Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 169-180
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USE OF SPIN TRAPS IN INTACT ANIMALS UNDERGOING MYOCARDIAL ISCHEMIA/ REPERFUSION: A NEW APPROACH TO ASSESSING THE ROLE OF OXYGEN RADICALS IN MYOCARDIAL "STUNNING"

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Numerous studies have *indirectly* suggested that oxygen-derived free radicals play an important pathogenetic role in the prolonged depression of contractile function observed in myocardium reperfused after reversible ischemia (myocardial "stunning"). In order to provide *direct* evidence for the oxy-radical hypothesis of stunning, we administered the spin trap, α-phenyl N-*tert*-butyl nitrone (PBN), to open-chest dogs undergoing a 15-min coronary artery occlusion followed by reperfusion. Plasma of local coronary venous blood was analyzed by electron paramagnetic resonance (EPR) spectroscopy. EPR signals characteristic of radical adducts of PBN appeared during ischemia and increased dramatically in the first few minutes after reperfusion. After this initial burst, the production of adducts abated but did not cease, persisting up to 3 h after reflow. The production of PBN adducts after reperfusion was inversely related to collateral flow during ischemia. PBN itself enhanced recovery of contractile function, indicating that the radicals trapped may play a pathogenetic role in myocardial stunning. Superoxide dismutase plus catalase attenuated PBN adduct production and, at the same time, improved recovery of contractile function. Antioxidant therapy given 1 min before reperfusion suppressed PBN adduct production and improved contractile recovery; however, the same therapy given 1 min *after* reperfusion did not suppress early radical production and did not attenuate contractile dysfunction. After i.v. administration, the elimination half-life of PBN was estimated to be approximately 4–5 h. The results demonstrate that 1) free radicals are produced in the stunned myocardium in intact animals; 2) inhibition of free radical production results in improved contractile recovery; and 3) the free radicals important in causing dysfunction are produced in the first few minutes of reperfusion. Taken together, these studies provide cogent evidence supporting the oxy-radical hypothesis of stunning in open-chest dogs. It is now critical to determine whether these results can be reproduced in *conscious* animal preparations.

KEY WORDS: Free oxygen radicals, coronary artery reperfusion, spin traps, electron paramagnetic resonance spectroscopy, α-phenyl N-*tert*-butyl nitrone, myocardial dysfunction.

INTRODUCTION

Myocardium reperfused after brief, reversible ischemia exhibits prolonged depression of contractile function[1-4] or "stunning",[5] which is associated with a variety of func-

This study was supported in part by a Grant-in-Aid 88R-171 from the American Heart Association, Texas Affiliate, NIH Grant GM36512, Biomedical Research Support Grant 2507RR05538-25, NHLBI Grants HL36277 and HL43151, and by the SCOR in Heart Failure Grant HL42267 from NIH. R.B. was a recipient of the Physician-Scientist Award of the American College of Chest Physicians.

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tional abnormalities.[1,2,6,7] In recent years, a number of studies have suggested that postischemic myocardial dysfunction may be mediated in part by the generation of reactive oxygen species, such as $\cdot O_2^-$, H_2O_2 and $\cdot OH$. Thus, it has been shown that the recovery of the stunned myocardium is enhanced by agents that either scavenge oxygen metabolites, such as superoxide dismutase (SOD) and catalase,[8-10] N-2-mercaptopropionyl glycine (MPG),[11] and dimethylthiourea,[12] or prevent their generation, such as allopurinol[13] and desferrioxamine.[14] All the evidence provided by these studies, however, is indirect and, therefore, inconclusive. Clearly, in order to definitely establish a role of oxygen metabolites in postischemic dysfunction, it is necessary to develop techniques that can directly demonstrate free radical production in the stunned myocardium in the presence and absence of antioxidant interventions.

Indeed, despite the numerous studies[8-14] demonstrating beneficial effects of antioxidants in the stunned myocardium, the *mechanism* of such effects has not been conclusively established. As a consequence, unequivocal demonstration of a causal role of oxygen metabolites has not been possible. The major problem is that thus far anti-free radical activity has been assumed to occur *in vivo* without direct evidence because the antioxidants that attenuate stunning have not been proven to scavenge radicals in a complex system such as the intact animal. The indirect nature of the evidence available is undoubtedly the major reason why to date the free radical hypothesis of myocardial stunning remains unproven.

In an effort to overcome these limitations, we have developed a new approach based on spin-trapping techniques. The rationale for using spin traps was that these agents react with free radicals to form relatively stable adducts that can be detected, identified, and quantitated. The major goals of the studies reported herein were the following: 1) to demonstrate production of free radicals in the stunned myocardium in intact animals with the use of the spin trap α-phenyl N-*tert*-butyl nitrone (PBN). To this end, PBN was administered in our canine model of postischemic dysfunction and the venous effluent was analyzed by EPR spectroscopy. 2) To determine whether the free radicals generated in the stunned myocardium are derived from univalent reduction of oxygen. To this end, we assessed the ability of SOD plus catalase (the two most specific scavengers of $\cdot O_2^-$ and H_2O_2 available) to inhibit PBN adduct production. 3) To determine whether attenuation of myocardial stunning by antioxidants is associated with attenuation of free radical reactions in the intact animal. To this end, we examined the effect of either SOD plus catalase or MPG on postischemic dysfunction at the *same doses* and under the *same conditions* in which the agents were found to suppress PBN adduct production. 4) To develop a new, more direct technique – spin trap administration – for the study of free radical processes in ischemic/reperfused myocardium in intact animals. 5) To determine the PBN concentration achieved with our protocols in the systemic blood and to estimate the elimination half-life of this spin trap.

MATERIALS AND METHODS

The techniques have been previously detailed.[15-17] Briefly, pentobarbital-anesthetized dogs were instrumented with a snare around a coronary artery and epicardial Doppler wall thickening probes. For sampling of local coronary venous effluent blood, a catheter was advanced into the vein which accompanied the artery to be occluded. The selected coronary artery was occluded for 15 min and then reperfused for 3 h. This

duration of ischemia was selected because it is well established that it does not result in myocardial necrosis in the dog,[6] but nevertheless it does produce prolonged depression of contractility.[1–4,7] Regional myocardial function was assessed as systolic thickening fraction.[3] Three experiments were performed.

Experiment 1

In these studies PBN was given by the intracoronary route to achieve a coronary arterial concentration of 1.6 mM. Details of this experiment have been published.[15] Five groups of dogs were studied: *Group I.* In these animals (n = 5) PBN was infused beginning 5 min before coronary occlusion and ending 10 min after reperfusion. *Group II.* In these dogs (n = 5) PBN was infused beginning 20 s prior to coronary reperfusion and ending 10 min thereafter. *Group III.* PBN was infused for 10 min beginning at 30 min (n = 3), 1 h (n = 3) or 2 h (n = 3) after coronary reperfusion. *Group IV.* In these animals (n = 11), PBN was infused as in groups I, II and III, but no coronary artery occlusion was performed. *Group V.* These dogs (n = 8) underwent the same experimental protocol as groups I, II and III except for the fact that PBN was not administered.

Experiment 2

In these studies PBN was infused by the intravenous route beginning 5 min before coronary occlusion and continuing until 10 min after reperfusion (total dose: 50 mg/kg). Details of this experiment have been published.[16] Five groups of dogs were studied: *Group I.* These animals (n = 6) received PBN as described. *Group II.* In these dogs (n = 6) PBN was infused as in group I; in addition, the animals received 16,000 U/kg of recombinant human SOD (Pharmacia-Chiron Partnership) and 12,000 U/kg of catalase (Sigma). SOD and catalase were infused i.v. starting 15 min before occlusion and continuing until 30 min after reperfusion. *Group III.* These animals (n = 8) were given SOD and catalase as in group II, but without PBN. *Group IV.* These dogs (n = 7) did not receive either PBN or SOD/catalase. *Group V.* These animals (n = 3) received PBN but did not undergo coronary occlusion.

Experiment 3

In these studies PBN was infused by the intracoronary route as in experiment 1. MPG, a potent and cell-permeable ·OH scavenger, was used as an antioxidant at a dose of 8 mg/kg/h. Details of this experiment have been published.[17] Seven groups of dogs were studied: *Group I.* These dogs (n = 8) received an intracoronary infusion of MPG starting 15 min before coronary occlusion and ending 2 h after reperfusion. *Group II.* In these dogs (n = 9) the infusion of MPG was started 1 min before reperfusion and ended 2 h thereafter. *Group III.* In these dogs (n = 10) the infusion of MPG was started 1 min after reperfusion and ended 2 h and 15 min thereafter. *Group IV.* In these dogs (n = 10) no MPG was given. *Group V.* In these dogs (n = 5) PBN was infused starting 20 s before coronary artery reperfusion and ending 10 min thereafter. In addition, MPG was given at the same dose previously used for group II, i.e., 8 mg/kg/h starting 1 min before reperfusion and ending 2 h thereafter. *Group VI.* IN these dogs (n = 5) PBN was given as in group V. In addition, MPG was given at the same dose previously used for group III, i.e., 8 mg/kg/h starting 1 min after

reperfusion and ending 2 h and 15 min thereafter. *Group VII.* In these dogs (n = 6) PBN was given as in the previous groups but no MPG was administered.

In all experiments, blood samples (6 ml) were drawn from the aorta or from the appropriate coronary vein and immediately centrifuged. The plasma specimens were frozen at −70°C for subsequent analysis by EPR spectroscopy. The techniques used to detect PBN spin adducts and to quantify their production have been detailed.[15–18] Myocardial production of spin adducts at a specific time point was expressed in U/mg/g (arbitrary units per minute per gram of reperfused myocardium). Values are mean ± SEM. Analysis of variance with Bonferroni correction was used for inter- and intra-group comparisons.

RESULTS

Experiment 1

EPR signals characteristic of radical adducts of PBN appeared in the coronary effluent after coronary occlusion, but the signal was weak (Figure 1). Upon reperfusion, however, there was an immediate, dramatic increase in spin adduct release (Figures 1 and 2). The release of PBN adducts peaked at 2 min after reperfusion and then declined markedly over the ensuing 20 min but continued (albeit much less intensely) up to 3 h after reperfusion (Figure 1). In group I, as well as in groups II and III, no EPR signals were observed in any of the arterial blood samples, which were obtained 5 and 13 min into the occlusion phase and 2, 5, 20, 60 and 180 min into the

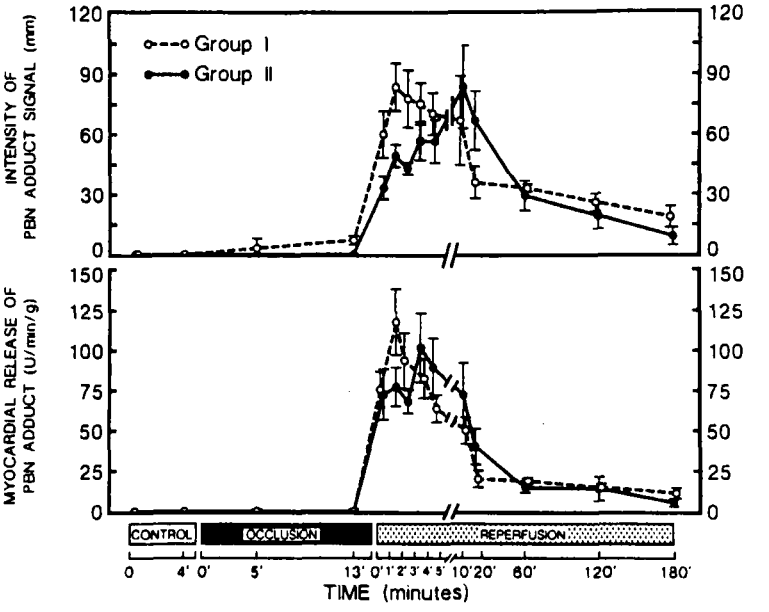

FIGURE 1 *Upper panel.* Intensity of the EPR signals detected in the coronary venous effluent blood in groups I and II. *Lower Panel.* Time course of myocardial release of PBN adducts in groups I and II. Data are mean ± SEM. See text for explanation of units used. (Reproduced from the *J. Clin. Invest.*, **82**, 476–485, (1988), by copyright permission of the American Society for Clinical Investigation).

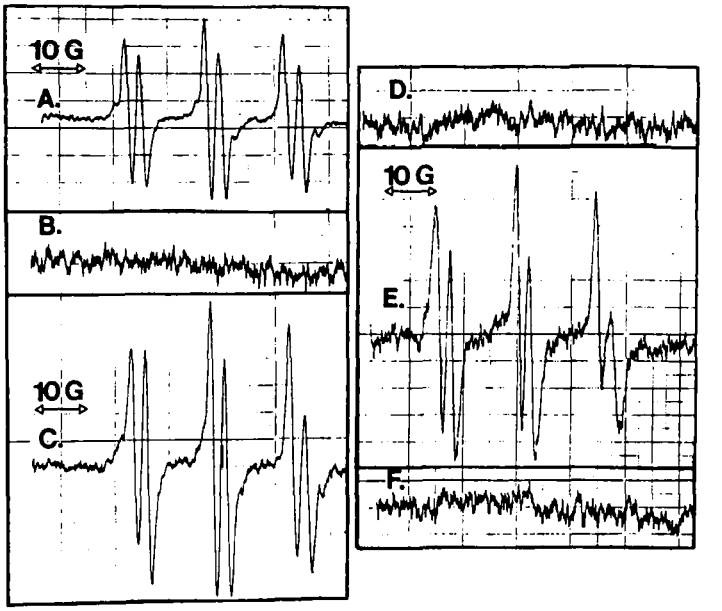

FIGURE 2 Representative EPR spectra of PBN radical adducts detected in the coronary venous effluent blood. Shown in this figure are signals from plasma samples obtained: (A) 3 min after reperfusion in a dog from group I (PBN infusion started 5 min before ischemia) (a_N = 14.75, a_β^H = 2.69 G; gain, 5 × 10^5); (B) at corresponding time after start of PBN infusion in a control dog (gain, 1 × 10^6); (C) 5 min after reperfusion in a dog in group II (PBN infusion started 20 s before reperfusion) (a_N = 14.77, a_β^H = 2.69 G; gain, 5 × 10^5); (D) at corresponding time after start of PBN in a second control dog (gain, 1 × 10^6); (E) 35 min after reperfusion in a dog in group III (PBN infusion started 30 min after reperfusion) (a_N = 15.00, a_β^H = 2.78 G; gain, 5 × 10^6); (F) at corresponding time after start of PBN in a third control dog (gain, 1 × 10^6). (Reproduced from the *J. Clin. Invest.*, **82**, 476–485, (1988), by copyright permission of the American Society for Clinical Investigation).

reperfusion phase. Thus, the presence of an EPR signal in venous effluent blood cannot be ascribed to recirculation of the PBN adducts. In group II, as expected, no EPR signal was detected during the occlusion phase. After reperfusion, however, there was marked production of PBN adducts (Figure 2), the magnitude and time course of which were comparable to those observed in group I (Figure 1). These findings demonstrate that the abrupt increase in the release of PBN adducts observed after reflow in group I does indeed represent free radicals production by the reperfused myocardium, and is not primarily due to "wash out" of spin trapped radicals accumulated during ischemia. In group III, as expected, no EPR signal was observed until PBN was administered. Immediately after the start of PBN infusion a radical adduct appeared in the venous effluent, even when the spin trap was given as late as 2 h after reperfusion. The magnitude of adduct release was similar to that observed in groups I and II at corresponding times after reflow. These results demonstrate that the prolonged release of PBN adducts observed after reperfusion in groups I and II is due to continued production of free radicals rather than to slow removal of adducts accumulated during the early phase of reflow. In groups I and II, there was a linear and inverse relationship between myocardial production of PBN adducts after coronary reperfusion and collateral flow to the ischemic region during occlusion. Thus, the

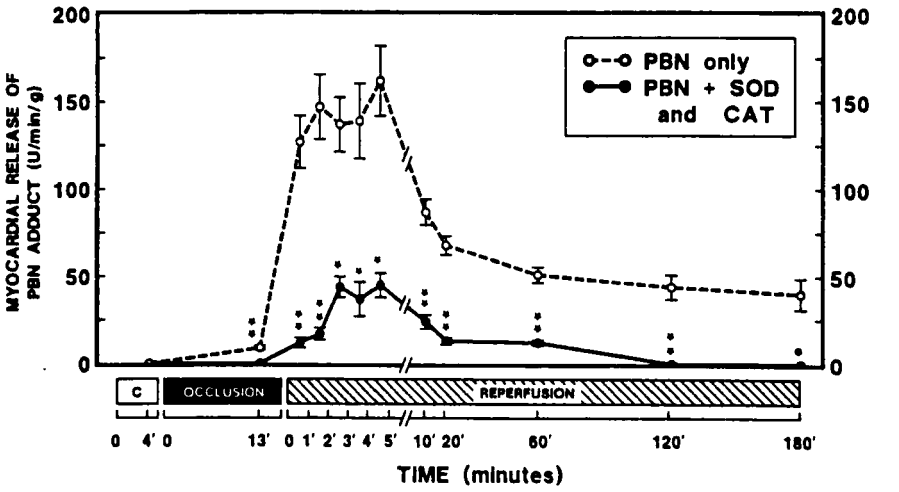

FIGURE 3 Time-course of myocardial release of PBN adducts in group I (PBN only, n = 6) and group II (PBN plus SOD and CAT, n = 6). Data are mean ± SEM. See text for explanation of units used. C, control. *P < 0.05, **P < 0.01 vs. group I. (Reproduced with permission from ref.[16]).

intensity of free radical generation after reperfusion is determined by the severity of the antecedent ischemic insult: the greater the degree of hypoperfusion, the greater the production of radicals.

In group IV, the infusion of PBN did not produce any appreciable effect on regional contractile function or hemodynamic variables. Furthermore, no EPR signal was detected in the coronary venous blood samples throughout the administration of the spin trap. The consistent absence of spin adduct release, coupled with the lack of deterioration of regional function, indicates that the production of adducts in groups I, II, and III was not due to nonspecific toxic effects of PBN. Finally, we found that, in both group I and II, PBN significantly enhanced the recovery of thickening fraction in the stunned myocardium as compared with group V (dogs that did not receive PBN).

Experiment 2

Intravenous administration of PBN did not produce any discernible toxicity. In *group I*, EPR signals characteristic of radical adducts of PBN appeared in the coronary effluent after coronary occlusion. Upon reperfusion, however, there was a dramatic burst of spin adduct release (Figure 3). The release of PBN adducts peaked 5 min after reperfusion and then declined but continued up to 3 h (Figure 3). Thus, the results obtained in this group were similar to those observed in Experiment 1 with intracoronary PBN. In *group II*, SOD and catalase markedly reduced PBN adduct production during both the occlusion and the reperfusion phase (Figure 3). The total cumulative release of adducts during the 3 h of reperfusion was decreased by an average of 86% ($p < 0.0001$). In *group V* (controls), administration of PBN in the absence of coronary occlusion/reperfusion did not produce any changes in hemodynamics or wall thickening, nor did it produce any EPR signal in any of the blood samples. These results indicate that the generation of radicals observed in groups I

and II was not due to nonspecific toxic effects of PBN. Finally, in group III, the administration of SOD and catalase significantly enhanced the recovery of contractile function as compared with control dogs (group IV). The intravenous administration of PBN alone (group I) also enhanced the recovery, which is consistent with the results obtained with intracoronary PBN in Experiment 1. The finding that PBN enhances postischemic recovery of function further corroborates the hypothesis that free radical species contribute to myocardial stunning, since, like other spin traps, PBN in effect acts as a free radical scavenger.

In three dogs, the concentration of PBN in the arterial plasma was measured by high performance liquid chromatography. PBN concentration averaged 0.32 ± 0.11 mM 1 min after coronary occlusion, 0.58 ± 0.19 mM 1 min after reperfusion, and 0.56 ± 0.15 mM 10 min after reperfusion (end of PBN infusion). After the end of the infusion, PBN levels declined slowly (0.41 ± 0.13 mM at 20 min, 0.26 ± 0.07 mM at 1 h, 0.16 ± 0.05 mM at 4 h of reperfusion). On the basis of these data, the elimination half-life of PBN in the anesthetized dogs was estimated to be approximately 4–5 h.

Experiment 3

In control dogs (group IV) there was little recovery of contractile function after reperfusion, and 4 h after restoration of flow the previously ischemic region was still

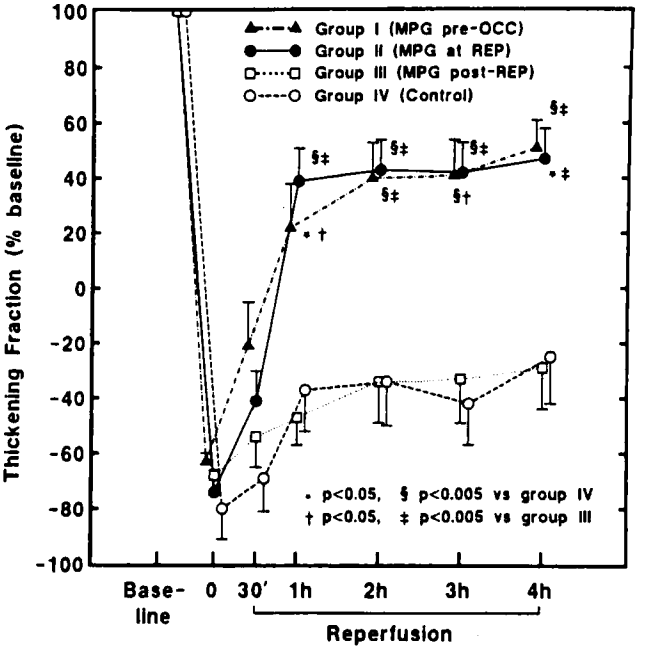

FIGURE 4 Systolic thickening fraction in the ischemic/reperfused region 5 min after coronary occlusion (O) and at selected times after reperfusion in the following groups: group IV (controls, n = 10 [open circles]), group I (MPG infusion started 15 min before ischemia, n = 8 [solid triangles]), group II (MPG infusion started 1 min before reperfusion, n = 9 [solid circles]), and group III (MPG infusion started 1 min after reperfusion, n = 10 [open squares]). Thickening fraction is expressed as percent of baseline values. Data are mean ± SEM. *P < 0.05, §P < 0.005 vs group IV (controls); †P < 0.05, ‡P < 0.005 vs group III. (reproduced from ref.[17] by permission of the American Heart Association).

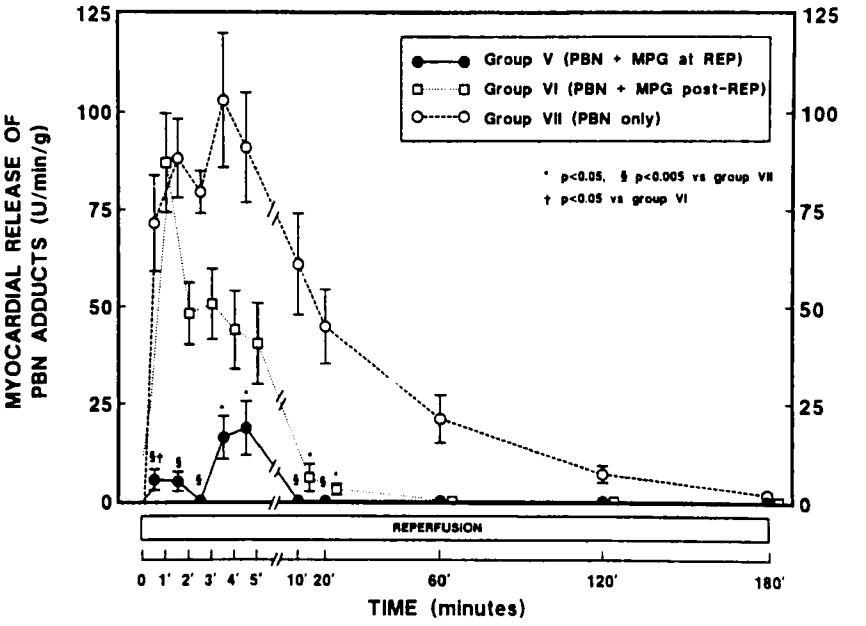

FIGURE 5 Time-course of myocardial release of PBN adducts in group VII (controls, n = 6), group V (MPG infusion started 1 min before reperfusion, n = 5), and group VI (MPG infusion started 1 min after reperfusion, n = 5). Data are mean ± SEM. See text for explanation of units used. *P < 0.05, §P < 0.005 vs group VII (controls); †P < 0.05 vs group VI. (Reproduced from ref.[17] by permission of the American Heart Association).

dyskinetic, indicating severe myocardial stunning (Figure 4). In group I, which received MPG beginning before occlusion, recovery of function was substantially greater (Figure 4). In group II, which received MPG starting 1 min before reperfusion, recovery of function was also substantially greater than in controls, and essentially equivalent to group I (Figure 4). In contrast, in group III, which received MPG beginning 1 min after reperfusion, there was no appreciable improvement in recovery of function (Figure 4). Figure 5 summarizes the time course of release of PBN adducts from the ischemic/reperfused region in groups V, VI and VII. In group VII (controls), the appearance of EPR signals was similar to that previously observed in experiment 1. In group V, which received MPG beginning 1 min before reperfusion, PBN adduct production was markedly reduced as compared with control dogs (group VII) (Figure 5). This effect was evident immediately after release of coronary occlusion ($\Delta = -92\%$ in the initial 60 s of reflow). In group VI, which received MPG beginning 1 min after reperfusion, PBN adduct production before the start of MPG infusion (i.e., in the initial 60 s of reflow) was, as expected, similar to control dogs (group VII). Following the administration of MPG, the release of spin trapped radicals decreased rapidly (Figure 5). In summary, production of spin trapped radicals was markedly inhibited by MPG both in group V and in group VI; the difference between the two groups was that this inhibition began in the initial seconds of reperfusion in group V whereas it was delayed in group VI.

DISCUSSION

Because of lack of direct evidence, at least three fundamental postulates underlying the oxy-radical hypothesis of postischemic dysfunction remain to be proven: a) free radicals are generated in the stunned myocardium *in vivo*; b) the free radicals produced are derived from univalent reduction of oxygen; and c) inhibition of free radical reactions results in enhanced recovery of contractility (i.e., the radical reactions are *necessary* for the development of postischemic dysfunction). These postulates are critical because the oxy-radical hypothesis of stunning cannot be definitively accepted until all three of these assumptions are demonstrated.

The results of the present studies suggest that postulates a), b) and c) are correct. Specifically, the studies demonstrate that a brief episode of regional ischemia and the subsequent reperfusion are associated with the appearance of PBN adducts, indicating that free radicals are produced in the stunned myocardium in the intact dog. The studies further demonstrate that PBN adduct production in the stunned myocardium is markedly inhibited by the administration of SOD and catalase, indicating that the univalent pathway of reduction of oxygen is the source of the radicals. In addition, the experiments demonstrate that when PBN adduct production is inhibited either by SOD and catalase (Experiment 2) or by MPG (Experiment 3), the concomitant contractile derangements are greatly diminished, indicating that radical reactions are necessary for severe myocardial stunning to occur. Prior studies[8-14] 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 observed after reversible myocardial ischemia (myocardial stunning).

Previous investigations using either direct EPR spectroscopy of myocardial tissue[19] or spin trapping techniques[20,21] have demonstrated production of free radicals in isolated hearts subjected to global ischemia and reperfusion. These data have provided direct evidence that cardiac ischemia and reperfusion are associated with free radical generation and have yielded important insights into the free radical reactions in this setting. 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 cannot necessarily be extrapolated to the latter. Further, isolated heart preparations have not been specifically demonstrated to be associated with myocardial stunning, which is defined as a persistent but ultimately reversible depression of contractility.[5] The present studies extend these prior obserations[19-21] by demonstrating, in the intact animal subjected to reversible regional ischemia, a) that free radicals are produced, b) that antioxidant enzymes actually block production of these radicals, and c) that this decrease in radical production results in a decrease in postischemic mechanical dysfunction. Further, the present studies define the time-course of free radical production in the stunned myocardium and characterize the relation of radical generation to the severity of the ischemic insult.

The nature of the radicals trapped by PBN remains to be determined. Analysis of the hyperfine coupling constants suggests that the signals are due to a mixture of different radical adducts, since (with chloroform as the solvent) the a_{β}^{H} values (2.67–2.79 G) are smaller than those generally observed with carbon-centered radical adducts of PBN (> 3.0 G), whereas the a_N values (14.75–15.00 G) are larger than those characteristic of oxygen-centered radical adducts of PBN (< 14.50 G)[18] (Figure 2). In

addition to the intermediate values of the coupling constants, the asymmetry of the central doublets (Figure 1) implies the presence of at least two radical adducts with different g values. Since these adducts are soluble in nonaqueous solvents and resemble the lipid radical adducts of PBN observed in other systems,[18] they may be derived from membrane lipids. The PBN adducts observed are not those of $\cdot O_2^-$ or $\cdot OH$. The EPR spectra (Figure 2) are consistent with a mixture of different secondary radicals, which could be generated by various mechanisms. For example, it is well known that initially-formed oxygen radicals can react with membrane lipids; this oxy-radical-initiated lipid peroxidation gives rise to alkyl as well as alkoxy radicals,[22] both of which can be trapped by PBN forming reasonably persistent adducts.[18] Thus, as has been suggested,[22] the generation of initial oxygen radicals may be in itself a relatively brief event, but these species could initiate a self-propagating process of lipid peroxidation that could continue in the membrane lipids even after the production of the initiating radical species has ceased. By scavenging the initially-formed oxygen metabolites, SOD plus catalase and MPG may prevent the initiation of these secondary reactions and thus decrease the production of the secondary radicals trapped by PBN.

The results of Experiment 3 demonstrate that a substantial portion (apparently most) of the cellular damage responsible for myocardial stunning develops in the initial second of reperfusion. The results further demonstrate that production of free radicals immediately after reflow is necessary for severe stunning to develop. MPG enhanced the recovery of function to a similar extent when it was administered before ischemia (group I) or immediately before reperfusion (group II). However, no functional improvement was observed when MPG was administered 1 min after reperfusion (group III). Further, the enhancement of functional recovery effected by MPG given immediately before reperfusion (group II) was associated with marked suppression of free radical production *in vivo* during the initial seconds of reflow (group V). The failure of MPG given 1 min after reperfusion to improve functional recovery (group III) was associated with a delay in the suppression of free radical production, such that the initial reflow-induced burst was unaffected (group VI). Thus, Experiment 3 identifies a narrow time-window (first 60 s of reperfusion) during which most of the injury responsible for stunning apparently develops. Interestingly, experiment 3 shows that when antioxidant therapy is delayed until 1 min after reflow, radical generation during the rest of the reperfusion phase can still be suppressed (group VI) but mechanical dysfunction is not attenuated (group III). This suggests that the free radicals critical in the pathogenesis of myocardial stunning are those produced immediately after restoration of flow.

The measurements of PBN concentration demonstrate that, with our intravenous administration protocol (Experiment 2), reasonably steady levels > 0.5 mM are achieved in the systemic blood plasma during the early phase of reperfusion (i.e., between 1 and 10 min of reperfusion). The relatively long elimination half-life of PBN (4–5 h) explains the fact that PBN radical adducts continue to be produced in the stunned myocardium for hours after the infusion of the spin trap is discontinued (Figures 1, 2, 3, 5). Our data regarding the kinetics of PBN should be useful for designing and interpreting experiments in which the spin trap is administered to anesthetized dogs.

The studies reported herein must be interpreted with caution because they were performed in open-chest preparations, in which anesthesia, surgical trauma, and the attending neuro-humoral perturbations can potentially interfere with the results. It is, therefore, critical to determine whether the findings obtained in open-chest prepara-

tions can be reproduced in conscious preparations, in which any potential artifact due to anesthesia, trauma, and other neuro-humoral abnormalities is eliminated. In our opinion, the oxygen radical hypothesis of stunning must be tested in conscious animals before it can be definitively accepted.

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Accepted by Prof. E.G. Janzen