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INHIBITORY EFFECT OF A HYDROPHILIC α-TOCOPHEROL ANALOGUE, MDL 74,405, ON GENERATION OF FREE RADICALS IN STUNNED MYOCARDIUM IN DOGS

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A previous study has demonstrated that the hydrophilic (α-tocopherol analogue, MDL 74,405, attenuates postischemic myocardial dysfunction ("stunning") in dogs. The present study was undertaken to determine directly whether the salutary effect of this drug on myocardial stunning results from inhibition of the generation of oxygen-derived free radicals. Open-chest dogs undergoing a 15-min coronary artery occlusion and 3 h of reperfusion received an intravenous infusion of either saline (controls, $n = 7$) or MDL 74,405 ($n = 6$) starting 30 min before coronary occlusion and ending 60 min after reflow at a dose of 0.3 mg/kg/h. To measure free radical production, all dogs received an intravenous infusion of the spin trap α-phenyl *N-tert*-butyl nitrone (PBN) and local coronary venous plasma was analyzed by electron paramagnetic resonance (EPR). In control dogs, the myocardial production of PBN adducts exhibited an initial burst immediately after the onset of reflow and remained elevated until 10 min after reperfusion. Dogs treated with MDL 74,405 demonstrated a marked decrease in PBN adduct production. This effect of MDL 74,405 could not be attributed to nonspecific factors such as differences in ischemic zone size, collateral flow, arterial pressure, heart rate, coronary flow or other hemodynamic variables. These results demonstrate that the hydrophilic vitamin E analogue, MDL 74,405, inhibits free radical generation after myocardial ischemia-reperfusion *in vivo*. This finding provides direct evidence that the salutary effects of MDL 74,405 on myocardial stunning are due to attenuation of oxidative stress.

KEY WORDS: Free radical generation, hydrophilic α-tocopherol analogue, myocardial stunning, open-chest dogs.

INTRODUCTION

It is now widely accepted that oxygen-derived free radicals play an important role in the pathogenesis of postischemic myocardial dysfunction, or myocardial "stunning," after brief, reversible ischemia.[1-8] Oxygen-derived free radicals generated during conditions of oxidative stress can react with a variety of cellular components, leading

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to alterations in membrane permeability and enzyme activity. An important step in the toxic action of free radicals on myocardial (and other) tissues is the peroxidation of polyunsaturated fatty acids in cellular membrane, with consequent loss of cellular or organelle function.[9] α-Tocopherol (vitamin E) is the most important naturally occurring, lipid soluble antioxidant in human blood and tissues.[10–12] It plays an important role in protecting tissues against the deleterious consequences of oxidative injury and especially against lipid peroxidation.[13,14] Prolonged myocardial ischemia is associated with a decrease in tissue α-tocopherol levels,[15,16] probably because of a consumption of the natural defense systems against oxidative injury. These considerations suggest that vitamin E should be beneficial in myocardial ischemia-reperfusion injury. However, due to the marked lipophilicity of vitamin E and the consequent slow incorporation into tissues, acute administration of this agent may produce very low levels of the antioxidant in the ischemic-reperfused tissue, necessitating either protracted pretreatment or infusion of pharmacologic (and potentially toxic) doses.[17] To overcome this problem, an N,N,N,-trimethyl-ethanaminium analogue of α-tocopherol, MDL 74,405 (2S-(-)-3,4 dihydro-6-hydroxy-N,N,N,2,5,7,8,-heptamethyl-2H-1-benzopyran-2-ethanaminium, 4-methylbenzenesulfonate), has been recently developed (Figure 1). This compound retains the antioxidant properties of α-tocopherol but is hydrophilic and, in addition, is preferentially taken up in the myocardium as a result of the introduction of quaternary ammonium terminals.[18,19]

In a previous study,[20] we have demonstrated that MDL 74,405 produces a significant attenuation of myocardial stunning after a 15-min coronary occlusion in open-chest dogs. In that study, we hypothesized that MDL 74,405 exerts its protective effects by attenuating oxyradical-mediated injury. However, there is no information concerning the ability of MDL 74,405 to interfere with free radical reactions *in vivo*. Furthermore, to our knowledge, there has been no direct demonstration that vitamin E or any of its analogues inhibits free radical generation after myocardial ischemia-reperfusion injury. The purpose of this study was to determine whether MDL 74,405 inhibits the generation of oxygen-derived free radicals in the same model of myocar-

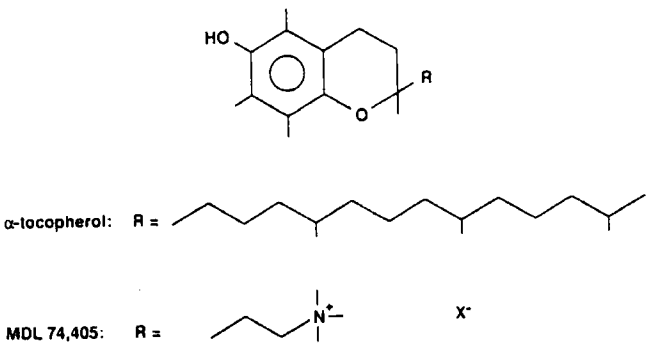

FIGURE 1 Molecular structure of α-tocopherol and MDL 74,405 (2S-(-)-3,4 dihydro-6-hydroxy-N,N,N,2,5,7,8,-heptamethyl-2H-1-benzopyran-2-ethanaminium,4-methylbenzenesulfonate), an N,N,N,-trimethyl-ethanaminium analogue of α-tocopherol. Note that in MDL 74,405 the 2-position lipophilic side chain that is responsible for the intercalation of α-tocopherol into lipid layers is replaced by a short chain (which imparts greater hydrophilicity) containing a quaternary ammonium end (which imparts cardioselectivity).

dial stunning in which it was previously found to improve the recovery of function. To this end, we directly measured free radical production with the spin trap α-phenyl *N-tert*-butyl nitrone (PBN) and electron paramagnetic resonance (EPR) spectroscopy in the presence or absence of MDL 74,405.

METHODS

Experimental Preparation The experimental preparation and techniques employed in this study have been described in detail previously.[4-7] Briefly, pentobarbital-anesthetized dogs of either sex weighing 17–29 kg were instrumented with a snare and a Doppler flow probe around the mid left anterior descending coronary artery (LAD), catheters in the left carotid artery, left atrial appendage, and jugular vein, and a 6-F Millar pressure transducer in the left ventricular (LV) cavity via an apical stab wound. A 27-gauge intracoronary needle was placed just distal to the snare occluder for infusion of PBN. To prevent clotting, heparin was given immediately after insertion of the needle (3,000 U iv) and continuously thereafter (500 U/h). An 8-F Sones catheter was introduced into the coronary sinus and advanced into the anterior interventricular vein. In its final position, the tip of the catheter was at least 0.5 cm distal to the intracoronary needle, so that any contamination of blood samples with venous effluent from other vascular beds was minimized.[5]

Protocol for Administration of MDL 74,405 and PBN MDL 74,405 was supplied in powder form by Marion Merrell Dow Inc. (Merrell Dow Research Institute, Strasburg, France) and was dissolved in normal saline. The drug was infused for 105 minutes at a dose of 0.3 mg/kg/h (at a rate of 1.0 ml/min) via a jugular vein starting 30 min before coronary occlusion, continuing during 15 minutes of occlusion and ending 60 min after reperfusion (total dose: 0.53 mg/kg). This is the same dose that was shown to attenuate myocardial stunning in this model.[20] PBN (Sigma; purity, 99% by gas chromatographic analysis) was dissolved in normal saline at a concentration of 4 mg/ml and infused through the intracoronary needle directly into the artery to be occluded. The infusion was started 5 min before occlusion and ended 10 min after reperfusion. Since coronary blood flow varied from dog to dog and from time to time during the course of PBN infusion, the infusion rate was normalized to flow, so as to achieve a similar concentration of PBN (240 μg/ml or 1.3 mM) in the coronary arterial blood in all dogs.[5] The volume of PBN solution infused was equal to 6% of coronary blood flow. During occlusion, the rate of infusion was decreased to 10% of preocclusion values until 20 s before reperfusion, when the preocclusion rate was resumed.

Experimental Protocol Dogs in the treated group received MDL 74,405 whereas control dogs received vehicle at the same rate and with the same timing as the MDL 74,405 solution. All dogs in both groups were given PBN and subjected to a 15-min LAD occlusion followed by 3 h of reperfusion. To measure regional myocardial blood flow, radioactive microspheres were injected 10 min into the coronary occlusion.[21] At the end of the study, the size of the occluded coronary vascular bed was determined by a previously described postmortem perfusion technique.[21]

Serial blood samples for measurement of plasma MDL 74,405 levels were collected from the arterial catheter at baseline (5 min before drug infusion), 5 min before coronary occlusion (25 min into drug infusion), 3 min after reperfusion, and

1 h after reperfusion (immediately before the end of the infusion). Blood samples were centrifuged and the plasma frozen at −70°C. Myocardial biopsies were obtained with a Tru-Cut needle from the ischemic and nonischemic regions after the animals were sacrificed (3 h after reperfusion) and immediately frozen in liquid nitrogen. MDL 74,405 level in plasma and tissue was determined with high-performance liquid chromatography; the results were expressed as ng/ml of plasma and $\mu g/g$ of tissue, respectively.

EPR Analysis Blood samples (6 ml each) were simultaneously drawn from the Sones catheter (anterior interventricular vein) and the arterial catheter over 60 s and immediately centrifuged. The plasma specimens were frozen at −70°C for subsequent analysis by EPR spectroscopy. The techniques used to detect spin adducts of free radicals and to quantity the myocardial release of these species into the coronary venous effluent blood have been described in detail.[5,22] The myocardial production of spin adducts at a specific time point was expressed in $U \cdot min^{-1} \cdot g^{-1}$ (arbitrary units per minute per gram of reperfused myocardium);[4-7] the total cumulative myocardial production of PBN adducts over a given interval of time was calculated by integrating the measurements obtained at individual time points.[4-7]

It is unlikely that the freezing and thawing of the samples caused significant changes in the EPR signals observed. Although the ·OH adducts of PBN are known to be unstable, the EPR spectra seen in this study are not those of the ·OH radicals. As elaborated in the Discussion, the PBN adducts observed in our system are probably those of alkyl radicals; PBN adducts of alkyl radicals are known to be reasonably persistent.[22,29,31,32] Most importantly, in selected samples that were thawed with an aliquot examined by EPR spectroscopy, frozen and thawed again and re-examined a few days later, we found no significant changes in adduct intensity.

Statistical Analysis All values are reported as mean ± SEM. Means were compared with the two-tailed Student's *t* test for unpaired data. Myocardial production of PBN adducts was analyzed by nonparametric methods (Wilcoxon's signed-rank test) because these data did not follow a normal distribution.[23] A P value <0.05 was considered statistically significant.

RESULTS

Arterial Blood Gases, Hematocrit and Body Temperature As shown in Table 1, arterial PO_2, pH and hematocrit and esophageal temperature were within physiological range in both groups throughout the experimental protocol.

TABLE 3
Regional myocardial blood flow ($ml \cdot min \cdot^{-1} \cdot g^{-1}$)

Group	N	Ischemic zone flow			Nonischemic zone flow			IZF/NZF
		Epi	Endo	TM	Epi	Endo	TM	(×100)
Control	7	0.35 ± 0.21	0.14 ± 0.09	0.24 ± 0.15	1.62 ± 0.20	1.77 ± 0.24	1.69 ± 0.22	16.2 ± 11.3%
Treated	6	0.15 ± 0.06	0.17 ± 0.07	0.16 ± 0.06	1.08 ± 0.13	1.36 ± 0.13	1.22 ± 0.17	12.5 ● 4.6%

Data are means ± SEM. Epi, epicardium; Endo, endocardium; TM, transmural; IZF/NZF, ratio of average transmural ischemic zone flow to simultanous average transmural nonischemic zone flow.

Hemodynamic Variables Heart rate, LAD flow, and peak positive and negative LVdP/d*t* did not differ significantly between the two groups at baseline, before coronary occlusion, during occlusion, or at any time point after reperfusion (Table 2). Mean arterial pressure tended to be lower in the treated group, but the differences were statistically significant only at 2 h ($P < 0.05$) and 3 h ($P < 0.05$) after reperfusion.

Occluded Bed Size and Regional Myocardial Blood Flow The size of the occluded vascular bed was similar in the treated group (29.5 ± 4.3 g; 25.4 ± 0.9% of LV weight) and in the control group (22.7 ± 2.6 g; 26.7 ± 3.6% of LV weight). Collateral blood flow to the ischemic zone during LAD occlusion did not differ significantly between the two groups ($P = 0.76$), although it tended to be lower in the treated group (Table 3). Since MDL 74,405 had no hemodynamic effects, the trend for collateral flow to be lower in treated dogs most likely reflected random differences in native collaterals between the two groups.

Myocardial Production of PBN Adducts Figure 2 shows the time course of release of PBN adducts from the ischemic-reperfused region. In control dogs, EPR signals characteristic of radical adducts of PBN appeared in the coronary venous effluent during occlusion. The release of PBN adducts exhibited an initial burst immediately after the onset of reperfusion, peaking at 4 min after reperfusion, and then declined, but continued up to 3 h thereafter (Figure 2). In the dogs treated with MDL 74,405, PBN adduct production was markedly reduced compared with the control group (Figure 2); the differences were statistically significant ($P < 0.05$) at 1, 2, 4, 5, and 10 min of reperfusion. In the treated group, the total cumulative release of PBN adducts in the first 5 min, 10 min, and 3 h of reperfusion was decreased by 68%, 73% and 83%, respectively, compared with the control group (Figure 3).

A free radical generating mixture [plasma supplemented with ethanol 100 mM, Fe^{2+} 5 mM, H_2O_2 10 mM (all final concentrations)] was incubated with PBN (1 mM) in the presence or absence of MDL 74,405 (0.43 μM). No difference was

TABLE 1
Basic physiological variables

Group	N	Baseline	Reperfusion		
			1 h	2 h	3 h
pH					
Control	7	7.48 ± 0.02	7.39 ± 0.01	7.38 ± 0.01	-
Treated	6	7.42 ± 0.03	7.39 ± 0.04	7.42 ± 0.03	-
PO_2, mmHg					
Control	7	82 ● 9	111 ± 9	102 ± 3	-
Treated	6	96 ± 3	97 ± 2	94 ● 7	-
Hematocrit, %					
Control	7	44.0 ± 2.4	-	-	40.7 ± 2.4
Treated	6	47.2 ± 1.4	-	-	42.3 ± 5.6
Esophageal Temperature, °C					
Control	7	37.0 ± 0.4	37.0 ± 0.4	37.1 ± 0.3	37.3 ± 0.4
Treated	6	36.9 ± 1.1	37.6 ± 0.2	37.9 ± 0.1	37.9 ± 0.1

Data are means ± SEM.

TABLE 2
Effect of MDL 74,405 on Hemodynamic Variables

Group	N	BSL	POC	OCC	Reperfusion			
					30 min	1 h	2 h	3 h
HR, beats/min								
Control	7	159 ± 6	163 ± 6	163 ± 4	164 ± 6	159 ± 10	167 ± 4	171 ± 3
Treated	6	176 ± 7	170 ± 12	170 ± 9	166 ± 14	167 ± 10	163 ± 7	167 ± 9
MAP, mmHg								
Control	7	117 ± 11	115 ± 11	109 ± 9	110 ± 10	108 ± 14	113 ± 4	112 ± 5
Treated	6	95 ± 7	105 ± 9	92 ± 7	98 ± 9	91 ± 10	94 ± 6*	90 ± 5*
Coronary Flow, ml/min								
Control	7	18.8 ± 2.7	18.1 ± 3.6	0	14.4 ± 1.7	15.1 ± 1.6	14.0 ± 2.2	13.2 ± 2.1
Treated	6	18.9 ± 3.1	18.1 ± 3.3	0	21.0 ± 4.7	18.2 ± 4.5	19.1 ± 4.3	17.6 ± 2.9
LV dP/dt_{max}, mmHg/s								
Control	7	2000 ± 149	1867 ± 126	1817 ± 118	1717 ± 193	1633 ± 241	1900 ± 154	1980 ± 149
Treated	6	1788 ± 189	1988 ± 233	1650 ± 241	1725 ± 281	1650 ± 230	1938 ± 346	1713 ± 344
LV dP/dt_{min}, mmHg/s								
Control	7	2975 ± 324	2892 ± 290	2475 ± 321	2608 ± 396	2542 ± 450	2880 ± 281	3110 ± 323
Treated	6	2413 ± 357	2689 ± 376	2413 ± 364	2550 ± 523	2413 ± 458	2489 ± 413	2413 ± 394

Data are means ± SEM. BSL, baseline; POC, pre-occlusion; OCC, during occlusion; HR, heart rate; MAP, mean arterial pressure; LV dP/dt_{max}, maximal rate of left ventricular pressure rise; LV dP/dt_{min}, maximal rate of left ventricular pressure fall. $^*P < 0.05$ vs. Control.

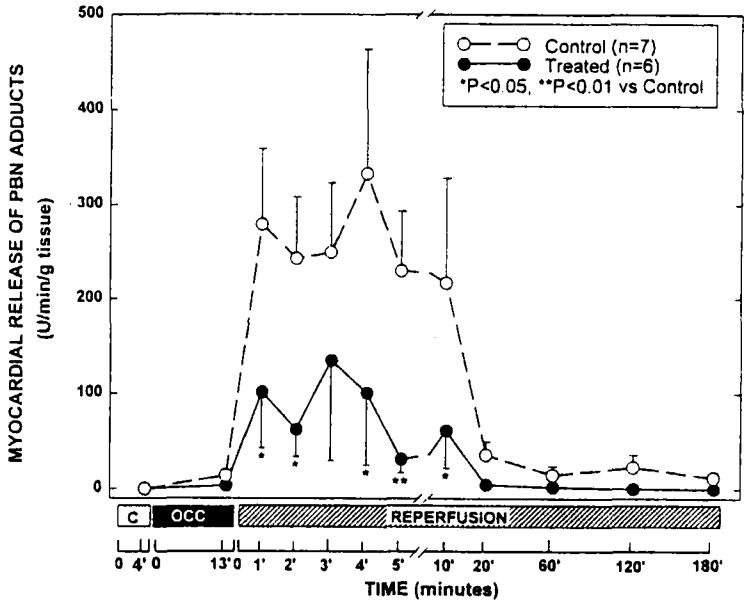

FIGURE 2 Graph showing the time course of myocardial release of α-phenyl *N-tert*-butyl nitrone (PBN) adducts in control dogs (dashed line with open circles, $n = 7$) and in MDL 74,405 treated dogs (continuous line with solid circles, $n = 6$). Data are mean ± SEM. See text for explanation of units used. C, control; OCC, occlusion; U, units.

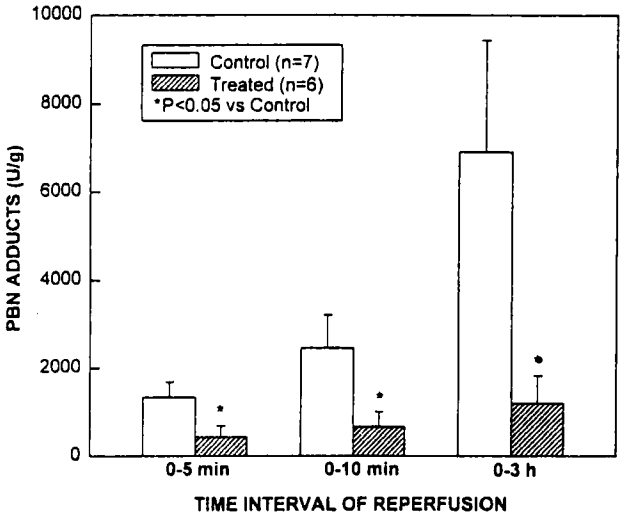

FIGURE 3 Bar graph showing the total cumulative myocardial release of α-phenyl *N-tert*-butyl nitrone (PBN) adducts during the first 5 minutes, 10 minutes, and 3 hours of reperfusion in control dogs (open bar, $n = 7$) and MDL 74,405 treated dogs (hatched bar, $n = 6$). The cumulative release of PBN adducts is expressed in arbitrary units per gram of myocardium (see text for explanation). Data are mean ± SEM.

observed in the magnitude of the EPR signals, indicating that MDL 74,405 does not react directly with the spin adducts of PBN.

Plasma and Tissue Concentration of MDL 74,405 The concentration of MDL 74,405 in the plasma was 0.20 ± 0.05 μM at 5 min before occlusion, 0.21 ± 0.05 μM at 3 min after reperfusion, and 0.28 ± 0.06 μM at 1 h after reperfusion. Thus, the plasma concentration of MDL 74,405 achieved a steady state soon after the start of the infusion. At the end of the reperfusion phase, MDL 74,405 levels in the ischemic-reperfused and nonischemic myocardium were 4.75 ± 0.43 and 5.62 ± 0.65 nmol/g, respectively. Thus, similar to our previous study,[20] the myocardial tissue concentration was much higher than the plasma concentration, confirming the preferential uptake of this drug into myocardial tissue.

DISCUSSION

This study demonstrates that the intravenous administration of MDL 74,405, a hydrophilic α-tocopherol analogue, reduces the production of PBN adducts in the stunned myocardium in the intact dog. The reduction of PBN adduct production became significant immediately after the onset of reperfusion and persisted for the rest of the reperfusion phase. The decrease in PBN adduct production was not associated with alterations in systemic hemodynamics or coronary flow, excluding the possibility that it may have been caused by changes of the extrinsic variables that may modulate the generation of free radicals. Thus, the inhibitory effect of MDL 74,405 on the production of PBN adducts indicates a direct inhibition of free radical reactions in this model. These data demonstrate for the first time that MDL

74,405 can effectively scavenge free radicals *in vivo* and provide direct evidence that the previously observed[20] salutary effect of this drug on postischemic myocardial dysfunction is associated with attenuation of oxyradical-mediated injury.

Vitamin E is the most important lipid-soluble antioxidant in human blood and tissues.[10–12] Together with the water-soluble antioxidant ascorbic acid (vitamin C), it constitutes a regenerative system that is effective in suppressing peroxidation of membrane lipids.[24] Several studies[13,14,17,25] have suggested that administration of vitamin E in the setting of myocardial ischemia and reperfusion is beneficial, and endogenous α-tocopherol levels have been found to be decreased in settings characterized by free radical induced injury.[16] Acute administration of α-tocopherol, however, is not feasible due to its high lipophilicity and resulting slow tissue incorporation.[17] Consequently, hydrophilic vitamin E analogues, such as MDL 74,405, have been developed[18,19,26,27] by replacing the 2-position lipophilic side-chain (responsible for intercalation of α-tocopherol into lipid layers) with a short chain that is likely to impart greater hydrophilicity while leaving the antioxidant properties of α-tocopherol intact.[18] In addition, it has been found that the addition of a quaternary amine end resulted in preferential myocardial accumulation after systemic administration.[18,28] These analogues were reported to diffuse into the intracellular space, in contrast to vitamin E which remains limited to cellular membranes due to its lipophilicity.[28]

Grisar and Petty[18,19,26,27] have previously shown salutary effects of compounds structurally related to MDL 74,405 in rat models of myocardial infarction. We[20] studied MDL 74,405 in a canine model of transient ischemia (15-minute coronary occlusion in open chest dogs) and found it exerts a significant beneficial effect on myocardial stunning. In the present study, in which we used the same model of myocardial stunning and the same dose of MDL 74,405, we observed a significant decrease in the production of PBN adducts, indicating an inhibitory effect of MDL 74,405 on free radical generation *in vivo*. 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 MDL 74,405 suppressed the formation of PBN adducts at the same dose and under the same experimental conditions in which it inhibited postischemic dysfunction. The correlation between these two effects further corroborates the concept that free radical species play a causative role in the depression of contractility associated with myocardial stunning.

As discussed previously,[4–7,29] the precise nature of the radicals trapped by PBN in our system remains to be determined. The spectral features of the EPR signals observed are not those of $\cdot$OH adducts. Analysis of the EPR spectra suggests the trapping by PBN of a mixture of carbon-centered lipid (alkyl) radicals.[4–7,29] In this regard, it is well established that initially-formed oxyradicals can react with membrane lipids to produce secondary carbon-centered radicals,[9] which can be trapped by PBN forming reasonably persistent adducts.[22] The fact that the PBN adducts are soluble in nonaqueous solvents and resemble the lipid radical adducts of PBN observed in other systems[22] further supports the concept that they are derived from membrane lipids. Accordingly, the marked suppression of PBN adduct formation provides evidence that MDL 74,405 decreased the rate of lipid peroxidation. The ability of MDL 74,405 to scavenge $\cdot$OH[30] suggests that the drug may be acting to prevent the initiation of the lipid peroxidation process by $\cdot$OH. It is also possible, however, that MDL 74,405 prevented lipid peroxidation (and, consequently, formation of PBN radical adducts) by scavenging $\cdot O_2^-$[30] and/or lipid peroxy radicals.[30]

In summary, our results demonstrate that the hydrophilic vitamin E analogue, MDL 74,405, reduces the production of PBN radical adducts after myocardial ischemia-reperfusion *in vivo*. This finding provides direct evidence that the salutary effect of MDL 74,405 on myocardial stunning[20] is due to attenuation of oxidative stress. In view of the beneficial effects of hydrophilic α-tocopherol analogues, including MDL 74,405, on myocardial ischemia-reperfusion injury,[18-20,26-28] these drugs may have therapeutic potential in clinical settings in which oxyradical-induced damage may contribute to the development of myocardial stunning, such as acute myocardial infarction, coronary bypass surgery, and cardiac transplantation.[14] The hydrophilic properties of these drugs, the preferential myocardial accumulation,[18,28] the intracellular penetration,[28] and the relatively long plasma half-life (Marrion Merrell Dow, data on file) may provide distinct advantages for clinical use in these situations.

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