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Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog

(ischemia/reperfusion/heart/magnetic resonance)

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ABSTRACT **Electron paramagnetic resonance (EPR) spectroscopy was used to investigate whether (*i*) the free radicals produced in the "stunned" myocardium (myocardium with postischemic contractile dysfunction) are derived from O_2, (*ii*) inhibition of radical reactions improves function, and (*iii*) i.v. spin traps are effective. Open-chest dogs undergoing a 15-min coronary occlusion received an i.v. infusion of the spin trap, α-phenyl *N-tert*-butylnitrone (PBN) (50 mg/kg). In group I ($n = 6$), EPR signals characteristic of radical adducts of PBN appeared in the coronary venous blood during ischemia and increased dramatically after reperfusion. In group II ($n = 6$), which received PBN and i.v. superoxide dismutase (SOD; 16,000 units/kg) plus catalase (12,000 units/kg), myocardial production of PBN adducts was undetectable during ischemia ($\Delta = -100\%$, $P < 0.01$ vs. group I) and markedly inhibited after reperfusion ($\Delta = -86\%$, $P < 0.001$). This effect was seen at all levels of ischemic zone flow but was relatively greater in the low-flow range. In group III ($n = 8$), the same dosages of SOD and catalase without PBN markedly enhanced contractile recovery (measured as systolic wall thickening) after reperfusion [$P < 0.01$ at 3 hr vs. controls (group IV, $n = 7$)]. Systemic plasma activity of SOD and catalase averaged 127 ± 24 and 123 ± 82 units/ml, respectively, 2 min after reperfusion. PBN produced no apparent adverse effects and actually improved postischemic contractile recovery in group I ($P < 0.05$ at 3 hr vs. controls). This study shows that (*i*) SOD and catalase are highly effective in blocking free radical reactions *in vivo*, (*ii*) the radicals generated in the "stunned" myocardium are derived from univalent reduction of O_2, and (*iii*) inhibition of radical reactions improves functional recovery. The results provide direct, *in vivo* evidence to support the hypothesis that reactive oxygen metabolites play a causal role in the myocardial "stunning" seen after brief ischemia.**

Periods of myocardial ischemia that are too brief to cause necrosis are nevertheless followed by prolonged depression of contractility (1–3) or "stunning" (4), which is associated with numerous functional abnormalities (1–4). Recent studies have shown that myocardial stunning can be attenuated by antioxidants (5–11), suggesting that the accumulation of reactive oxygen metabolites, such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$), may play an important role in the pathogenesis of postischemic dysfunction. However, demonstration of the free radical hypothesis of myocardial stunning remains inconclusive because the evidence is indirect. In particular, it has not been determined whether oxygen-derived radicals are actually generated after a brief coronary occlusion and, if so, whether their generation is actually necessary for dysfunction to occur. An unambiguous link *in vivo* between oxyradical formation and myocardial stunning could be established if one could directly quantitate free radical generation with and without antioxidants in intact animals and then correlate the changes in radical production with the changes in contractility.

Using electron paramagnetic resonance (EPR) spectroscopy and the spin trap α-phenyl *N-tert*-butylnitrone (PBN), generation of free radical species in the stunned myocardium *in vivo* has been recently observed (12). Several important problems, however, remain to be addressed. First, the precise nature of the PBN radical adducts has not been identified. It is, therefore, unknown whether the radicals trapped by PBN in the stunned myocardium are derived from partially reduced oxygen species (i.e., from $O_2^{\cdot-}$ and H_2O_2) or from the numerous other nonspecific sources that could potentially generate reactive free radicals. This issue could be resolved by examining the effect of superoxide dismutase (SOD) ($O_2^{\cdot-}$:$O_2^{\cdot-}$ oxidoreductase; EC 1.15.1.1) and catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) (the two most specific scavengers of $O_2^{\cdot-}$ and H_2O_2 available) on production of PBN adducts.

Second, although free radicals are produced in the stunned myocardium, the fundamental question remains: are these species actually responsible for the contractile abnormality? Insights into this problem could be gained by manipulating PBN adduct formation and by assessing the effects on contractility. If inhibition of radical production results in enhanced recovery of function, this would be strong, direct evidence for a causal role of oxygen species in myocardial stunning.

Finally, in our initial experiments (12), PBN was given by the intracoronary route, which has technical drawbacks and, in addition, is extremely difficult to use in conscious animals. This point is important because in order to confirm the free radical hypothesis, it will eventually be necessary to demonstrate that the findings obtained in open-chest preparations are applicable to more physiological conditions (i.e., awake animals). These limitations could be overcome by administering PBN i.v., but it is unknown whether i.v. administration would cause systemic toxicity and whether it would be effective in trapping radicals.

The present study had four objectives: (*i*) To determine whether the free radicals generated in the stunned myocardium derive from univalent reduction of oxygen. To this end, we investigated the ability of SOD plus catalase to inhibit PBN radical adduct production in our *in vivo* model of postischemic dysfunction. (*ii*) To determine whether inhibition of free radical reactions by SOD plus catalase enhances

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Abbreviations: EPR, electron paramagnetic resonance; LV, left ventricular; PBN, α-phenyl *N-tert*-butylnitrone; REP, reperfusion; SOD, superoxide dismutase.
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functional recovery. To this end, we examined the effect of SOD plus catalase on postischemic dysfunction at the *same doses* and under the *same conditions* in which the enzymes were found to suppress PBN adduct production. (*iii*) To analyze the efficacy of SOD plus catalase in relation to the severity of ischemia. (*iv*) To develop an improved technique—i.v. spin trap administration—for the study of free radical processes *in vivo*.

METHODS

The techniques have been previously described in detail (3, 5, 8–12). Briefly, pentobarbital-anesthetized dogs (22 ± 2 kg) were instrumented with a snare around the mid left anterior descending coronary artery and epicardial Doppler wall thickening probes (3, 5, 8–12). A catheter was advanced into the anterior interventricular vein, and the tip was positioned at least 0.5 cm distal to the snare, so as to minimize any contamination of blood samples with venous effluent from other vascular beds. The left anterior descending coronary artery was occluded for 15 min and reperfused for 3 hr. Regional myocardial blood flow was determined with radioactive microspheres (11). Regional myocardial function was assessed as systolic thickening fraction [i.e., (systolic wall thickening/end-diastolic thickness) × 100] (3). Absence of irreversible tissue damage was confirmed in all animals by triphenyltetrazolium staining (3).

PBN (Sigma) was dissolved in normal saline (11 mg/ml) and infused via a central venous line beginning 5 min before coronary occlusion and continuing until 10 min after reperfusion (REP) (total dose, 50 mg/kg). Dogs (n = 34) were assigned to one of six groups. (*i*) In group I, animals (n = 6) received PBN as described. (*ii*) In group II, dogs (n = 6) were infused with PBN as in group I; in addition, the animals received 16,000 units/kg of recombinant human SOD (3200 units/mg; Pharmacia–Chiron Partnership) and 12,000 units/kg of catalase (47,000 units/mg; Sigma). SOD and catalase were infused i.v. starting 15 min before occlusion and continuing until 30 min after REP. (*iii*) Group III animals (n = 8) were given SOD and catalase as in group II. In three dogs, the plasma activity of SOD and catalase was determined with described assays (13, 14); hemolysis was excluded by hemoglobin determination (15). (*iv*) Group IV dogs (n = 7) did not receive either PBN or SOD/catalase. (*v*) Group V animals (n = 3) received PBN (100 mg/kg i.v. over 60 min) but underwent no coronary occlusion. (*vi*) Group VI animals (n = 4) underwent the same protocol as group I. In each dog, four 12-ml plasma samples were collected after REP; one-half of the samples was processed as usual, whereas SOD and catalase were added to the other sample half to a final concentration of 1300 units/ml and 1700 units/ml, respectively.

Blood samples (6 ml) were drawn over 60 s from the anterior interventricular vein and immediately centrifuged. Plasma specimens were analyzed by EPR spectroscopy as has been described (12, 16–19). The spectrometer settings are specified in the legend to Fig. 1. The myocardial production of spin adducts at a specific time point was expressed in arbitrary units per min per g of myocardium (12); *total* cumulative myocardial production of PBN adducts over the 3 hr of REP was calculated by integrating the measurements at the various time points (12). Values are mean ± SEM. Analysis of variance with Bonferroni correction was used for inter- and intragroup comparisons.

RESULTS

As shown in Table 1, hemodynamic variables and occluded bed size did not differ among groups I, II, III, and IV. Two observations indicate that our dose of PBN did not produce toxicity: (*i*) there were no consistent differences in hemodynamics, coronary flow, and wall thickening in the nonischemic region between groups I and II (which received PBN) and groups III and IV (which did not receive PBN); and (*ii*) in groups I and II these variables did not change before (baseline) and after PBN infusion (Table 1).

Table 1. Hemodynamic variables, regional myocardial blood flow and occluded bed size in groups I–IV

Variable	Baseline	Occlusion	Reperfusion	
			1 hr	3 hr
HR, beats/min				
Group I	142 ± 3	146 ± 3	148 ± 5	146 ± 6
Group II	153 ± 5	163 ± 5	154 ± 7	155 ± 9
Group III	150 ± 5	158 ± 8	153 ± 5	162 ± 7
Group IV	157 ± 4	162 ± 3	159 ± 6	161 ± 7
MAP, mmHg				
Group I	106 ± 7	109 ± 6	102 ± 6	100 ± 5
Group II	110 ± 7	108 ± 8	125 ± 9	115 ± 9
Group III	106 ± 4	100 ± 4	101 ± 5	110 ± 7
Group IV	115 ± 7	108 ± 5	106 ± 3	105 ± 6
LAP, mmHg				
Group I	4 ± 1	6 ± 2	3 ± 1	3 ± 1
Group II	5 ± 1	5 ± 1	6 ± 2	6 ± 1
Group III	4 ± 1	4 ± 1	3 ± 1	4 ± 1
Group IV	5 ± 1	6 ± 1	6 ± 1	6 ± 1
CBF, ml/min				
Group I	20 ± 2	0	18 ± 2	20 ± 2
Group II	20 ± 3	0	17 ± 2	18 ± 3
Group III	16 ± 2	0	14 ± 2	17 ± 3
Group IV	18 ± 2	0	15 ± 3	15 ± 3
TF/NZ,* %				
Group I	20 ± 3	114 ± 8	81 ± 13	93 ± 12
Group II	22 ± 3	104 ± 2	87 ± 3	80 ± 7
Group III	17 ± 2	94 ± 5	90 ± 4	84 ± 4
Group IV	20 ± 2	121 ± 17	91 ± 12	87 ± 7
RMBF/IZ,† $ml \cdot min^{-1} \cdot g^{-1}$				
Group I	1.33 ± 0.22	0.33 ± 0.08 (19 ± 4)		
Group II	1.48 ± 0.16	0.30 ± 0.10 (20 ± 6)		
Group III	1.28 ± 0.11	0.20 ± 0.03 (14 ± 2)		
Group IV	1.47 ± 0.11	0.23 ± 0.03 (17 ± 3)		
RMBF/NZ, $ml \cdot min^{-1} \cdot g^{-1}$				
Group I	1.59 ± 0.25	1.89 ± 0.20		
Group II	1.85 ± 0.25	1.64 ± 0.16		
Group III	1.33 ± 0.10	1.50 ± 0.14		
Group IV	1.23 ± 0.11	1.41 ± 0.10		
OB,‡ g				
Group I		18.2 ± 2.4 (20.8 ± 2.5)		
Group II		21.9 ± 3.3 (23.6 ± 3.4)		
Group III		13.4 ± 1.1 (21.8 ± 1.3)		
Group IV		18.0 ± 2.0 (20.3 ± 2.0)		

Group I received PBN; group II received PBN plus SOD and catalase; group III received SOD and catalase; group IV did not receive either PBN or SOD/catalase. Baseline measurements were obtained before SOD/catalase or PBN. Data are mean ± SEM. HR, heart rate; MAP, mean arterial pressure; LAP, mean left atrial pressure; CBF, coronary blood flow in the occluded/reperfused artery; TF/NZ, thickening fraction in the nonischemic zone; RMBF, transmural regional myocardial blood flow in the ischemic/reperfused zone (IZ) and in the nonischemic zone (NZ); NZF, nonischemic zone flow; OB, occluded vascular bed.

*All measurements subsequent to baseline are expressed as percent of baseline values.

†Numbers in parentheses represent occlusion flow expressed as % NZF.

‡Numbers in parentheses represent occluded bed expressed as % LV weight.

Group I (n = 6): PBN Only. No spin adducts were detected in the venous effluent before coronary occlusion. EPR signals

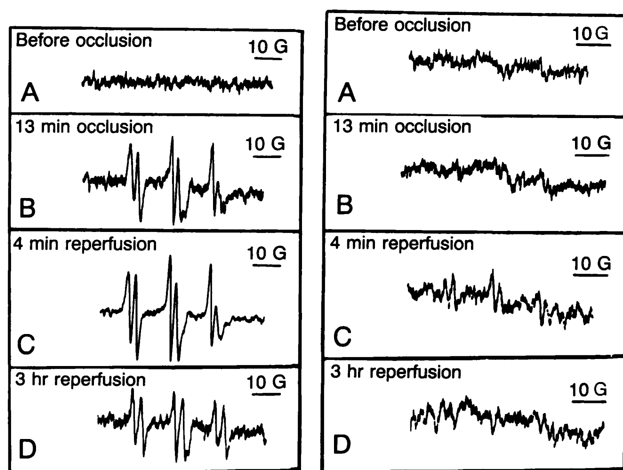

FIG. 1. Representative EPR spectra of PBN radical adducts detected in the coronary venous effluent blood in group I (PBN only). The horizontal bar above each spectrum indicates 10 G. Shown in this figure are signals from plasma samples obtained 4 min after starting PBN infusion but before coronary occlusion (*A*) (gain, 2×10^6) and 13 min after coronary occlusion (*B*) ($a_N = 14.6$ G, $a_\beta^H = 2.4$ G in chloroform; gain, 2×10^6). Visual inspection of the spectrum indicates a mixture of radical adducts. Computer simulation of the spectrum (performed as in ref. 20) suggests the presence of at least two different adducts in a ratio of ≈3:1 with hyperfine coupling constants as follows: adduct 1 (the major component), $a_N = 14.63$ G, $a_\beta^H = 2.35$ G; adduct 2 (the minor component), $a_N = 14.90$ G, $a_\beta^H = 3.20$ G. The spectrum is consistent with adduct 2 being carbon-centered, whereas the identity of adduct 1 remains to be assigned. (*C*) Four minutes after REP ($a_N = 14.8$ G, $a_\beta^H = 2.5$ G; gain, 1×10^6). At this time computer simulation suggests the same two radical adducts (adducts 1 and 2) are present in a ratio of ≈1:1. (*D*) Three hours after REP ($a_N = 14.9$ G, $a_\beta^H = 3.2$ G; gain, 2×10^6). Primarily carbon-centered radical adducts (adduct 2) are seen at this time. The spectrometer settings were as follows: microwave power, 19.7 mW; modulation amplitude, 1 G; time constant, 1.25 s; scan range, 100 G; and scan time, 8 min. All spectra were recorded at room temperature (25°C). (*Right*) Representative EPR spectra of adducts detected in the coronary venous blood in group II (PBN plus SOD and catalase). Shown in this figure are signals from plasma samples obtained 4 min after starting PBN infusion but before coronary occlusion (*A*), 13 min after coronary occlusion (*B*), 4 min after REP (*C*), and 3 hr after REP (*D*). The gain for all scans was 2×10^6. No signal was of sufficient strength for accurate determination of coupling constants.

characteristic of radical adducts of PBN appeared in the coronary effluent after occlusion (Fig. 1). Upon REP, however, there was an immediate, dramatic burst of spin-adduct release (127 ± 30 units/min per g in the first min) (Figs. 1 and 2). The release of PBN adducts peaked 5 min following REP (162 ± 40 units/min per g) and then declined markedly but continued up to 3 hr, when it averaged 40 ± 18 units/min per g (Figs. 1 and 2).

Visual inspection of the EPR spectra in Fig. 1 (*Left*) indicates that the signals are likely due to a mixture of different radical adducts. At 180 min of REP (Fig. 1*D*), the hyperfine coupling constants ($a_N = 14.9$ G, $a_\beta^H = 3.2$ G with chloroform as a solvent) are consistent with the trapping by PBN of mostly carbon-centered radicals (although carbon-centered adducts of PBN usually have $a_N = 14.4$–14.5 G in benzene, chloroform increases this value by ≈0.4 G). The hyperfine coupling constants during coronary occlusion ($a_N = 14.6$ G, $a_\beta^H = 2.4$ G in chloroform, Fig. 1*B*) and at 4 min of REP ($a_N = 14.8$ G, $a_\beta^H = 2.5$ G in chloroform, Fig. 1*C*) cannot be readily ascribed to a single spin adduct of PBN and may be produced by multiple components; the identity of the free radicals trapped at these times remains to be determined.

Group II (n = 6): PBN Plus SOD and Catalase. SOD and catalase markedly reduced PBN spin adduct production during both the occlusion and the REP phase (Figs. 1 and 2). During occlusion, no radical adducts were detected (Δ = −100% vs. group I). The total cumulative release of adducts during the 3 hr of REP was decreased by an average of 86% (10,039 ± 1,873 units/g in group I vs. 1409 ± 325 units/g in group II, $P < 0.001$). In both group I and II, there was a linear, inverse relationship between the total myocardial production of PBN adducts over the 3-hr REP period and the collateral flow to the ischemic region during the occlusion phase (Fig. 3). However, treatment with SOD and catalase markedly decreased the slope of this correlation (288 in group I vs. 26 in group II), so that at any given level of ischemic zone flow, there was less production of PBN adducts (Fig. 3). Multiple regression analysis and analysis of covariance demonstrated a significant difference ($P < 0.001$) between the two regression lines in both the estimated y values for $x = 0$ (y intercepts) and the estimated y values for $x > 0$. Importantly, the effect of the enzymes was more pronounced in the low-flow range (Fig. 3). Thus, the greater the severity of ischemia (and hence the propensity to generate radicals upon REP), the greater the protection afforded by SOD and catalase.

Group V (n = 3): Control Group for EPR Signals. The samples obtained from this control group were processed in a manner identical to that used for groups I and II. Despite the fact that the dose of PBN administered to these control dogs (100 mg/kg) was *twice* that administered to groups I and II (50 mg/kg), no EPR signal of any kind was detected in any of the blood samples, which were obtained at 10-min intervals during infusion of the spin trap and for 2 hr thereafter (total, 48 samples). Further, PBN caused no changes in hemodynamics or wall thickening. The following measurements (average values) were obtained before and after the 60-min

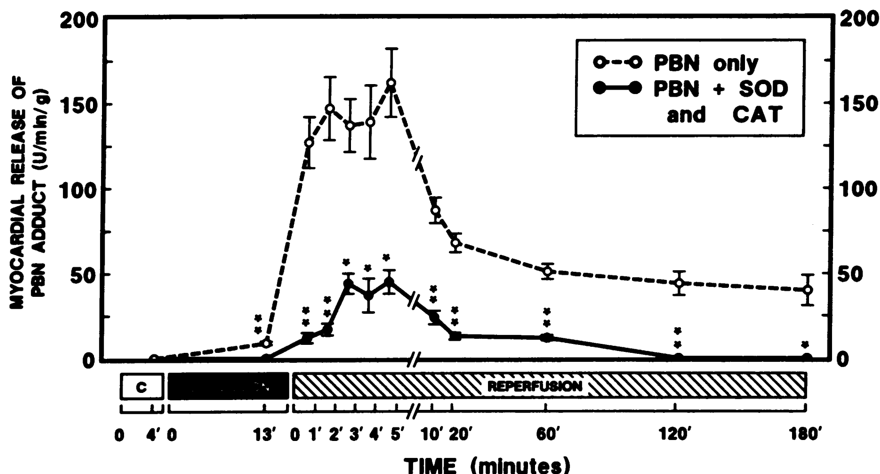

FIG. 2. Time course of myocardial release of PBN adducts in group I (PBN only, n = 6) and group II (PBN plus SOD and catalase, n = 6). Data are mean ± SEM. See text for explanation of units used. C, control; U, units; CAT, catalase; *, $P < 0.05$; **, $P < 0.01$ vs. group I.

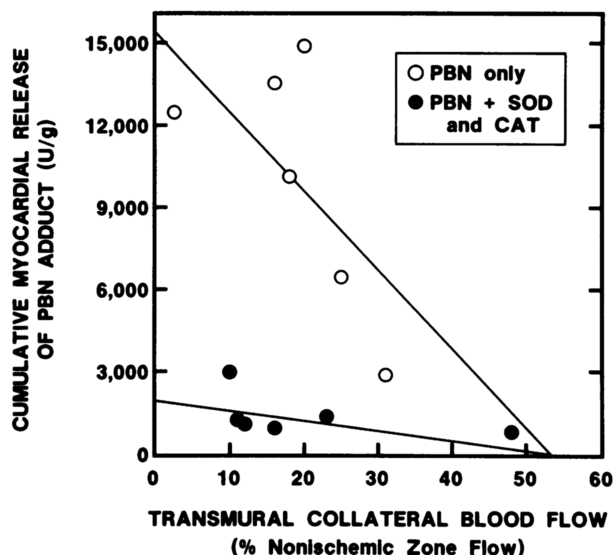

FIG. 3. Relationship between mean transmural collateral blood flow to the ischemic region during coronary occlusion and total cumulative myocardial release of PBN adducts during the 3 hr of REP in group I [PBN only (○)] and group II [PBN plus SOD and catalase (CAT) (●)]. Collateral flow is expressed as percent of simultaneous nonischemic zone flow; adduct release is expressed in arbitrary units per g of myocardium (U/g) (see text). Regression equations and r values (obtained by linear regression analysis) were as follows: PBN only, $y = 15{,}353 - 288x$ and $r = -0.67$; PBN plus SOD/CAT, $y = 1934 - 26x$ and $r = -0.47$.

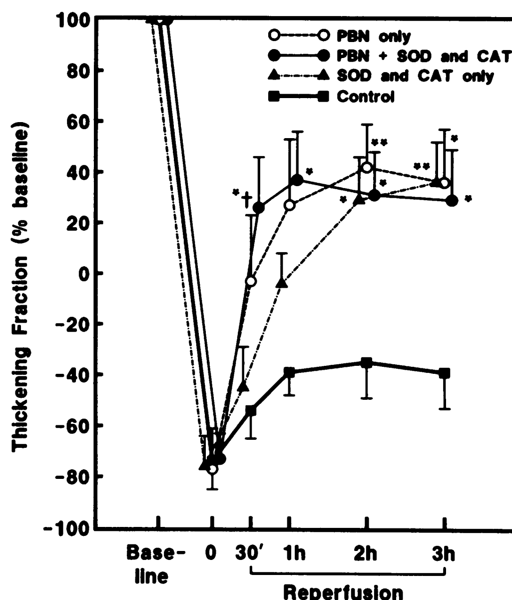

FIG. 4. Systolic thickening fraction in the ischemic/REP region 5 min after coronary occlusion (0) and at selected times after REP in the following groups: group IV [no PBN given (controls), $n = 7$ (■)], group I [PBN only, $n = 6$ (○)], group II (PBN plus SOD and catalase (CAT), $n = 6$ (●)], and group III (SOD and CAT, $n = 8$ (▲)]. Thickening fraction is expressed as percent of baseline values. Data are mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ vs. control (group IV); †, $P < 0.05$ vs. SOD and CAT (group III).

infusion of PBN, respectively: heart rate, 142 and 145 beats per min; mean arterial pressure, 98 and 103 mmHg; mean left atrial pressure, 4 and 4 mmHg; peak positive left ventricular (LV) dP/dt, 2516 and 2872 mmHg/s; peak negative LV dP/dt, 2872 and 2689 mmHg/s. Taken together, these results indicate that the generation of radicals seen in groups I and II was not due to nonspecific toxic effects of PBN.

Group VI ($n = 4$): Effect of SOD and Catalase on PBN Adducts *in Vitro*. *In vitro* addition of SOD and catalase to 16 plasma samples containing PBN adducts produced no discernible effect on EPR signal intensity (data not shown). Thus, the suppression of radical production by SOD and catalase *in vivo* cannot be ascribed to nonspecific effects of the enzymes on the PBN adducts.

Effects of SOD/Catalase and PBN on Contractile Function. Baseline systolic thickening fraction in the left anterior descending coronary artery region did not differ among group I (18 ± 4%), group II (19 ± 4%), group III (23 ± 2%), and group IV (23 ± 2%). Control dogs (group IV) exhibited minimal recovery of contractile function after REP, and at 3 hr the previously ischemic region was still dyskinetic, indicating severe myocardial stunning (Fig. 4). However, in the dogs receiving SOD and catalase (group III), recovery of function was significantly greater ($P < 0.01$ at 3 hr) (Fig. 4). PBN alone (group I) also enhanced recovery (Fig. 4), which is consistent with the notion that spin traps act as free radical scavengers (21). The addition of PBN to SOD/catalase (group II) resulted in further improvement of contractility at 30 min ($P < 0.05$ vs. SOD/catalase) but not at 1, 2, or 3 hr ($P > 0.15$) (Fig. 4). This lack of additive effect suggests that PBN traps the same damaging radicals the formation of which is prevented by SOD and catalase.

The activity of SOD and catalase in the arterial plasma averaged 89 ± 16 and 111 ± 38 units/ml, respectively, 5 min after coronary occlusion, and 127 ± 24 and 123 ± 82 units/ml, respectively, at 2 min, 147 ± 23 and 218 ± 105 units/ml at 20 min, 44 ± 9 and 38 ± 22 units/ml at 1 hr, and 6 ± 2 and 8 ± 4 units/ml at 2 hr of REP.

DISCUSSION

A previous study (12) has shown that free radicals are generated in the stunned myocardium *in vivo*. However, this finding does not, in itself, establish the validity of the oxygen radical hypothesis of postischemic dysfunction. At least two other fundamental postulates remain to be proven: (*i*) the free radicals produced are derived from oxygen, and (*ii*) inhibition of free radical reactions enhances recovery of contractility (i.e., the radical reactions are necessary for the development of postischemic dysfunction).

Our findings suggest that both postulate *i* and postulate *ii* are correct. Specifically, PBN radical adduct production in the stunned myocardium was found to be markedly inhibited by SOD and catalase, indicating that the univalent pathway of reduction of oxygen is the source of the radicals. Further, when PBN radical adduct production was inhibited by SOD and catalase (group II), the concomitant contractile derangements were found to be greatly diminished (group III), indicating that radical reactions are necessary for severe myocardial stunning to occur. Prior studies (5–11) have indirectly suggested that oxygen radicals contribute to myocardial stunning. The present observations provide direct, *in vivo* evidence supporting the hypothesis that reactive oxygen species play a causal role in the persistent contractile dysfunction seen after reversible myocardial ischemia.

It has been previously shown that SOD (22, 23) or catalase (24) decreases the concentration of free radicals in isolated hearts subjected to global ischemia and REP. These data have provided important insights into the free radical reactions associated with REP. However, because of the numerous differences between the buffer-perfused heart undergoing global ischemia *in vitro* and the blood-perfused heart subjected to regional ischemia in the intact animal, results obtained in the former preparation cannot necessarily be extrapolated to the latter. Further, the isolated heart preparations used in these investigations (22–24) have not been specifically demonstrated to be associated with myocardial stunning, which is defined as a persistent but ultimately

reversible depression of contractility (4). The present study expands these prior observations (22–24) by demonstrating that antioxidant enzymes actually block production of secondary radicals in myocardium subjected to reversible regional ischemia in the intact animal and that this decrease in radical production results in a decrease in postischemic mechanical dysfunction.

Our results indicate that SOD and catalase, given i.v., are remarkably effective: production of PBN radical adducts became undetectable during ischemia ($\Delta = -100\%$) and was markedly inhibited following REP ($\Delta = -86\%$). Although infusion of SOD and catalase was discontinued 30 min after REP, PBN adduct production was still decreased at 1 hr and ceased completely by 2 hr; in contrast, in group I PBN adduct production continued up to 3 hr after REP (Fig. 2). Thus, our data indicate that SOD and catalase confer sustained protection against free radical reactions, which continues for hours after the infusion of these short-lived enzymes is terminated. The most likely explanation is that SOD and catalase prevent the initiation of lipid peroxidation, which can continue as a chain reaction even after production of $O_2^{\cdot -}$ and H_2O_2 has ceased (25). Importantly, we found that the efficacy of SOD and catalase increases as the severity of ischemia—and, therefore, the tendency to generate radicals—increases (Fig. 3).

Because SOD and catalase are highly specific enzymes, our findings indicate that the radicals trapped by PBN are derived from reactions initiated by $O_2^{\cdot -}$ and/or H_2O_2. Definitive identification of these radicals will require further complex studies involving adduct isolation and mass spectrometric analysis; thus, only speculations are possible. The PBN adducts observed are not those of $O_2^{\cdot -}$ or HO·. As elaborated in *Results*, the EPR spectra suggest a mixture of different secondary radicals (Fig. 1), which could be generated by various mechanisms. For example, it is well known that initially formed oxygen radicals can react with membrane lipids to produce secondary oxygen- and carbon-centered radicals (25), both of which can be trapped by PBN forming reasonably persistent adducts (17, 21). By scavenging $O_2^{\cdot -}$ and H_2O_2, SOD and catalase may prevent the initiation of these secondary reactions and thus decrease the production of the secondary radicals trapped by PBN.

To establish an unambiguous link between attenuation of radical reactions and enhancement of contractility, the two effects must be seen under identical conditions. The present protocol was designed accordingly. Our results demonstrate that SOD and catalase reduce postischemic dysfunction at the same doses and under the same experimental conditions in which they reduce formation of PBN adducts. The correlation between the two effects suggests that the production of free radical species in the stunned myocardium plays a causal role in the depression of contractility. The demonstration that SOD and catalase prevent free radical reactions *in vivo* is also important for interpreting the protective effects exerted by these enzymes in various other experimental settings (25, 26).

The present data also have important methodological implications. To our knowledge, no previous study has used i.v. spin traps to detect radicals in ischemia/REP. This experiment demonstrates that i.v. PBN effectively traps free radicals and that, at this dose, no apparent toxicity occurs [PBN actually exerted a beneficial effect on the postischemic myocardium (Fig. 4)]. The i.v. route has many significant advantages over the intracoronary route previously employed (12). Because the spin trap is uniformly distributed in the intravascular volume, its concentration in the coronary arterial inflow during occlusion and early after REP should be homogeneous in different dogs. In contrast, when spin traps are given by the intracoronary route, their concentration in the coronary inflow may vary from dog to dog depending on the degree of collateral flow and reactive hyperemia, two variables which cannot be predicted. Hence, i.v. administration of spin traps may facilitate comparisons among different animals. Furthermore, unlike the intracoronary route, the i.v. route can be readily used in the awake animal. Intravenous administration also makes it possible to use spin trapping in various organs/tissues and in numerous experimental settings of radical-mediated damage in which selective intraarterial administration would not be feasible. Therefore, the use of i.v. spin traps may significantly expand the applications of the technique and should provide an invaluable tool for exploring the role of free radicals *in vivo* under physiological experimental conditions. It is recognized, however, that the present results were obtained in an open-chest preparation in which confounding effects of anesthesia, surgical trauma, and associated neurohumoral perturbations cannot be ruled out. The applicability of i.v. PBN to *conscious* animal preparations remains to be established.

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