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MK Sharma, GR Buettner, KT Spencer and RE Kerber

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Ascorbyl Free Radical as a Real-time Marker of Free Radical Generation in Briefly Ischemic and Reperfused Hearts

An Electron Paramagnetic Resonance Study

Mukesh K. Sharma, Garry R. Buettner, Kirk T. Spencer, Richard E. Kerber

Abstract The role of free radicals in myocardial reperfusion injury remains controversial. We have developed a new method using ascorbyl free radical (AFR) as a real-time, quantitative marker of free radical generation during myocardial reperfusion. A total of 35 dogs were studied. Twelve open-chest dogs underwent either 5 minutes (n=5) or 20 minutes (n=7) of coronary artery occlusion and 30 minutes of reperfusion. Seven additional animals undergoing 20 minutes of coronary occlusion also received the antioxidant enzymes superoxide dismutase and catalase, beginning 10 minutes before occlusion through the end of reperfusion. Exogenous ascorbate was infused intravenously, and the concentration of AFR in the great cardiac vein was continuously measured by electron paramagnetic resonance spectroscopy. Preocclusion AFR concentration was similar in the three groups. Upon reperfusion, AFR rose significantly in each animal group ($P<.05$). However, the AFR rise in the 20-minute-occlusion group, 38±17%, was significantly greater than in the 5-minute-occlusion group, 27±14% ($P<.002$). In addition, in the animals that received superoxide dismutase and catalase, the rise in the AFR was markedly attenuated, 13±6% ($P<.002$). Two dogs that received ascorbate but did not undergo coronary artery occlusion/reperfusion sequences showed no change in coronary venous AFR signal, indicating the stability of the signal over time. Five dogs received ascorbate while undergoing interventions to alter coronary venous flow: intravenous saline, dobutamine, dipyridamole, and nitroglycerin. Coronary venous AFR changes were minimal despite large coronary flow alterations, indicating that the AFR signal is independent of changes in coronary venous flow. An additional 5 dogs, which underwent a 20-minute occlusion/reperfusion sequence without receiving ascorbic acid infusion, showed myocardial dyskinesis at 30 minutes after reperfusion that was similar in extent and severity to that in the dogs that received ascorbic acid for AFR measurement, suggesting that exogenous ascorbate at the doses given does not attenuate stunning during the initial minutes of reperfusion. In 4 dogs undergoing prolonged (60-minute) coronary occlusion, the rise in AFR was attenuated, 13±9% versus 38±17% in the 20-minute coronary occlusion group ($P<.05$), possibly as a result of necrosis of the radical-generating cellular components. We conclude that AFR is a reliable, real-time, quantitative marker of free radical generation during myocardial reperfusion after brief coronary occlusion. (***Circ Res.* 1994;74:650-658.**)

Key Words • ascorbyl free radical • free radicals • myocardium • reperfusion • electron paramagnetic resonance • ischemia • stunning

Reperfusion of the myocardium after a brief period of ischemia has been shown to result in cellular damage and dysfunction.[1] It has been hypothesized that free radicals play a central role in the production of this damage.[2-6] However, this remains controversial. The chemical nature of free radicals makes them extremely reactive and short-lived, which renders their direct detection and quantification very difficult. As a result, until recently, the support for the free radical hypothesis of myocardial ischemia/reperfusion injury has been indirect, that is, either by in vitro tests for lipid peroxidation[7,8] or by showing myocardial protection with antioxidants, such as superoxide dismutase (SOD) and catalase.[9-12] Recently, with the use of "spin-traps," free radical generation has been demonstrated by electron paramagnetic resonance (EPR) spectroscopy.[13-16] Spin-traps are exogenously administered chemical probes that react with free radicals to form stable nitroxide compounds called spin adducts.[17] These spin adducts are then quantified by EPR after in vitro processing of blood samples. Although spin-trapping provides an estimate of free radical generation, the sample processing is tedious and time-consuming. Moreover, this technique does not detect free radicals directly during myocardial reperfusion in real time.

For the present study, we have developed a new EPR method that allows real-time, ex vivo quantification of free radical generation during myocardial reperfusion. The method uses the steady-state ascorbyl free radical (AFR) concentration as a marker and does not require any spin-traps.

Vitamin C is widely distributed in the body as ascorbate monoanion. Ascorbate is a reducing agent that is capable of readily donating electrons to repair highly reactive free radicals, such as superoxide, hydroxyl, and lipid peroxyl radicals.[18] In doing so, however, ascorbate is itself converted into a free radical, AFR (Fig 1). The unpaired electron in the AFR molecule is delocalized

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From the Cardiovascular Division (M.K.S., K.T.S., R.E.K), Department of Internal Medicine, and ESR Center (G.R.B.), College of Medicine, the University of Iowa, Iowa City.

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Correspondence to Richard E. Kerber, MD, Department of Medicine, University of Iowa Hospital, 200 Hawkins Drive, Iowa City, IA 52242.

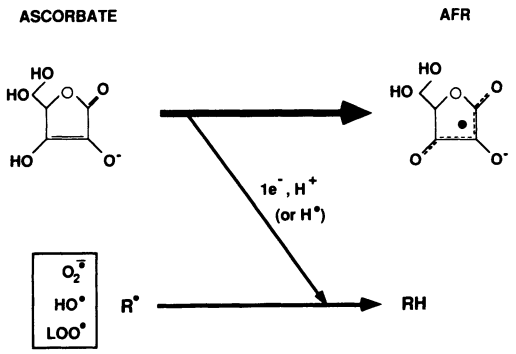

FIG 1. Diagram of reaction showing the conversion of ascorbate monoanion to ascorbyl free radical (AFR).

over a highly conjugated tricarbonyl system, which makes it a resonance-stabilized radical with very low reactivity.[18,19] These properties extend the lifetime of this radical, allowing its direct detection by EPR spectroscopy.[20,21] In our laboratory, we have demonstrated that in biochemical systems, at a given ascorbate level and fixed pH, the concentration of AFR directly correlates with the magnitude of free radical oxidative stress.[22]

Using an open-chest canine model of myocardial ischemia/reperfusion, we performed an EPR study with the objective of determining whether AFR could serve as a marker of free radical generation in vivo. Three sets of experiments were performed. The purpose of the first set was to determine the effect of duration of myocardial ischemia on the magnitude of AFR generation, and that of the second set, to demonstrate the effect of the antioxidant enzymes SOD and catalase on the amount of AFR produced. The third set was designed to establish the relation, if any, between coronary sinus flow and AFR, the stability of the AFR signal over time, and the possible protective effect of exogenous ascorbate.

Materials and Methods

Animal Preparation

An open-chest canine model of ischemia was used. General anesthesia was achieved with fentanyl-droperidal (0.13 mL/kg), followed by intravenous pentobarbital (20 mg/kg), which was supplemented as necessary. Respiratory support was provided with a volume-cycled respirator after endotracheal intubation. Tidal volume, rate, and F_{IO_2} were adjusted according to arterial blood gases to maintain physiological arterial pH and P_{O_2}.

Femoral arteries and veins were cannulated in both limbs. The left femoral artery was used to monitor blood pressure, and the left femoral vein was used for infusions. Through a left lateral thoracotomy, the pericardial sac was opened and the heart was suspended in a cradle. The left anterior descending coronary artery (LAD) was isolated in its most proximal portion, and the great cardiac vein was cannulated in its most distal portion. A snare was placed around the isolated portion of the LAD to perform coronary occlusions. A venous-venous shunt was fashioned between the great cardiac vein and the right femoral vein.

EPR Methods

A Varian E-4 spectrometer, with a TM_{110} cavity and an aqueous flat cell, was used to monitor AFR (see Fig 2). The lower end of the flat cell was connected to the coronary venous cannula using thin (0.05-mm outer diameter) Teflon tubing, and its upper end was connected to the right femoral vein.

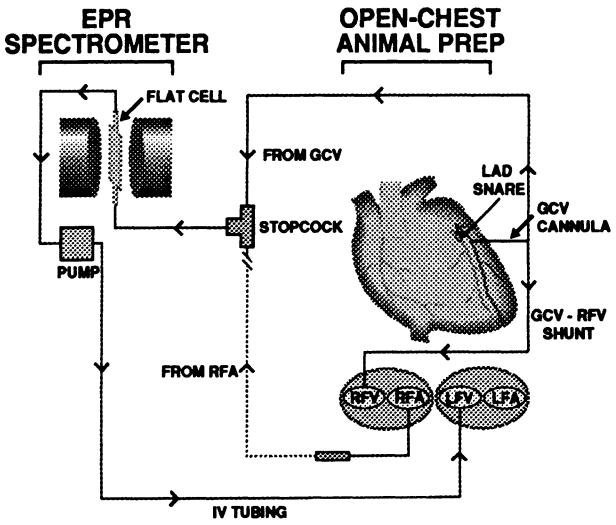

FIG 2. Schematic of the experimental preparation. EPR indicates electron paramagnetic resonance spectrometer; RFA, right femoral artery; RFV, right femoral vein; LFA, left femoral artery; LFV, left femoral vein; LAD, left anterior descending coronary artery; GCV, great cardiac vein; and IV, intravenous. Arrows indicate direction of blood flow.

Thus, coronary venous blood could be continuously withdrawn from the heart, scanned for AFR in the EPR spectrometer, and returned to the animal. Similarly, with a simple turn of the stopcock, the EPR sampling could be switched from coronary venous to femoral arterial blood. With this experimental setup, the blood could be scanned for AFR within 4 seconds of leaving either the coronary vein or the femoral artery.

The EPR spectrum of AFR is shown in Fig 3. The following EPR instrument settings were used to acquire optimum AFR spectra: nominal power, 40 mW; modulation amplitude, 0.63 G; time constant, 1 second; and scan rate, 1 G per 24 seconds.[23]

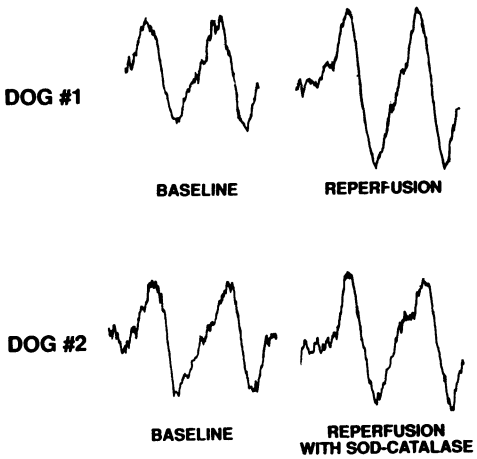

FIG 3. Tracings showing morphology of electron paramagnetic resonance (EPR) spectrum of ascorbyl free radical (AFR). At our spectrometer settings, AFR is a doublet signal with a^H=1.8 G. Signal height was measured. The peak-to-peak signal height is directly proportional to the concentration of AFR in the sample. The actual concentration of AFR was determined as previously described using 3-carboxyproxyl as a standard,[21] accounting for saturation effects.[23] For our experimental conditions, 1 mm of normalized AFR signal height =0.073 nmol/L AFR in the blood being sampled. The morphology of the EPR spectrum was not changed by the addition of superoxide dismutase (SOD) and catalase.

The ascorbate radical concentration was determined using 3-carboxy proxyl (Aldrich Chemical Co, Milwaukee, Wis) as a standard.[21] Briefly, the ascorbate radical concentration was determined from signal height measurements. Standardization was accomplished by use of identical physical arrangements of the samples and identical instrument settings, except for receiver gain. After saturation effects were accounted for,[23] when appropriate, we found that for our experimental conditions, 1 mm of signal height corresponds to 0.073 nmol/L AFR; all signal heights were normalized to full gain of the instrument, 10^5.

Normally, the concentration of AFR in the whole canine blood is too low for detection by EPR under the constraints of our need to examine AFR concentration versus time. Therefore, after the experimental preparation, ascorbic acid was infused intravenously to amplify the endogenous AFR signal. The infusion was begun 30 minutes before the expected initiation of LAD occlusion. While developing this technique, we learned that the level of arterial AFR could be kept constant by very-low-dose supplementation with intravenous ascorbate (the range being 7.6 to 30.4 mg/min after an initial 1-g slow intravenous bolus). Once a stable AFR concentration had been reached, the rate of ascorbate infusion needed only minor adjustment in a given animal. After a steady arterial AFR concentration was reached (approximately 16±4 nmol/L), the monitoring was switched to coronary venous blood. The ascorbate infusion was then fine-tuned to achieve a stable coronary venous AFR concentration (8±3 nmol/L). Consecutive EPR scans of the coronary venous AFR signal were collected approximately every 90 seconds. In addition, intermittent EPR scans for arterial AFR were obtained to confirm the stability of systemic AFR concentration.

Echocardiography

Two-dimensional echocardiography, using an Advanced Technology Laboratories Ultramark-4 device with a 5-MHz transducer, was performed on each dog at baseline, during occlusion, and at 30 minutes of reperfusion. Right parasternal short-axis views were obtained at the left ventricular (LV) midpapillary level. The portion of LV wall showing endocardial wall motion abnormality (severe hypokinesis, akinesis, or dyskinesis) after reperfusion was measured by planimetry, as was the total myocardial area. This area of myocardial stunning was then expressed as a percentage of the total area of the myocardium. In five dogs that did not receive ascorbate and six dogs that did receive ascorbate, we also measured systolic thickening in the center of the abnormally contracting myocardium and, for comparison, in the opposite never-ischemic myocardium. To do this we measured end-diastolic and end-systolic thickness and derived percent thickening by the formula

$$\frac{\text{End-Diastolic Thickness} - \text{End-Systolic Thickness}}{\text{End-Diastolic Thickness}} \times 100$$

A negative number indicates systolic thinning.

Protocol

Experiment 1

After the completion of preocclusion monitoring, 5 minutes (five dogs) or 20 minutes (seven dogs) of coronary occlusion was performed with the preplaced LAD snare. This was followed by 30 minutes of reperfusion. Coronary occlusion was verified by the development of ECG changes, epicardial cyanosis, and systolic bulging of the myocardium perfused by the occluded artery.

Experiment 2

A group of seven dogs received the antioxidant enzymes SOD and catalase during a 20-minute occlusion/reperfusion sequence. Through a left atrial catheter, the animals received a combination of 15 000 U/kg SOD (bovine erythrocyte SOD, 4200 U/mg protein, Sigma Chemical Co, St Louis, Mo) and 55 000 U/kg catalase (bovine liver suspension, 58 000 U/mg protein, Sigma) as a continuous infusion over a period of 1 hour, beginning 10 minutes before occlusion through the end of the 30-minute reperfusion period.

Experiment 3

Twelve dogs were studied to learn more about the characteristics of ascorbyl free radical and its effect on myocardial stunning.

Experiment 3a. To demonstrate the stability of AFR concentration in coronary venous blood over time and without an ischemia/reperfusion sequence, we conducted a sham occlusion study on two control dogs that underwent open-chest preparation, including the isolation of the LAD and placement of a snare around it. Ascorbate infusion and the measurement of AFR were performed as in the previous experiments. However, LAD occlusion was not performed. The AFR measurements were continued for a total of 50 minutes after the preparation was stabilized, equivalent to the 20-minute occlusion and 30-minute reperfusion sequence of experiment 1.

Experiment 3b. Next, to study the effect of exogenous ascorbic acid on the extent of myocardial stunning, we subjected five additional dogs to a 20-minute LAD occlusion/30-minute reperfusion protocol, without the infusion of exogenous ascorbate during the study. The circumferential extent of myocardial dyskinesis and presence or absence of systolic thickening after reperfusion were measured by echocardiography and compared with similar measurements in the dogs in experiment 1, which underwent a similar 20-minute occlusion/30-minute reperfusion while receiving exogenous ascorbate.

Experiment 3c. To demonstrate that AFR production in myocardium was independent of changes in coronary venous flow, five dogs underwent interventions designed to increase or decrease coronary venous flow to observe the effect of an acute increase in blood flow on AFR. These interventions included a bolus of normal saline (250 cm^3), intravenous dipyridamole (15 mg IV), and intravenous infusions of dobutamine (10 $\mu g \cdot kg^{-1} \cdot min$) and nitroglycerin (100 μg/min). AFR was monitored as before. Coronary venous flow was measured by collecting coronary venous drainage from the coronary venous cannula over a 2-minute period during the peak hemodynamic effect of each intervention.

Experiment 4

Finally, to evaluate the effect of prolonged ischemia on AFR generation, four dogs underwent 60 minutes of coronary occlusion followed by 30 minutes of reperfusion. Ascorbate infusion and AFR monitoring were performed as in experiment 1.

Statistical Analysis

In the three groups of animals of experiments 1 and 2, the baseline and peak values of AFR concentration in coronary venous blood were compared by paired t test. Repeated-measures analysis of variance was used to analyze raw AFR data in nanomolar concentrations as well as the data expressed as a percent increase over baseline. The overall analysis was followed by pairwise comparisons between groups at each time point using a Bonferroni correction to control the overall experimental error at 0.05.[24] The results are expressed as mean±SD.

Results

Experiment 1

The data on changes in coronary venous AFR concentration during ischemia/reperfusion sequences in the three groups of dogs from experiments 1 and 2 are presented in Tables 1 through 3 and summarized in Figs 4 through 6. The baseline (preocclusion) concentrations

TABLE 1. Changes in Coronary Venous AFR Concentration After 5 Minutes of Coronary Occlusion and Reperfusion

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Mean	SD
Coronary venous AFR (nM)							
Preocclusion	4.53	9.34	6.42	6.13	7.30	6.75	1.77
Minutes of reperfusion							
1	5.10	11.80	5.50	7.30	7.40	7.42	2.66
3	6.10	13.90	7.60	7.60	8.20	8.68	3.02
5	5.80	9.60	7.20	6.60	8.90	7.62	1.59
7	5.70	9.10	6.40	6.70	7.90	7.16	1.34
10	5.00	9.30	6.30	6.00	7.70	6.86	1.67
15	4.50	8.80	6.30	5.50	7.30	6.48	1.66
20	3.80	8.80	6.30	5.80	7.00	6.34	1.82
25	3.70	8.80	6.10	6.00	7.20	6.36	1.87
30	3.70	8.80	6.00	5.70	7.30	6.30	1.90
Percent change in AFR concentration							
Minutes of reperfusion							
1	12.68	26.28	−14.38	19.05	1.37	9.00	15.96
3	34.78	48.76	18.31	23.94	12.33	27.62	14.42
5	28.15	2.74	12.08	7.63	21.92	14.50	10.40
7	25.94	−2.61	−0.37	9.26	8.22	8.09	11.25
10	10.47	−0.47	−1.93	−2.15	5.48	2.28	5.53
15	−0.57	−5.82	−1.93	−10.31	0.00	−3.73	4.32
20	−16.04	−5.82	−1.93	−5.41	−4.11	−6.66	5.46
25	−18.25	−5.82	−5.04	−2.15	−1.37	−6.53	6.82
30	−18.25	−5.82	−6.60	−7.05	0.00	−7.54	6.63

AFR indicates ascorbyl free radical.

were similar in the three groups of animals studied (see Table 1). On reperfusion, the AFR concentration rose significantly ($P<.05$) in each group. In the 5-minute-occlusion group, the peak AFR rise, 27±14%, was reached at 3 minutes of reperfusion, returning to preocclusion levels within 15 minutes of reperfusion. In comparison, the rise of AFR in the 20-minute-occlusion group was greater, reaching a peak of 38±17% at 7 minutes of reperfusion and returning to preocclusion levels only after 30 minutes of reperfusion ($P<.002$). On pairwise comparisons, the rise in coronary venous AFR concentration in the two groups was significantly different at 7 and 10 minutes of reperfusion ($P<.002$) (Fig 4). The baseline arterial concentration of AFR, on the other hand, was similar in the three groups of dogs of experiments 1 and 2 (16±4, 16±4, and 15±4 nmol/L, respectively, P=NS) and showed no significant rise during the occlusion/reperfusion sequences. After 30 minutes of reperfusion, the 5-minute-occlusion group had no residual wall motion abnormality by echocardiography, whereas each dog in the 20-minute-occlusion group had persistent wall motion abnormality in the form of hypokinesis, akinesis, or dyskinesis.

Experiment 2

In the 20-minute coronary occlusion animals that received SOD and catalase, the rise in AFR concentration from preocclusion levels was only 13±6% versus 38±17% in the 20-minute-occlusion group that did not receive these antioxidant enzymes ($P<.002$). Once again, on pairwise comparisons, the differences between the two groups were significant at 7 and 10 minutes of reperfusion ($P<.002$) (Fig 5).

When the area under the curve of percent change in AFR was analyzed for each animal in the three groups, the differences in the magnitude of AFR generation over a 30-minute period were even more pronounced. As shown in Fig 6, AFR generation was significantly greater in the 20-minute-occlusion group than in the 5-minute-occlusion group (440±236% versus 134±73%; $P<.005$). In addition, SOD and catalase markedly attenuated the AFR rise after 20 minutes of coronary occlusion (136±94% versus 440±236%; $P<.005$).

Experiment 3a

In the sham occlusion dogs that underwent no ischemia/reperfusion sequence, the AFR concentration showed minimal change over the 50-minute period of observation (maximum change ±0.4 nmol/L or 6.7% versus 38±17% peak rise in the 20-minute-occlusion dogs of experiment 1).

Experiment 3b

By echocardiography, the area of postreperfusion severe hypokinesis, akinesis, or dyskinesis (stunning) after 20 minutes of coronary occlusion and 30 minutes of reperfusion was 16±1% in the animals that received exogenous ascorbate and 11±4% (P=NS) in the ani-

TABLE 2. Changes in Coronary Venous AFR Concentration After 20 Minutes of Coronary Occlusion and Reperfusion

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Mean	SD
Coronary venous AFR (nM)									
Preocclusion	6.72	8.32	4.96	6.60	3.70	8.80	7.60	6.67	1.82
Minutes of reperfusion									
1	7.01	11.39	5.84	7.30	3.40	11.40	8.00	7.76	2.89
3	7.88	10.22	7.01	8.20	3.50	11.70	8.80	8.19	2.59
5	7.88	9.93	7.88	9.20	4.40	11.10	8.90	8.47	2.12
7	8.47	10.80	7.30	8.20	6.40	11.70	10.10	9.00	1.93
10	7.59	11.10	7.30	8.50	4.20	11.40	8.20	8.33	2.44
15	7.67	9.93	6.42	6.70	3.80	9.60	7.90	7.43	2.08
20	6.72	9.64	6.13	6.60	3.90	8.50	7.70	7.03	1.84
25	7.01	8.91	5.84	6.60	3.50	8.20	8.30	6.91	1.85
30	6.72	7.74	5.55	6.70	3.10	7.90	7.70	6.49	1.71
Percent change in AFR concentration									
Minutes of reperfusion									
1	4.35	36.84	17.65	10.61	−8.11	29.55	5.26	13.73	15.52
3	17.39	22.81	41.18	24.24	−5.41	32.95	15.79	21.28	14.72
5	17.39	19.30	58.82	39.39	18.92	26.14	17.11	28.15	15.67
7	26.09	29.82	47.06	24.24	72.97	32.95	32.89	38.01	17.10
10	13.04	33.33	47.06	28.79	13.51	29.55	7.89	24.74	13.89
15	14.13	19.30	29.41	1.52	2.70	9.09	3.95	11.44	10.23
20	0.00	15.79	23.53	0.00	5.41	−3.41	1.32	6.09	9.88
25	4.35	7.02	17.65	0.00	−5.41	−6.82	9.21	3.71	8.60
30	0.00	−7.02	11.76	1.52	−16.22	−10.23	1.32	−2.70	9.21

AFR indicates ascorbyl free radical.

mals that did not receive ascorbate. As expected, coronary venous AFR signals were virtually undetectable ($<\approx 1$ nmol/L with our instrumental conditions) in the animals that received no exogenous ascorbate.

Experiment 3c

The coronary blood flow in response to the various interventions varied by 60±32% (range, −51% to +133%), whereas the coronary venous AFR varied by only 6±3% (range, −9% to +8%). This change in AFR was random without relation to the change in flow ($r=.24$) or the specific drug intervention used.

Experiment 4

After 60 minutes of occlusion, the peak rise in AFR during the reperfusion period was 13±9% versus 38±17% in the 20-minute-occlusion group ($P<.05$). Echocardiography showed persistent wall motion abnormalities in all four dogs.

Discussion

Our study demonstrates that AFR rises reproducibly after an occlusion/reperfusion sequence. The magnitude of AFR rise during reperfusion depends on the duration of coronary occlusion; AFR rise was greater after a 20-minute occlusion than after a 5-minute occlusion. The greater rise in AFR was also accompanied by persistent dyskinesis, indicating myocardial stunning. We showed further that the antioxidant enzymes SOD and catalase markedly attenuated the increase in AFR concentration during reperfusion. These observations strongly suggest that the change in AFR concentration in coronary venous blood can be used as a real-time marker of free radical generation during myocardial ischemia/reperfusion.

The study also demonstrates the stability of AFR concentration over time in the absence of ischemia/reperfusion, independent of the changes in coronary blood flow.

Initial studies directed at establishing a causal relation between postischemic dyskinesis and free radicals used scavengers or agents that would prevent the formation of free radicals to reduce reperfusion injury.[4,5] Such evidence was indirect. With the use of spin-trapping, several investigators have demonstrated the release of free radicals during postischemic reperfusion in isolated hearts and Langendorff preparations.[13,16] Bolli et al[14] were the first to show a temporal correlation between free radical production and postischemic dyskinesis in intact animals by using the EPR spin-trapping technique; the spin-trap *N-tert*-phenylbutyl nitrone was infused into the coronary artery. Weglicki and Kramer have published somewhat similar results by simple mixing of *N-tert*-phenylbutyl nitrone with samples of the coronary sinus blood.[25,26]

Spin-traps are chemical compounds that may have significant cardiac effects[27] when given intracoronary or systemically, especially at the higher concentrations

TABLE 3. Changes in Coronary Venous AFR After 20 Minutes of Coronary Occlusion and Reperfusion After Superoxide Dismutase Catalase

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Mean	SD
Coronary venous AFR (nM)									
Preocclusion	15.00	8.60	9.60	6.20	5.80	5.80	8.80	8.54	3.25
Minutes of reperfusion									
1	14.70	9.60	11.30	5.00	5.10	5.50	8.20	8.49	3.66
3	14.90	12.00	13.10	4.80	5.00	5.80	8.50	9.16	4.17
5	17.50	10.40	12.10	5.80	5.80	6.10	10.20	9.70	4.30
7	17.50	10.50	11.00	6.90	6.40	5.90	9.90	9.73	4.01
10	17.30	8.80	7.50	6.50	6.60	5.80	9.30	8.83	3.94
15	16.10	8.30	8.10	6.00	6.30	5.50	8.10	8.34	3.61
20	14.90	8.50	8.10	5.80	6.70	5.50	7.90	8.20	3.17
25	13.90	8.80	8.00	5.50	6.10	5.30	7.90	7.93	2.96
30	13.70	8.80	8.90	5.40	6.70	4.80	7.90	8.03	2.96
Percent change in AFR concentration									
Minutes of reperfusion									
1	−2.00	11.63	17.71	−19.35	−12.07	−5.17	−6.82	−2.30	12.97
3	−0.67	39.53	36.46	−22.58	−13.79	0.00	−3.41	5.08	23.89
5	16.67	20.93	26.04	−6.45	0.00	5.17	15.91	11.18	11.83
7	16.67	22.09	14.58	11.29	10.34	1.72	12.50	12.74	6.27
10	15.33	2.33	−21.88	4.84	13.79	0.00	5.68	2.87	12.30
15	7.33	−3.49	−15.63	−3.23	8.62	−5.17	−7.95	−2.79	8.47
20	−0.67	−1.16	−15.63	−6.45	15.52	−5.17	−10.23	−3.40	9.82
25	−7.33	2.33	−16.67	−11.29	5.17	−8.62	−10.23	−6.66	7.74
30	−8.67	2.33	−7.29	−12.90	15.52	−17.24	−10.23	−5.50	11.04

AFR indicates ascorbyl free radical.

required to achieve a detectable EPR signal. Moreover, the spin-trap technique cannot provide on-line temporal and quantitative information, because elaborate processing of plasma samples is necessary for successful EPR analysis of spin adducts. During this processing, chain reactions, including lipid peroxidation within the samples, cannot always be completely prevented. By using AFR as a marker of free radical production, we have overcome this limitation of the spin-trapping technique.

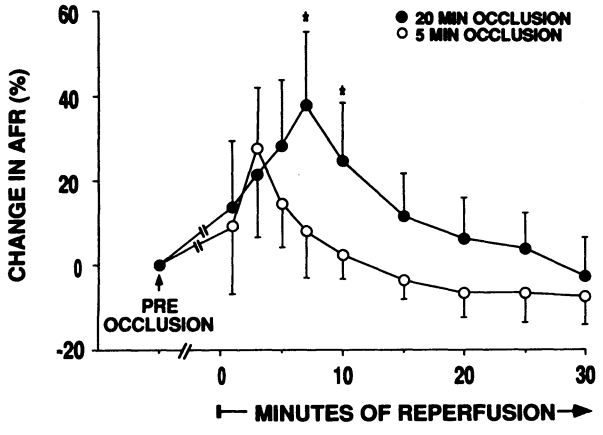

FIG 4. Graph showing percent change in ascorbyl free radical (AFR) concentration during reperfusion in animals undergoing 5 minutes versus 20 minutes of left anterior descending coronary artery occlusion. There is a significant ($P<.002$) overall difference between the two groups. *Significant difference ($P<.002$) on pairwise comparisons between the two groups at different stages of reperfusion. Error bars represent 1 SD.

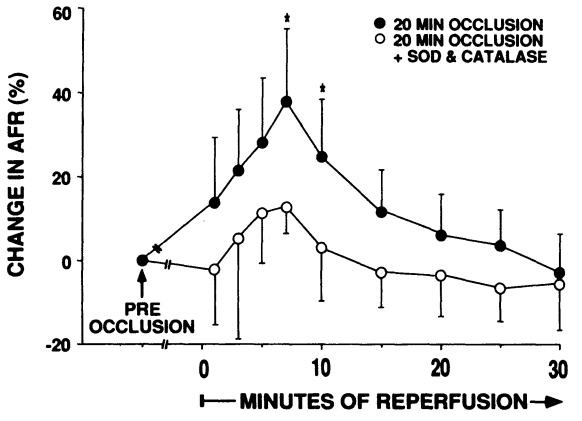

FIG 5. Graph showing percent change in ascorbyl free radical (AFR) concentration during reperfusion in animals undergoing 20 minutes of left anterior descending coronary artery occlusion with and without superoxide dismutase (SOD) and catalase. *Significant difference ($P<.002$) between the two groups on pairwise comparisons at different stages of reperfusion. Error bars represent 1 SD.

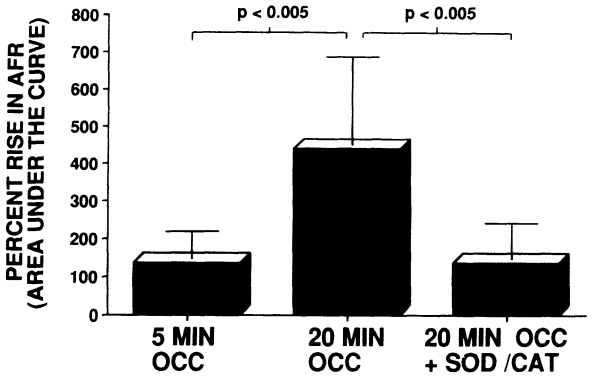

FIG 6. Bar graph showing area under the curve of percent increase in ascorbyl free radical (AFR) concentration during reperfusion. Each bar represents the mean and SD for that group. OCC indicates occlusion; SOD, superoxide dismutase; and CAT, catalase.

The technique described in this study evolved through a series of experimental developments in our laboratory. We were unable to detect an AFR signal in test-tube samples of whole canine blood without supplementation with ascorbate, but we could easily do so in unsupplemented plasma. Using serially collected plasma samples, we observed an increase in the coronary venous AFR concentration when ischemic myocardium was reperfused in an open-chest dog preparation, whereas the arterial AFR concentration did not change. In addition, the concentration of total ascorbate in both the arterial and the coronary venous blood remained unchanged during occlusion/reperfusion sequences. This suggested that the reperfused myocardium was the site of increased AFR production.

Although the results were encouraging, the increase in the coronary venous AFR concentration was small, and we could not exclude the possibility of changes caused by sample manipulation, even though the samples were kept near 4°C. We discovered that systemic supplementation with ascorbic acid resulted in an easily detectable AFR signal when whole blood from an intact animal was circulated through an EPR spectrometer. Once a steady-state concentration of AFR has been reached by adjustment of the amount of ascorbate being infused, both venous and arterial concentrations remain fairly stable in the absence of any physiological stress. We further studied the stability of AFR concentrations in two sham-occlusion studies. The maximum change in AFR concentration without alteration of the infusion rate in both of these animals was 0.4 nmol/L or 6.7%, thus demonstrating the stability of AFR concentration over time in the absence of ischemia/reperfusion.

The very low one-electron reduction potential of the AFR/ascorbate monoanion couple, $E^{o'} = +282$ mV,[28] results in ascorbate being the terminal reductant in oxidizing free radical chain reactions.[18] Thus, nearly every oxidizing radical that could arise in a biological system (Table 4, References 29 through 32) will bring about the one-electron oxidation of ascorbate, forming AFR. Ascorbate reacts rapidly with superoxide and hydroxyl radicals as well as with alkyl, peroxyl, and alkoxyl radicals,[19,33] thereby "repairing" these radicals and stopping the chain initiation and propagation reactions that may begin on reoxygenation of ischemic tissue. Because the resonance-stabilized AFR is relatively unreactive (compared with hydroxyl or lipid alkoxyl and peroxyl radicals), the steady-state concentration of AFR serves as a measure for the degree of ongoing oxidative stress.[21,22,34]

TABLE 4. One-Electron Reduction Potentials of Selected Radicals

Thermodynamic Couple	$E^{o'}$, mV	Reference
$HO\cdot, H^+/H_2O$	2310	29
RO ·, H^+/ROH (aliphatic alkoxyl radical)	1600	30
ROO ·, H^+/ROOH (alkyl peroxyl radical)	1000	30
$O_2\cdot^-, 2H^+/H_2O_2$	940	29
GS ·/GS^- (glutathione)	920	31
PUFA ·, H^+/PUFA-H (polyunsaturated fatty acid, bis-allylic-H)	600	30
α-Tocopheroxyl ·, H^+/α-tocopherol (TO ·, H^+/TOH)	480	32
Ascorbyl $\cdot^-$, H^+/ascorbate monoanion	282	28

Although the reperfusion-induced rise in AFR was greater after 20 minutes of coronary occlusion than after 5 minutes of occlusion, a longer coronary occlusion, 60 minutes, resulted in an attenuated rise in AFR, only a 13% rise versus 38% rise in the 20-minute-occlusion group. This observation is also in agreement with a recent study that found maximal radical production after 15 minutes of global ischemia compared with 60 minutes of ischemia, followed by reperfusion.[35] One-hour coronary occlusions have been shown to result in irreversible myocardial necrosis, as opposed to the reversible changes induced by a 20-minute period of ischemia. The more prolonged ischemia may injure the free radical–generating cellular components, thereby reducing AFR generation on reperfusion. Since only a small number of prolonged-occlusion dogs have been studied, these results must be regarded as preliminary.

Could ascorbate, working as an antioxidant, attenuate myocardial stunning? To address this issue, we studied five dogs to which no ascorbate was administered during the ischemia/reperfusion sequence. The extent and severity of postreperfusion dyskinesis or stunning in the animals during an initial 30-minute period after the start of experiments was no different from the animals that received ascorbate during a similar occlusion/reperfusion sequence. Thus, in the doses used for this method, ascorbate does not attenuate myocardial stunning during the first 30 minutes of reperfusion. However, the monitoring period may have been insufficient; other studies that looked at the effects of antioxidants in models of stunning found that the differences between the treated and untreated groups were apparent only after 1 hour or more of reperfusion.[36] Whether exogenous ascorbate, at the level we use, attenuates myocardial stunning over a longer period of reperfusion is not established by these experiments and remains a question for further study.

To evaluate the stability of the AFR signal in the face of altered coronary venous flow, we altered coronary

flow by various pharmacological interventions and showed that the concentration of AFR in the coronary venous blood is independent of changes in coronary flow and bears no relation to the pharmacological mechanism of flow alteration in the absence of oxidative stress after ischemia and reperfusion.

Other investigators have observed AFR in the perfusate from isolated rat hearts undergoing ischemia and reperfusion.[37-39] Pietri et al[38] provided data on the kinetics of ascorbate leakage and the associated levels of AFR from isolated hearts submitted to ischemia and reperfusion. Their study showed that the release of AFR into the coronary perfusate as detected by EPR occurred mainly on reperfusion after a period of low-flow or no-flow ischemia. There was a positive correlation between the magnitude of AFR release and the duration of ischemia. The measurement of residual ascorbic acid in the reperfused myocardium revealed marked ascorbate depletion of the myocardium related to the duration of ischemia. Perfusion of hearts with SOD during ischemia and the first 10 minutes of reperfusion greatly inhibited both the endogenous depletion of ascorbate and the concentration of AFR in the perfusate. Since no exogenous ascorbate was used, the source of AFR could be the intramyocardial ascorbate that leaks into the perfusate during an ischemia/reperfusion sequence, which subsequently undergoes metal-catalyzed oxidation.[21] Adventitious catalytic metals are naturally present in buffers.[40]

The technique described in our study has the advantage of being on-line, providing real-time measurement of AFR concentration and its changes; the lag between the appearance of blood from the reperfused myocardium in the coronary vein to its arrival in the EPR flat cell for scanning is only 4 seconds. No exogenous chemical spin-traps are required; hence, tedious processing of blood samples is unnecessary, and potential artifacts are avoided. Also, the animals do not suffer from blood loss from frequent sampling, so the preparation remains robust longer.

A potential disadvantage of the AFR technique is that the maximum postreperfusion increase in coronary venous concentration was relatively modest, 38±17%. Other investigators, using spin-trapping techniques, have reported much more impressive rises in free radical adducts after reperfusion.[5] Whether the relatively small AFR rise will prove a significant limitation of this technique in investigating the role of free radicals in various states of myocardial injury will be established with further experience.

In summary, we have shown a significantly greater rise in AFR peak concentration after 20 minutes of coronary artery occlusion compared with 5 minutes; the total free radical generation (area under the curve, Fig 5) was also greater. With the same duration of ischemia (20 minutes), the attenuation of AFR rise by the antioxidant enzymes SOD and catalase further suggests that the change in AFR concentration truly reflects the change in the production of free radicals. We conclude that AFR is a reliable, real-time, quantitative marker of free radical generation during myocardial reperfusion after brief coronary occlusion.

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