jugular vein into the coronary sinus. The catheter was then secured with a ligature in the great cardiac vein at a point proximal to the site of LAD occlusion. A venovenous shunt was constructed between the great cardiac vein and the right femoral vein. Heparin was administered to prevent blood clotting in the indwelling cannulas and tubing that conducted blood to and from the electron paramagnetic resonance apparatus.

Electron paramagnetic resonance method for determination of ascorbate free radicals. The measurement of ascorbate free radicals has been validated previously as a

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measure of total oxidative stress.[6] Ascorbate is the terminal small-molecule antioxidant; it reduces more highly oxidizing radicals such as hydroxyl, superoxide, and lipid peroxyl radicals. As it donates an electron (hydrogen atom) to reduce these oxidizing radicals, ascorbate itself is converted to a free radical, the ascorbate radical. This relatively stable π-radical can be detected and quantitated by the electron paramagnetic resonance technique. The steady-state ascorbate free radical concentration is proportional to the total oxidative stress of the interventions evaluated.[5-7]

Electron paramagnetic resonance spectra were obtained with a spectrometer (Varian E-4, Palo Alto, Calif.) with a TM_{110} cavity and an aqueous flat cell. Scans were collected serially on blood being continuously withdrawn from the great cardiac vein or the femoral artery. An infusion pump was used to circulate the sample through the spectrometer and return it to the animal.[5] In this way blood could be scanned for ascorbate free radical within several seconds of its leaving the animal. The following instrument settings were used for all studies because they provide the largest ascorbate radical signal: nominal power 40 mW, modulation amplitude 0.63 G, time constant 1 second, and scan rate 1 G/24 sec. Nominal microwave power of 40 mW was used because it gives the greatest peak-to-peak $Asc^{\cdot-}$ electron paramagnetic resonance signal height. The $Asc^{\cdot-}$ electron paramagnetic resonance signal is partially saturated at the power level. Appropriate corrections were made to determine steady state $Asc^{\cdot-}$ concentrations. The power from the microwave bridge of the Varian spectrometer is calibrated. The power saturation curve of $Asc^{\cdot-}$ in the region of 40 mW has very little gradient; thus a small error in the nominal power setting produces very little change in $Asc^{\cdot-}$ signal height.

The concentration of ascorbate free radical was determined from the signal height of the ascorbate electron paramagnetic resonance spectrum after calibration by double-integration techniques and 3-carboxy proxyl as the standard.[6, 7] For our experimental conditions, 1 mm of signal height corresponded to 0.0734 nmol/L ascorbate free radical after saturation effects were accounted for.[7] All signal heights were normalized to the full gain of the instrument, 10^5.

Echocardiography. Two-dimensional echocardiograms were obtained to determine the functional injury to the myocardium as a result of the ischemia–reperfusion sequences and to correlate this injury with the ascorbate radical generation. An echocardiograph (ATL Ultramark-4, Advanced Technologies Laboratories, Bothel, Wash.) with a 5 MHz transducer was used to obtain echocardiograms through the chest wall from the right parasternal short-axis view, at the papillary muscle level at baseline, during occlusion, and after 30 minutes of reperfusion. The portion of the left ventricular wall displaying a regional wall motion abnormality (hypokinesis, akinesis, or dyskinesis) was determined. Planimetric measurements of the entire myocardial area and the abnormally contracting region were made by one observer (P.D.L.) on echocardiograms performed after 30 minutes of reperfusion, at end-diastole (defined as maximum ventricular cavity area). The abnormal area was expressed as a percentage of the total myocardial area (including the papillary muscles). In addition, the percentage wall thickening was measured in the midportion of the area of regional wall motion abnormality and in a normal segment opposite this region. Percentage wall thickening was calculated with the formula ([ES – ED]/ED) × 100%, where ES is the wall thickness at end-systole (defined as minimum ventricular cavity area) and ED is the wall thickness at end-diastole (maximum ventricular cavity area). The intraobserver variability was assessed by remeasuring eight randomly selected studies after 30 minutes of reperfusion for the 20-minute–occlusion group (two studies), the 40-minute–occlusion group (three studies), and the 60-minute–occlusion group (three studies). The observer attempted to identify and measure the same echocardiographic frame twice, 48 hours apart.

Protocol. The animals were prepared as described earlier in this section. Normally, the concentration of ascorbate free radical in whole canine blood is too low for detection by electron paramagnetic resonance spectroscopy under the constraints of our need to examine ascorbate free radical concentration versus time. Therefore, approximately 30 minutes before occlusion of the LAD coronary artery, an intravenous infusion of ascorbate was given (1 gm bolus followed by 8 to 30 mg/min infusion) to amplify the endogenous ascorbate free radical signal. This infusion was continued throughout the experiment.

The technique of ascorbate loading was developed during our preliminary studies[5] with the intact animal preparations. Consecutive ascorbate free-radical electron paramagnetic resonance spectrum scans were obtained from the femoral artery until the signal amplitude of the spectrum was stable. The ascorbate free-radical concentration was then monitored from the coronary vein effluent. The arterial ascorbate free-radical concentration was rechecked periodically throughout the study to ensure the stability of the system and to maintain a steady-state arterial ascorbate concentration. In general, the arterial ascorbate free radical signal was stable; adjustments in the rate of the ascorbate infusion were made if the arterial signal varied more than 10%, to maintain the arterial ascorbate signal constant. Adjustments were not made during the 30-minute reperfusion period, but the arterial level was checked.

The LAD coronary artery was occluded with the snare for 20, 40, or 60 minutes and then released. Reperfusion was documented by resolution of electrocardiographic ST-segment elevation and of myocardial tissue cyanosis, with occasional dysrhythmias observed on the electrocardiogram monitor. The ascorbate free-radical concentration was measured with consecutive electron paramagnetic resonance scans at baseline, during coronary artery occlusion, and during reperfusion. An echocardiogram was performed at baseline, during occlusion, and again after 30 minutes of reperfusion for each animal.

Statistics. All data are expressed as a mean ± SD. Differences were considered significant at $p < 0.05$. One-factor analysis of variance was used to compare end-occlusion

Table I. Hemodynamic parameters, femoral artery ascorbate free radical concentration, and echocardiographic measurements for three ischemia groups

	Ischemia duration (min)		
	20 (n = 7)	*40 (n = 7)*	*60 (n = 8)*
End-occlusion hemodynamics			
MAP (mm Hg)	65 ± 8	80 ± 19	83 ± 11
HR (beats/min)	147 ± 13	141 ± 17	141 ± 27
RPP	12,850 ± 1350	14,367 ± 3972	14,861 ± 3547
Femoral artery ascorbate free radical concentration (nmol/L)			
End-occlusion	13.0 ± 2.9	12.0 ± 2.0	14.9 ± 2.5
After 30-min reperfusion	13.2 ± 2.9	11.2 ± 2.2	13.9 ± 2.1
Percentage regional wall motion abnormality after 30-min reperfusion	4.8 ± 5.6	9.7 ± 5.8	9.0 ± 7.3
Percentage thickening in abnormal region at baseline	57 ± 17	50 ± 9	57 ± 25
Percentage thickening after 30-min reperfusion			
Abnormal region	29 ± 17	14 ± 27	7.7 ± 20
Normal region	50 ± 28	42 ± 16	35 ± 27

HR, Heart rate; *MAP*, mean arterial pressure; *RPP*, rate–pressure product (HR × systolic blood pressure).

hemodynamics, ascorbate free-radical concentration, area under the curve of the ascorbate free-radical percentage change, and other parameters among the three occlusion groups. Follow-up pairwise comparisons of means were conducted with Bonferroni's correction to control the overall experimental error at 0.05. Because of the nonnormal distribution of many of these variables, nonparametric techniques also were used to compare the three groups. The results of the parametric analyses are reported because they were consistent with the nonparametric results. Two-factor repeated measures analysis of variance was used to analyze ascorbate free radical data in nanomoles per liter and expressed as a percentage increase over baseline. The overall analysis of each variable was followed by pairwise comparisons among groups at each time point with Bonferroni's correction to control the overall experimental error at 0.05.

RESULTS

Twenty-two dogs were studied in the current series of experiments: seven dogs in the 20-minute–occlusion group, seven dogs in the 40-minute–occlusion group, and eight in the 60-minute–occlusion group. In eight dogs, ventricular fibrillation developed during the reperfusion sequence of the protocol: in one dog in the 20-minute occlusion group, three in the 40-minute group, and four in the 60-minute group. In all dogs defibrillation was successful with three or fewer 50 J countershocks. The end-occlusion hemodynamic parameters of heart rate, mean arterial pressure, and rate–pressure product are shown in Table I. There were no significant differences in these values among the three groups. The end-occlusion arterial concentration of ascorbate free radical also was similar in the three groups and showed no significant change from end-occlusion to the end of reperfusion ($p > 0.15$) (Table I).

The data for coronary venous ascorbate free-radical concentration and percentage change from baseline for the 20-, 40-, and 60-minute–occlusion groups during reperfusion are shown in Tables II through IV, respectively and Fig. 1. The end-occlusion values were similar in the three groups of animals studied ($p > 0.30$). On reperfusion, the ascorbate free radical increased significantly in the 20- and 40-minute–occlusion groups. In the 20-minute–occlusion group there was a significant difference in the mean ascorbate free radical from end-occlusion to 30 minutes of reperfusion ($p < 0.0001$). Pairwise comparisons of each reperfusion mean with the end-occlusion mean showed a significant increase in ascorbate free radical at 3, 5, 7, and 10 minutes ($p < 0.05$). In the 40-minute–occlusion group, there was also a significant change with time ($p < 0.0001$), with significant increase in ascorbate free radical at 5, 7, 10, 15, 20, and 25 minutes ($p < 0.05$). The 60-minute–occlusion group did not have a significant increase in ascorbate free-radical concentration to greater than the end-occlusion value ($p > 0.25$). The mean percentage change from end-occlusion in ascorbate free-radical concentration was highest in the 20-minute occlusion group at 10 minutes of reperfusion, 41.0% ± 23.8%. In contrast, the peak ascorbate free radical increase in the 40- and 60-minute–occlusion groups was significantly less, at 17.4% ± 10.1% at 10 min-

Table II. Changes in coronary venous ascorbate free radical concentration after 20 minutes of coronary artery occlusion and 30 minutes of reperfusion

	Dog								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*Mean*	*SD*
Coronary venous ascorbate free radical (nmol/L)									
End-occlusion	5.5	6.7	7.1	8.0	6.9	6.5	6.6	6.8	0.8
Minutes reperfused									
1	5.0	8.8	7.5	8.3	7.2	6.9	7.3	7.3	1.2
3	5.0	9.8	10.9	10.8	9.5	6.9	7.6	8.7*	2.2
5	6.8	9.8	13.8	11.3	9.8	6.9	7.5	9.4*	2.6
7	6.0	9.4	12.8	11.2	10.1	6.9	7.9	9.2*	2.4
10	6.9	8.2	13.1	11.9	9.8	ND	8.2	9.7*	2.4
15	6.0	7.8	10.4	11.0	6.8	7.5	8.7	8.3	1.8
20	5.6	8.0	9.8	10.2	6.6	7.8	8.4	8.1	1.6
25	4.8	8.2	9.5	9.6	6.5	7.5	8.4	7.8	1.7
30	4.6	8.6	9.4	8.2	6.8	6.8	7.1	7.4	1.6
Percentage change in ascorbate free radical compared with end-occlusion									
Minutes reperfused									
1	-9.1	31.3	5.6	3.6	4.2	6.6	10.9	7.6	12.2
3	-9.1	46.3	53.5	34.1	37.5	6.6	15.3	26.3	22.7
5	23.6	46.3	94.4	40.2	41.7	6.6	13.1	38.0	29.1
7	9.1	40.3	80.3	38.9	45.9	6.6	19.6	34.4	25.6
10	25.5	22.4	84.5	48.1	41.7	ND	24.0	41.0	23.8
15	9.1	16.4	46.5	36.6	-2.0	15.6	31.6	22.0	16.9
20	1.8	19.4	38.0	26.7	-4.2	20.1	26.1	18.3	14.7
25	-12.7	22.4	33.8	19.4	-6.2	15.6	27.2	14.2	17.3
30	-16.4	28.4	32.4	2.4	-2.0	4.5	6.7	8.0	17.1

*$p < 0.05$ vs end-occlusion. *ND*, No data obtained.

utes and 11.1% ± 15.8% at 5 minutes of reperfusion, respectively (Fig. 1). The two-factor repeated-measures analysis of variance of the percentage change in ascorbate free radical (Fig. 1) showed evidence of significant occlusion group–by–time interaction effects. Therefore the follow-up pairwise comparisons were made at each time point among the three occlusion groups. The 60-minute group had a significantly lower percentage change than the 20-minute group at 3, 5, 7, and 10 minutes. The 40-minute group's percentage change was significantly lower than the 20-minute group's percentage change at 3 and 10 minutes. Similarly, the area under the curve of percentage change in ascorbate free-radical concentration versus time also was different for the three groups: 658% ± 467%, 358% ± 237%, and 143% ± 163% for the 20-, 40-, and 60-minute occlusion groups (Fig. 2) ($p < 0.05$, 60 minutes vs 20 minutes).

After 30 minutes of reperfusion, all three groups had observable wall motion abnormalities (hypokinesis, akinesis, or dyskinesis) by echocardiography. The values for percentage regional wall motion abnormality, abnormal region percentage thickening, and normal region percentage thickening are provided in Table I. The percentage regional wall motion abnormality after reperfusion was not significantly different for the three groups but tended to increase with increasing duration of occlusion (Fig. 3). The systolic wall thickening for the abnormal (reperfused) region during reperfusion also was not significantly different for the three groups but tended to decrease with increasing duration of occlusion (Fig. 3). Similarly, the systolic wall thickening in the normal region was not significantly different for the three groups but tended to decrease with increasing duration of occlusion. The percentage regional wall motion abnormality as determined twice by a single observer (intraobserver variability) was 9.9 ± 5.5 (first tracing) and 8.5 ± 5.7 (second tracing) when traced 48 hours apart.

DISCUSSION

The major finding of this study is that myocardial free radical production during coronary occlusion–reperfusion sequences is attenuated after longer periods of arterial occlusion. The ascorbate radical is formed as ascorbate, the terminal small-molecule antioxidant, reduces higher radicals.[6] The steady-state concentration of ascorbate radical is proportional to the total oxidative stress engendered by the occlusion–reperfusion sequences we performed.[5-7]

Table III. Changes in coronary venous ascorbate free radical concentration after 40 minutes of coronary artery occlusion and 30 minutes of reperfusion

	Dog								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*Mean*	*SD*
Coronary venous ascorbate free radical (nmol/L)									
End-occlusion	7.5	6.3	6.6	6.3	6.9	6.3	8.1	6.9	0.7
Minutes reperfused									
1	7.5	6.3	6.5	6.3	7.8	7.5	8.9	7.3	1.0
3	8.1	6.3	6.6	6.9	7.5	7.5	8.1	7.3	0.7
5	9.5	6.5	ND	7.2	8.4	7.5	8.2	7.9*	1.1
7	9.2	6.6	7.6	6.9	8.4	ND	8.1	7.8*	1.0
10	9.2	6.9	8.4	6.9	8.5	8.1	8.3	8.0*	0.9
15	8.5	7.1	7.2	7.2	8.6	8.4	8.1	7.9*	0.7
20	8.4	6.9	6.8	7.2	8.6	8.4	7.8	7.8*	0.8
25	8.1	7.2	7.9	6.8	7.8	8.1	7.8	7.7*	0.5
30	8.1	6.3	6.9	6.6	6.9	8.2	7.8	7.3	0.7
Percentage change in ascorbate free radical compared with end-occlusion									
Minutes reperfused									
1	0.0	0.0	-1.1	0.0	12.6	18.1	10.8	5.8	7.9
3	7.6	0.0	1.1	9.0	8.4	18.1	0.0	6.3	6.6
5	26.8	2.2	ND	13.6	20.8	18.3	1.9	13.9	10.2
7	23.1	4.4	16.5	9.0	20.8	ND	0.0	12.3	9.3
10	23.1	9.0	27.5	9.0	23.0	27.1	2.7	17.4	10.1
15	13.5	11.4	9.9	13.6	25.0	31.7	0.0	15.0	10.4
20	11.5	9.0	4.4	13.6	25.0	31.7	-3.5	13.1	12.0
25	7.6	13.6	20.9	6.8	12.6	27.1	-3.5	12.2	10.0
30	7.6	0.0	5.5	4.4	0.0	29.5	-3.5	6.2	11.0

*$p < 0.05$ vs end-occlusion. *ND*, No data obtained.

The method cannot identify specific radicals such as hydroxyl, superoxide, or lipid peroxyl radicals; however, the presence of any of these radicals can ultimately lead to ascorbate radical formation.

It is not clear which cell populations in the myocardium are responsible for free radical production after prolonged coronary artery occlusion–reperfusion sequences. Previous investigators have demonstrated that myocardial ischemia durations of ≤20 minutes result primarily in stunned but viable cells, whereas ischemia durations of >20 minutes result in some degree of myocardial infarction and nonviability.[8] Free radicals may originate from any of the following cell subpopulations: (1) viable cells that will survive reperfusion; (2) viable cells that will undergo lethal injury on reperfusion; and (3) nonviable cells that are being lysed by the reperfusion process. Our study does not allow us to distinguish precisely among these potential sources. However, the attenuation of free-radical production as myocardial ischemia duration is extended from 20 minutes to >40 minutes suggests that viable cells are required to produce free radicals. Our data, however, do not exclude the possibility that the free radical–mediated reperfusion injury continues to occur even after prolonged coronary occlusion. We also cannot differentiate between radicals generated by myocardial cells and those resulting from cellular elements of the blood.

Previous investigations of free radical production after various duration of coronary occlusion followed by reperfusion. Several previous investigations have measured myocardial free radical production with electron paramagnetic resonance spectroscopy, high-pressure liquid chromatography, and enhanced chemiluminescence in isolated hearts undergoing variable duration ischemia–reperfusion sequences.[9-11] Using an isolated perfused rat heart model and electron paramagnetic resonance spectroscopy, Kramer et al.[9] found that free-radical production increased in direct proportion to an increase in global ischemia duration from 20 to 40 minutes. Longer periods of ischemia, however, were not studied. Takemura et al.[10] measured free-radical production with high-pressure liquid chromatography in isolated perfused rat hearts. Free-radical production was maximal at 15 minutes of global ischemia and was found to be significantly decreased after 60 minutes of ischemia. Henry et al.[11] also studied free-radical production in isolated perfused rat hearts, by using enhanced chemiluminescence. Oxygen radical generation was maximal with 11.5 minutes of global ischemia and was attenuated when ischemia

Table IV. Changes in coronary venous ascorbate free radical concentration after 60 minutes of coronary artery occlusion and 30 minutes of reperfusion

	Dog									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*Mean*	*SD*
Coronary venous ascorbate free radical (nmol/L)										
End-occlusion	8.9	7.8	6.0	6.8	9.8	7.9	7.1	5.5	7.5	1.4
Minutes reperfused										
1	10.2	8.6	5.6	7.9	11.1	8.4	7.2	5.3	8.0	2.0
3	10.4	8.2	5.6	8.2	10.5	7.8	6.6	5.9	7.9	1.9
5	9.5	7.8	6.5	8.8	11.1	6.9	7.6	7.5	8.2	1.5
7	9.1	8.6	6.5	7.6	10.8	7.6	7.1	7.2	8.1	1.4
10	8.9	8.1	6.6	8.1	10.7	7.8	6.9	5.4	7.8	1.6
15	8.7	9.0	6.0	7.3	10.7	8.4	7.2	5.3	7.8	1.7
20	8.6	9.2	5.4	7.1	10.7	8.1	7.1	6.3	7.8	1.7
25	8.1	8.9	5.9	ND	10.4	7.8	ND	6.1	7.9	1.7
30	8.1	8.9	5.9	ND	10.5	7.6	ND	5.5	7.8	1.9
Percentage change in ascorbate free radical compared with end-occlusion										
Minutes reperfused										
1	14.6	10.3	6.7	16.2	13.3	6.3	1.4	-2.6	6.6	8.5
3	16.9	5.1	-6.7	20.6	7.1	-1.3	-7.0	7.9	5.3	10.1
5	6.7	0.0	8.3	29.4	13.3	-12.7	7.0	36.9	11.1	15.8
7	2.3	10.3	8.3	11.8	10.2	-3.8	0.0	31.6	8.8	10.8
10	0.0	3.9	10.0	19.1	9.2	-1.3	-2.8	0.0	4.8	7.5
15	-2.3	15.4	0.0	7.4	9.2	6.3	1.4	-2.6	4.4	6.3
20	-3.4	18.0	-10.0	4.4	9.2	2.5	0.0	15.9	4.6	9.5
25	-9.0	14.1	-1.7	ND	6.1	-1.3	ND	10.6	3.2	8.7
30	-9.0	14.1	-1.7	ND	7.1	-3.8	ND	0.0	1.1	8.3

ND, No data obtained.

duration was extended to 40.8 minutes. The findings of these investigators are consistent with the results of our study.

Other investigators have measured free-radical production in whole animals undergoing variable duration coronary artery ischemia–reperfusion sequences. Arroyo et al.[12] showed that the concentration of conjugated dienes (lipid peroxidation products) increased progressively with up to 90 minutes of regional ischemia in a canine model. Conjugated dienes accumulate during lipid peroxidation. Whether transient free-radical production also was increased after prolonged ischemia–reperfusion was not determined. Mergner et al.[13] performed an electron paramagnetic resonance spectrum study in an intact porcine model and various durations of ischemia to as long as 90 minutes. Spin trapping ex vivo was carried out by using α-phenyl-tert-butylnitrone. The total postischemic α-phenyl-tert-butylnitrone adduct production increased with increasing duration of ischemia. Peak adduct production for the 30- and 40-minute–ischemia groups occurred at about 20 to 30 minutes of reperfusion, which is later than that for the 15-minute ischemia group. These findings are different from those in the current study but may be explained by differences in experimental models and techniques.

Interventions to modulate free-radical production. Many previous investigators have measured regional myocardial function or infarct size after ischemia–reperfusion sequences to assess the potential benefit offered by an antioxidant intervention: administration of free-radical scavenging agents,[14-27] neutrophil suppression or depletion,[28-36] or xanthine oxidase inhibition.[37-41] The results of these studies have been conflicting, demonstrating myocardial benefit as a result of free-radical modulation in some instances and no benefit in others. These discrepant findings may be related to a variety of factors including differences in experimental protocol; doses and preparations of free-radical scavenging agents; laboratory animal variabilities; methods of assessing myocardial injury; statistical analyses; and ischemia–reperfusion durations.[42-44] When early reperfusion is studied, benefit from these interventions is uniformly noted.[14-21, 28-40] Naslund et al.[45] performed a study in closed-chest pigs undergoing variable duration coronary artery occlusions for 30, 60, and 90 minutes and 24 hours of reperfusion. Pigs given superoxide dismutase were compared with a control

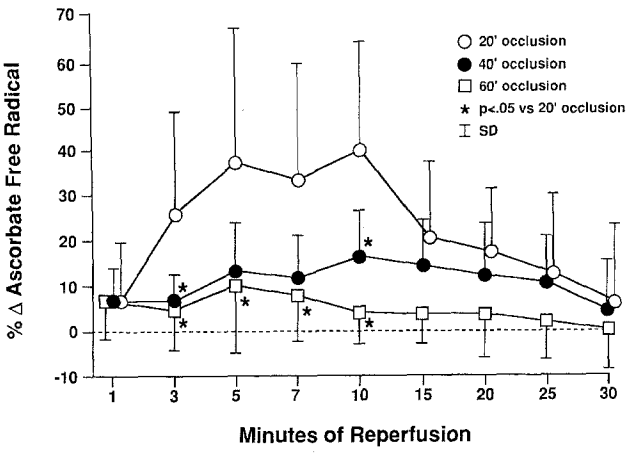

Fig. 1. Percentage change in ascorbate free radical above end-occlusion values during reperfusion in dogs undergoing various durations of coronary artery occlusions. Longer durations of occlusion were followed by smaller percentage increase in ascorbate free radical after reperfusion. *Open circles*, 20-minute occlusion group ($n = 7$); *solid circles*, 40-minute occlusion group ($n = 7$); *open squares*, 60-minute occlusion group ($n = 8$). *Significant difference ($p < 0.05$ vs 20-minute group by analysis of variance) between groups at different stages of reperfusion. *Error bars*, 1 SD.

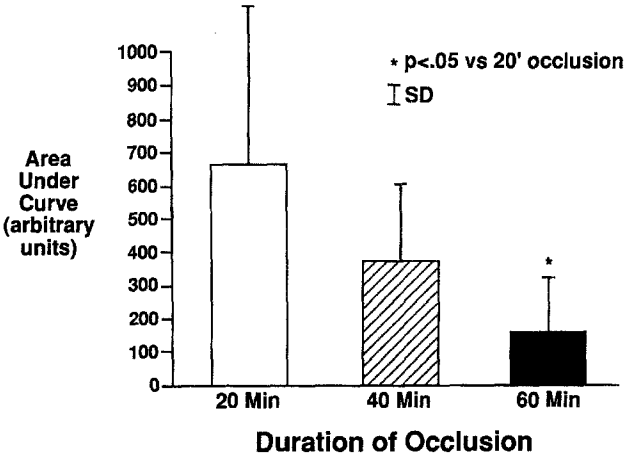

Fig. 2. Area under curve of percentage change in ascorbate free radical concentration versus time for three occlusion groups. Longer durations of occlusion resulted in less free radical generation after reperfusion. *Significant difference ($p < 0.05$ vs 20-minute group by analysis of variance).

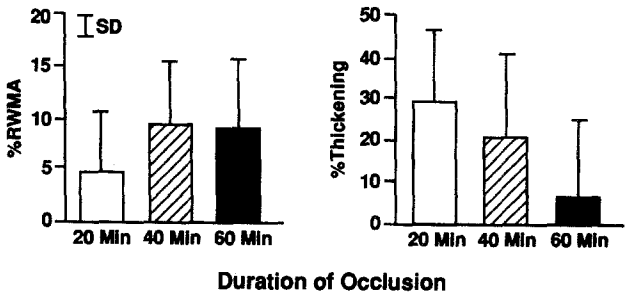

Fig. 3. Percentage regional wall motion abnormalities *(%RWMA)* and percentage systolic thickening in ischemic region after various durations of coronary artery occlusion and 30 minutes of reperfusion. Although differences are not significant, there is trend toward more extensive wall motion abnormality and reduced systolic thickening after longer durations of coronary occlusion.

group of animals. Infarct size was limited by superoxide dismutase in the 30- and 60-minute–occlusion groups but not in the 90-minute–occlusion group when compared with control animals.[45] This finding is consistent with diminished myocardial free radical production after prolonged coronary artery occlusion–reperfusion sequences.

Functional effects of various durations of occlusion–reperfusion and relation to ascorbate free radical generation. We performed echocardiography to evaluate the functional effects of the occlusion–reperfusion sequences on myocardial contractility and to correlate these effects with the ascorbate radical generation. Although the differences do not achieve statistical significance, the trend is clear: longer durations of occlusion preceding reperfusion were associated with more extensive and severe disturbances of ventricular contractility. Because ascorbate radical generation was attenuated when reperfusion occurred after longer durations of occlusion, these data suggest that the injury occurring after the longer durations resulted primarily not from the transient burst of free-radical production on reperfusion but rather from other metabolic and perhaps inflammatory processes.

Study limitations. It is possible that defibrillation in the dogs having ventricular fibrillation in the current study may have affected myocardial free-radical production. However, preliminary data from our laboratory demonstrate that direct current shocks to the myocardium actually increase ascorbate free-radical production.[46] A greater number of dogs in the 60-minute–occlusion group required defibrillation than in the 40- and 20-minute–occlusion groups. If free-radical production were substantially altered by defibrillation, the 60-minute–occlusion group would have been expected to have the highest ascorbate free-radical generation. In fact, the 60-minute–occlusion group had the lowest ascorbate free-radical generation. In this experiment, the duration of coronary artery occlusion seems to have been a more important determinant of ascorbate radical generation than defibrillation.

We did not perform collateral blood flow measure-

ments in our study and therefore cannot be certain that differences in ascorbate free-radical production observed were not due to random differences in myocardial collateral blood flow. Furthermore, it is possible that the blood sampled from the great cardiac vein may have been variably diluted by blood from surrounding nonischemic regions of the myocardium. However, tissue cyanosis, ST-segment changes, and regional wall motion abnormalities on echocardiography were noted to persist in the myocardial regions subjected to ischemia for as long as the coronary artery occlusion was maintained; the tissue cyanosis and ST-segment changes resolved or improved promptly with reperfusion. This observation suggests that there were not major changes in collateral blood flow to the ischemic regions during coronary occlusion. Other investigators, using a similar dog model, have shown only minimal changes in subendocardial and transmural blood flow in ischemic regions measured initially after 10 minutes of coronary occlusion and then repeated at 80 minutes,[25] 105 minutes,[47] or 150 minutes[22] of coronary occlusion. Therefore we believe it is unlikely that differences in collateral blood flow account for reduction in ascorbate free-radical generation after the longer coronary artery occlusions.

With longer durations of coronary artery occlusions the "no-reflow" phenomenon may have occurred, such that the more severely injured areas of the myocardium may not have been adequately reperfused. As a result, less ascorbate free radical from those areas may have been washed into the coronary venous effluent. Investigation of this possibility would necessitate serial measurements of ascorbate free radical in the myocardial tissue itself. This measurement would involve tissue sampling and processing techniques not currently developed for our ascorbate radical experimental system.

In this study, heparin was used to prevent blood clotting in the cannulas and tubing required to conduct the experiment. Heparin has been shown to inhibit activity of several pathways of complement, and complement inhibition may modify reperfusion injury.[48-50] Whether the heparin used in this study could have modified the free radical response to varying periods of coronary occlusion and resultant myocardial stunning is not known, and consideration of this possibility is beyond the scope of this paper. Whatever effect the heparin may have had in this regard would have been similar between the three groups of animals studied.

Clinical implications. The current study suggests that during early reperfusion, myocardial free-radical production after ischemic sequences is decreased with prolonged (greater than 20 to 40 minutes) coronary artery occlusion. Therefore the greatest benefit afforded by antioxidant therapy for myocardial ischemic events may be confined to the initial 20 minutes of ischemia. This possibility is consonant with conclusions from animal studies in which antioxidant therapy seems to have a time-limited benefit and is most effective for shorter duration ischemia –reperfusion sequences.

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