Evidence for an Essential Role of Reactive Oxygen Species in the Genesis of Late Preconditioning Against Myocardial Stunning in Conscious Pigs

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Abstract

Conscious pigs underwent a sequence of 10 2-min coronary occlusions, each separated by 2 min of reperfusion, for three consecutive days (days 1, 2, and 3). On day 1, pigs received an i.v. infusion of a combination of antioxidants (superoxide dismutase, catalase, and *N*-2 mercaptopropionyl glycine; group II, $n = 9$), nisoldipine (group III, $n = 6$), or vehicle (group I [controls], $n = 9$). In the control group, systolic wall thickening (WTh) in the ischemic-reperfused region on day 1 remained significantly depressed for 4 h after the 10th reperfusion, indicating myocardial "stunning." On days 2 and 3, however, the recovery of WTh improved markedly, so that the total deficit of WTh decreased by 53% on day 2 and 56% on day 3 compared with day 1 ($P < 0.01$), indicating the development of a powerful cardioprotective response (late preconditioning against stunning). In the antioxidant-treated group, the total deficit of WTh on day 1 was 54% less than in the control group ($P < 0.01$). On day 2, the total deficit of WTh was 85% greater than that observed on day 1 and similar to that observed on day 1 in the control group. On day 3, the total deficit of WTh was 58% less than that noted on day 2 ($P < 0.01$). In the nisoldipine-treated group, the total deficit of WTh on day 1 was 53% less than that noted in controls ($P < 0.01$). On days 2 and 3, the total deficit of WTh was similar to the corresponding values in the control group. These results demonstrate that: (*a*) in the conscious pig, antioxidant therapy completely blocks the development of late preconditioning against stunning, indicating that the production of reactive oxygen species (ROS) on day 1 is the mechanism whereby ischemia induces the protective response observed on day 2; (*b*) antioxidant therapy markedly attenuates myocardial stunning on day 1, indicating that ROS play an important pathogenetic role in postischemic dysfunction in the porcine heart despite the lack of xanthine oxidase; (*c*) although the administration of a calcium-channel antagonist (nisoldipine) is as effective as antioxidant therapy in attenuating myocardial stunning on day 1, it has no effect on late preconditioning on day 2, indicating that the ability of antioxidants to block late preconditioning is not a nonspecific result of the mitigation of postischemic dysfunction on day 1. Generation of ROS during reperfusion is generally viewed as a deleterious process. Our finding that ROS contribute to the genesis of myocardial stunning but, at the same time, trigger the development of late preconditioning against stunning supports a complex pathophysiological paradigm, in which ROS play an immediate injurious role (as mediators of stunning) followed by a useful function (as mediators of subsequent preconditioning). (*J. Clin. Invest.* 1996. 97:562–576.) Key words: superoxide dismutase • catalase • *N*-2-mercaptopropionyl glycine • nisoldipine • oxygen radicals

Introduction

A brief episode of myocardial ischemia renders the heart remarkably resistant to a subsequent episode of ischemia, a phenomenon termed ischemic "preconditioning" (1–5). The protection afforded by preconditioning develops within minutes after the first ischemic insult and lasts for $\sim$ 1 h (3, 4, 6, 7). This preconditioning phenomenon is highly effective in limiting necrosis (1, 6–8) and arrhythmias (9, 10), but has failed to attenuate postischemic dysfunction (myocardial "stunning") in studies (11–13) in which stunning was induced shortly after the preconditioning protocol.

Recently, we (14) have described a form of ischemia-induced protection that can be referred to as late preconditioning against stunning. Using conscious pigs, we found that a sequence of ten 2-min occlusion/2-min reperfusion cycles induces severe myocardial stunning, but when the same sequence is repeated 24 h later, the severity of stunning is markedly reduced (by $\sim$ 50%). The resistance to stunning is associated with an increase in the myocardial levels of heat stress protein (HSP)[1] 70 (14). The protection dissipates within 10 d but can be reinduced by another sequence of 10 2-min occlusion/2-min reperfusion cycles (14). Unlike early preconditioning against infarction, this form of protection does not appear to be mediated by activation of adenosine receptors, since it is not prevented by 8-*p*-sulfophenyl theophylline (SPT) (14). Thus, a powerful intrinsic cardioprotective response is activated by brief coronary occlusions in conscious pigs via mechanisms that are essentially unknown. The present study was undertaken to elucidate the mechanism of this newly discovered phenomenon of late preconditioning against stunning.

We postulated that oxidative stress may play a causative role. Myocardial reperfusion after a brief (15 min) episode of ischemia causes generation of oxygen-derived free radicals (15–22) and repetitive 5-min coronary occlusion/10-min reper-

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1. *Abbreviations used in this paper:* HSP, heat stress protein; LAD, left anterior descending; LV, left ventricular; MPG, mercaptopropionyl glycine; VF, ventricular fibrillation; WTh, wall thickening.

fusion cycles are associated with repetitive bursts of free radical production (23). Since oxidative stress is known to induce the synthesis of cardioprotective proteins, such as antioxidant enzymes (24–30) and HSPs (31–35), and since these proteins could theoretically mediate the protection observed 24 h after the initial ischemic challenge in our model, we hypothesized that the molecular adaptations that lead to late preconditioning against stunning are initiated by the exposure to increased levels of reactive oxygen species during the preconditioning ischemia. The primary goal of the present study was to test this hypothesis. To this end, we used the same model previously (14), i.e., conscious pigs subjected to a sequence of ten 2-min occlusion/2-min reperfusion cycles for three consecutive days (days 1, 2, and 3), and investigated whether administration of antioxidant therapy during the first ischemic challenge (day 1) prevented the protection observed during the second ischemic challenge (day 2).

An additional goal of the present study was to explore the role of oxygen radicals in myocardial stunning in the pig. Considerable evidence supports the concept that oxyradicals contribute to the pathogenesis of myocardial stunning in the dog (36) and that xanthine oxidase is an important source of reactive oxygen metabolites in this species (37). It is, however, controversial whether the human heart contains xanthine oxidase and, if so, how the levels of this enzyme compare to those found in the canine heart (38–42). Thus far, no report has suggested that oxyradicals contribute to stunning in large mammals that lack myocardial xanthine oxidase activity, and no information is available on the role of oxyradicals in conscious models other than the dog. Therefore, we sought to elucidate this issue by determining whether treatment with antioxidants attenuates stunning in the porcine heart.

The third goal of this study was to investigate whether the effects of antioxidant therapy on late preconditioning on day 2 reflect the decrease in wall motion abnormalities on day 1. Since we found that antioxidants alleviate myocardial stunning, we felt it was important to rule out the possibility that their ability to block late preconditioning may be simply a consequence of their ability to ameliorate the contractile dysfunction associated with stunning rather than a result of antioxidant activity. Accordingly, we determined whether nisoldipine, a calcium channel antagonist known to mitigate stunning, also blocks late preconditioning in our model.

Methods

A total of 40 pigs were used for this investigation. Since the experimental preparation and techniques have been previously described in detail (14), they will be briefly summarized here.

Experimental preparation. Domestic pigs of either sex (weight at surgery, 24.4±0.9 kg) were anesthetized with methoxyflurane and instrumented under sterile conditions with a hydraulic occluder and a Doppler flow velocity probe around the mid left anterior descending coronary artery (LAD), a Konigsberg (P7) high-fidelity micromanometer in the left ventricular (LV) cavity (in four pigs), three Doppler wall thickening (WTh) probes in the center of the region to be rendered ischemic and one in an area remote from it (posterior LV wall), and Tygon catheters in the left atrium, right ventricle and aorta (14). Two insulated copper wires were sutured to the right ventricle to record the electrocardiogram. The chest was closed in layers and a small tube was left in the thorax to evacuate air and fluid postoperatively. Antibiotics were administered i.v. before surgery and daily for 7 d thereafter (cefazolin 30 mg/kg b.i.d. and gentamicin 0.7 mg/kg b.i.d.). Arterial blood gases, hematocrit, rectal temperature, and heart rate were measured daily after instrumentation to ensure that the animals had fully recovered from the surgical procedure. The catheters were flushed daily till the end of the protocol. All pigs were allowed to recover for a minimum of 9 d (average, 14.1±3.9 d) after surgery and were trained for at least 6 d to lie quietly for 6 h in a specially designed cage.

General experimental protocol. The general protocol has been detailed previously (14). Throughout the experiment, pigs were studied while lying quietly in a cage in a quiet, dimly lit room. All pigs underwent 3 d of sham studies followed by 3 d of LAD occlusion studies (days 1, 2, and 3, respectively), so that the animals were studied for 6 consecutive days. On the first day of sham studies, the pigs were sedated with diazepam (initial dose: 1.5–2.5 mg/kg i.v. over 60 min; subsequent additional doses were given as needed to maintain sedation) and kept in the cage for 7 h (interval corresponding to the average duration of the study on the days when the LAD was occluded) while hemodynamic and WTh were monitored. This same protocol was repeated on the next 2 d. On the following day, i.e., on the first day of LAD occlusion (day 1), the same protocol used in the 3 d of sham studies was repeated but in addition, the pigs underwent a sequence of 10 2-min LAD occlusions, each separated by 2 min of reperfusion, starting 15 min after the administration of diazepam. This same protocol was repeated on the next 2 d (days 2 and 3). Thus, the difference between the 3 d of sham studies and the 3 d of coronary occlusion was the induction of myocardial ischemia and reperfusion. The purpose of performing sham studies for three consecutive days before coronary occlusion was to ensure that systemic hemodynamics and WTh would be stable from one day of LAD occlusion to the next, so that any changes in the duration and/or severity of myocardial stunning after the first day of LAD occlusion would not be ascribable to hemodynamic changes or to variability in regional myocardial function (14).

On each day of coronary occlusion, hemodynamic and WTh measurements were obtained before administration of diazepam and antioxidants (baseline), 14 min after administration of diazepam, i.e., immediately before LAD occlusion (preocclusion), 1 min into the 1st and 10th LAD occlusions, 1 min into each of the first nine reperfusions, and 5, 15, 30 min and 1, 2, 3, 4, and 5 h after the 10th reperfusion. To measure regional myocardial blood flow, radioactive microspheres were injected as previously described (43) 30–60 s into the 5th LAD occlusion.

Pilot studies. Our final protocol for administering superoxide dismutase (SOD) and catalase was selected on the basis of pilot studies in three pigs in which the enzymes were administered as a continuous i.v. infusion (270 U/kg per minute for SOD and 2,125 U/kg per minute for catalase), without bolus injections, from 30 min before the first occlusion to 30 min after the 10th reperfusion (these pigs also received *N*-2-mercaptopropionyl glycine (MPG, 100 mg/kg per hour) i.v. from 60 min before the 1st occlusion to 60 min after the 10th reperfusion). Although the total doses of SOD and catalase were the same as those given with our final protocol (27,000 U/kg and 212,500 U/kg, respectively), the infusion of the enzymes in these pilot studies failed to attenuate myocardial stunning on day 1 and to prevent the development of preconditioning against stunning on day 2 (as compared with a previously studied group of untreated pigs) (14). The plasma levels of SOD and catalase achieved in these three pilot pigs were lower than those measured in prior studies (16, 43) in dogs, in which SOD plus catalase effectively mitigated myocardial stunning. On the basis of these results, we postulated that higher circulating levels of the enzymes were necessary to attenuate postischemic myocardial dysfunction. Consequently, we developed a protocol consisting of two fast bolus injections combined with a continuous slower infusion, which was designed to rapidly raise the plasma levels of SOD and catalase just before the first coronary occlusion and to maintain such elevated levels throughout the entire sequence of 10 occlusion-reperfusion cycles. Measurements of plasma SOD and catalase demonstrated that the values attained with this protocol (Fig. 1) were much higher than those achieved in pilot studies.

Treatment protocol. Pigs were assigned to a control group (group I), an antioxidant-treated group (group II), and a nisoldipine-treated group (group III), all of which underwent the general protocol described above. On day 1, pigs in group II received a combination of three antioxidants: MPG, SOD, and catalase. MPG (Sigma Chemical Co., St. Louis, MO) was infused i.v. at a rate of 100 mg/kg per hour, starting 60 min before the 1st coronary occlusion and continuing until 1 h after the 10th reperfusion (total volume, 80 ml) (Fig. 1). This infusion rate has been previously found to attenuate myocardial stunning as well as production of spin-trapped radicals in conscious dogs (18). SOD and catalase were infused continuously at a rate of 270 U/kg per minute and 2,125 U/kg per minute, respectively, starting 5 min before the first coronary occlusion and ending 5 min after the 10th reperfusion. In addition, two boluses of SOD and catalase were given, one immediately before commencing the infusion, i.e., 5 min before the 1st occlusion (10,000 U/kg for SOD and 78,703 U/kg for catalase), and the other 1 min into the 4th reperfusion (3,500 U/kg for SOD and 27,625 U/kg for catalase) (Fig. 1). The total doses of SOD and catalase were 27,000 U/kg and 212,500 U/kg, respectively. The enzymes were dissolved in normal saline and given through the left atrial catheter (total volume, 100 ml). Human Cu,Zn SOD (expressed in yeast cells by recombinant DNA technology) was obtained courtesy of the Pharmacia-Chiron Partnership, Emeryville, CA (specific activity, 4,146 U/mg protein). Catalase (Sigma Chemical Co.) was purified from bovine liver (specific activity, 48,700 U/mg protein). On day 1, pigs in group III received an infusion of nisoldipine at a rate of 0.5 μg/kg per minute, starting 15 min before the first coronary occlusion and ending 30 min after the 10th reperfusion (total dose: 42.5 μg/kg; total volume: 180 ml). This dose was selected because in pilot studies it was found to be the highest dose that did not have appreciable hemodynamic effects. Nisoldipine was dissolved in polyethylene glycol 400 (0.5 mg/ml) and this solution was diluted with normal saline to the final volume (the vehicle used for nisoldipine has been previously shown not to affect myocardial stunning) (44). Throughout the study, nisoldipine was protected from light with aluminum foil. All solutions of antioxidants and nisoldipine were filtered through a 0.22 m Millipore filter to ensure sterility. Control pigs received an infusion of vehicle (normal saline) at the same rate and with the same timing as the solutions of antioxidants.

Postmortem tissue analysis. At the end of the study, the size of the occluded-reperfused coronary vascular bed was determined by a previously described postmortem perfusion technique (14). The heart was then cut into 1.0-cm-thick transverse slices, which were incubated for 20 min at 38°C in a 1% solution of triphenyltetrazolium chloride to verify the absence of infarction. Specimens for radioactivity counting were obtained as described (14); regional myocardial blood flow was calculated by standard methods (43).

Measurement of regional myocardial function. Regional myocardial function was assessed as systolic thickening fraction using a pulsed Doppler probe, as previously described (14–18, 22, 23, 43). In the four pigs instrumented with a Konigsberg pressure transducer in the left ventricle, the beginning and end of systole were determined from the onset of the rapid upstroke of the LV pressure tracing and the peak negative LV dP/dt, respectively (43). In the other pigs, the beginning of systole was determined from the peak of the QRS complex on the right ventricular electrogram and the end of systole from the onset of the rapid rise in LAD blood flow velocity after systole, as previously described (14). (In the four pigs instrumented with a Konigsberg transducer, the peak of the QRS complex was found to correspond exactly to the onset of the rapid upstroke of LV pressure, and the onset of the rapid rise in LAD flow velocity was found to occur within 1.3±1.2 ms from the peak negative LV dP/dt; in these four pigs, the measurements of WTh obtained using LV pressure and dP/dt as a reference system were identical to those obtained using the QRS complex and the LAD flow velocity.) Percent systolic thickening fraction was calculated as the ratio of net systolic thickening to end-diastolic wall thickness, multiplied by 100 (43). The total deficit of WTh after reperfusion (an integrative assessment of the severity of postischemic dysfunction) was calculated by measuring the area comprised between the WTh-vs.-time line and the baseline (100% line) during the recovery phase (14, 18, 22, 23); the recovery phase was defined as the interval between the 10th reperfusion and the time when thickening fraction returned to values > 90% of preocclusion values (14). In all animals, measurements from at least 10 beats were averaged at baseline and preocclusion, and from at least five beats at all subsequent time-points. As indicated above, three thickening Doppler probes were implanted in the potentially ischemic region. The measurements used for this study are those derived from the probe that gave the lower values of WTh (i.e., the most severe degree of myocardial stunning) after reperfusion.

Measurements of MPG, SOD, and catalase. In pigs treated with antioxidant therapy, heparinized arterial blood samples (4 ml) were obtained at selected times during the protocol. The plasma concentration of MPG was measured by a spectrophotometric method (18). The plasma activity of SOD was determined according to the method of McCord and Fridovich (45). The plasma activity of catalase was determined as previously described (46). Particular care was taken to avoid hemolysis in the blood samples; specimens showing any visible hemolysis were not analyzed.

Statistical analysis. Data are reported as means±SEM. For intragroup comparisons, hemodynamic variables and WTh were analyzed by a two-way repeated-measures ANOVA (time and day) to determine whether there was a main effect of time, a main effect of day, or a day-by-time interaction. If the global tests showed a significant main effect or interaction, post hoc contrasts between different time-points on the same day or between different days at the same time-point were performed with Student's *t* tests for paired data, and the resulting *P* values were adjusted according to the Bonferroni correction. For intergroup comparisons, continuous variables were analyzed by either a one-way or a two-way repeated-measures (time and group) ANOVA, as appropriate, followed by unpaired Student's *t* tests with Bonferroni correction. All statistical analyses were performed using the SAS software system. Two-way ANOVA was performed using the procedure GLM (General Linear Models).

Results

Exclusions and histochemical analysis. Of the 40 pigs entered into the study, four died because of technical problems during surgical instrumentation (laceration of a coronary artery, ventricular fibrillation [VF] secondary to coronary artery spasm, atrial fibrillation degenerating into VF). One pig died during the postoperative period. Two pigs were excluded because of malfunction of the WTh probes. The remaining 33 pigs form the basis of the present study. Of these, three were used for the pilot studies detailed above and the other 30 were assigned to a control group (group I, 11 pigs), an antioxidant-treated group (group II, 13 pigs), and a nisoldipine-treated group (group III, 6 pigs). Of the 11 pigs in the control group, one died of VF during the second reperfusion on day 3 and another could not complete day 3 because of failure of the balloon occluder. Therefore, a total of 11, 11, and 9 control pigs completed days 1, 2, and 3, respectively. Of the 13 pigs in the antioxidant-treated group, one could not complete day 1 because of failure of the balloon occluder; two died of VF on day 2 during the 2nd coronary occlusion and upon the 7th reperfusion, respectively; and one was found to have a small subendocardial infarction and was excluded. Therefore, 11 antioxidant-treated pigs completed day 1, and 9 pigs days 2 and 3. All six pigs in the nisoldipine-treated group completed days 1, 2, and 3.

Because the main focus of this study was to compare different days within the same group, we sought to avoid the potentially confounding effects of dropouts; accordingly, only those

pigs that completed the entire three-day protocol in the control and in the antioxidant-treated groups (9 pigs in each group) were included in the final analysis (thus, the final analysis included 9 of the 11 pigs studied on day 1 and day 2 in the control group and 9 of the 11 pigs studied on day 1 in the antioxidant-treated group). This approach decreases the number of pigs analyzed but has the advantage of enabling one to compare the same animals on days 1, 2, and 3.

Tetrazolium staining demonstrated absence of infarction in every pig included in the final analysis, indicating that the injury associated with the ten 2-min occlusion/2-min reperfusion cycles was completely reversible. In all animals, postmortem perfusion confirmed that the Doppler ultrasonic crystals were at least 1 cm away from the boundaries of the ischemic region.

Arterial blood gases, hematocrit, temperature, and diazepam dose. As in our previous investigation (14), in this study arterial pH, PO_2, hematocrit, and rectal temperature were within physiological limits throughout the study in all groups (data not reported for the sake of brevity). The doses of diazepam given to induce and maintain sedation were similar among the three groups and, within each group, among the three consecutive days of the study (2.70±0.29, 2.52±0.28, and 2.64±0.30 mg/kg on days 1, 2, and 3, respectively, in the control group; 2.75±0.24, 2.95±0.26, and 2.92±0.27 mg/kg, respectively, in the antioxidant-treated group; and 2.60±0.17, 2.54±0.11, and 2.49±0.13 mg/kg, respectively, in the nisoldipine-treated group).

Plasma levels of MPG, SOD, and catalase. These measurements are illustrated in Fig. 1. The plasma activity of SOD and catalase reached 951 and 576 U/ml, respectively, during the first reperfusion (8 min after the first bolus) and remained at these elevated levels throughout the sequence of occlusion-reperfusion cycles, ranging from 951 to 1396 U/ml and from 469 to 846 U/ml, respectively. Thus, our treatment protocol achieved the intended goal of rapidly raising plasma SOD and catalase to high levels at the beginning of the LAD occlusions and of maintaining these levels till the 10th reperfusion.

Hemodynamic variables. The administration of diazepam did not produce significant hemodynamic alterations, as indicated by the comparison of baseline (before diazepam) and preocclusion (after diazepam) measurements (Table I). All measured variables (heart rate, systolic arterial pressure, rate-pressure product, left atrial pressure, and LAD blood flow) remained stable within each day of the protocol, i.e., they did not change significantly from preocclusion values throughout the sequence of LAD occlusions and the subsequent 4 h of reperfusion, except for an increase in heart rate at 30 min, 2 h, 3 h, and 4 h on day 1 in the antioxidant-treated group, an increase in systolic arterial pressure at 3 h on day 1 in the antioxidant-treated group, and an increase in the rate-pressure product at 2 h, 3 h, and 4 h of reperfusion on day 1 in the antioxidant-treated group (Table I).

Probably as a result of the relatively long training period, of the 3 d of sham studies preceding day 1, and of the sedation with diazepam, the hemodynamic variables were also similar among the three groups and among different days within the same group, with few exceptions. In the antioxidant-treated group, the heart rate at 2 h and 3 h of reperfusion on day 1 and at preocclusion on day 3 was faster than the corresponding val-

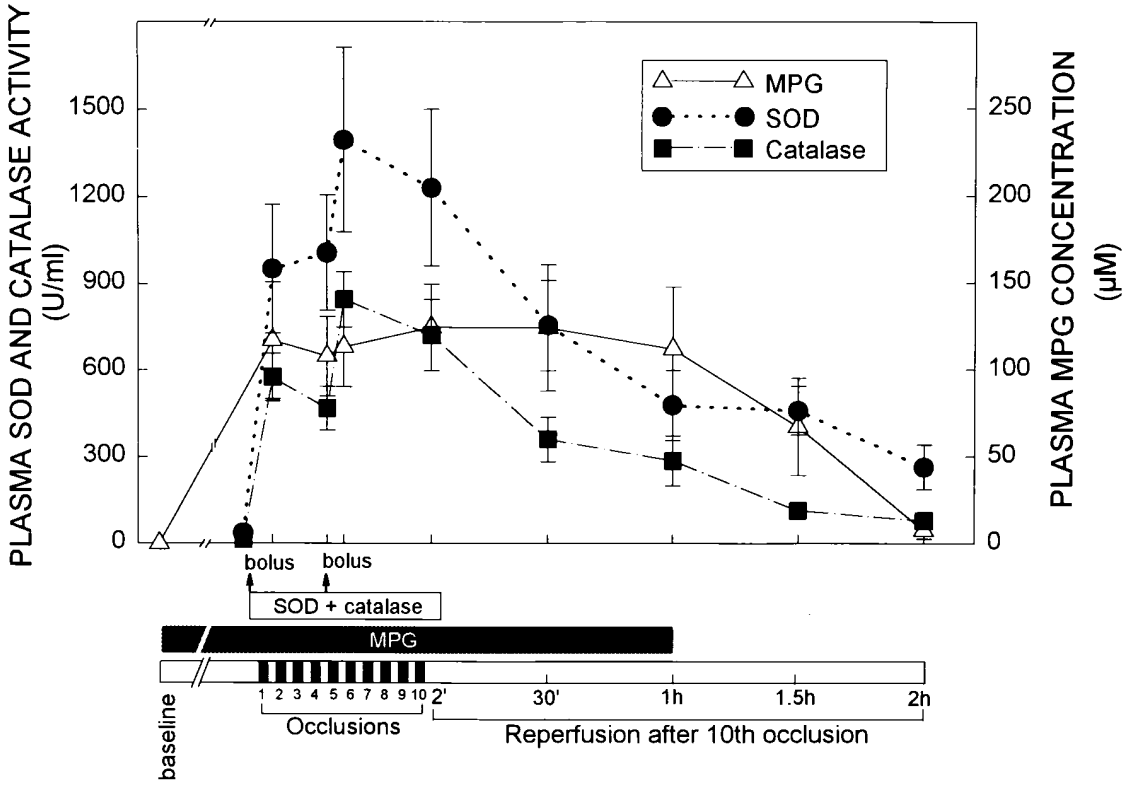

Figure 1. Graph showing the plasma concentration of MPG (*continuous line with open triangles*) and the plasma activity of SOD (*dotted line with solid circles*) and catalase (*dashed line with solid squares*) in group II. All 11 pigs that completed day 1 are included in this figure. Illustrated are measurements obtained at baseline (before infusion) and at selected times during the ten coronary occlusion-reperfusion cycles and during the final reperfusion after the 10th occlusion. Data are means±SEM.

ues in the control group, the heart rate at 2 h of reperfusion was greater on day 2 than on day 1, the systolic arterial pressure during the fourth reperfusion was higher on day 2 than on day 1, the rate-pressure product at 2 and 3 h of reperfusion on day 1 and at preocclusion on day 3 was higher than the corresponding values in the control group, the rate-pressure product at preocclusion was greater on days 2 and 3 compared with day 1, and the rate-pressure product during the fourth reperfusion and at 2 h was higher on day 2 than on day 1 (Table I). In the nisoldipine-treated group, the heart rate at 30 min of reperfusion on day 1 was faster than the corresponding value in the control group (Table I). Since a faster heart rate causes a decrease in diastolic time, and therefore in preload, these differences would not be expected to result in enhanced postischemic wall thickening. It should be noted that on day 1 antioxidant-treated pigs exhibited significantly greater wall thickening also at 30 min, 1 h, and 4 h of reperfusion, when the heart rate was not different from control pigs (Table I).

Occluded bed size and regional myocardial blood flow. The size of the occluded-reperfused vascular bed was similar in the three groups: 24.4±3.1 g (22.1±2.1% of LV weight) in the control group, 22.5±3.0 g (20.2±1.8% of LV weight) in the antioxidant-treated group, and 24.6±2.9 g (20.1±2.6% of LV weight) in the nisoldipine-treated group.

The measurements of regional myocardial blood flow are summarized in Table II. In all three groups, blood flow to the ischemic region (measured during the fifth LAD occlusion) was virtually zero in both the subepicardial and subendocardial layers of the LV wall on day 1; no increase in flow during coronary occlusion was observed on days 2 and 3. There were no statistically significant differences among the various days of the experimental protocol or among the three groups with respect to epicardial, endocardial, or mean transmural flow to the nonischemic zone (Table II).

Regional myocardial function. Fig. 2 shows representative examples of WTh tracings, whereas Figs. 3–6 illustrate the serial measurements of thickening fraction expressed as a percentage of preocclusion measurements. The administration of diazepam had no significant effect on WTh on any day of the protocol, either in the region to be rendered ischemic (Figs. 3–6) or in the nonischemic (control) region (Table I). Systolic thickening fraction in the nonischemic region remained stable within each day of the protocol during the sequence of LAD occlusions and the subsequent 4 h of reperfusion (Table I). In addition, thickening fraction in the nonischemic zone did not differ significantly among the three groups or among different days within the same group (Table I). These data indicate that the effects of repetitive coronary occlusions were evaluated in a preparation in which regional myocardial function was otherwise stable.

The baseline (pre-diazepam) systolic thickening fraction in the region to be rendered ischemic was 28.2±3.1%, 28.3±3.2%, and 26.5±3.1% on days 1, 2, and 3, respectively, in the control group; 25.7±2.0%, 28.4±2.9%, and 27.1±3.0%, on days 1, 2, and 3, respectively, in the antioxidant-treated group; and 34.0±1.9%, 33.9±1.0%, and 32.4±1.4% on days 1, 2, and 3, respectively, in the nisoldipine-treated group. After administration of diazepam (preocclusion measurements), the values of thickening fraction averaged 27.1±3.5%, 26.9±3.5%, and 27.9±3.2% on days 1, 2, and 3, respectively, in the control group; 24.4±2.0%, 27.7±3.1%, and 24.8±2.5%, on days 1, 2, and 3, respectively, in the antioxidant-treated group; and 33.6±1.2%, 34.5±1.3%, and 33.8±2.0% on days 1, 2, and 3, respectively, in the nisoldipine-treated group. There were no significant differences among the three groups on the same day or among different days within the same group. We shall first describe the control group and then the treated groups.

Control group (group I). On day 1, the extent of paradoxical systolic thinning during ischemia did not change significantly with subsequent occlusions, so that during the 10th occlusion it was similar to that measured during the 1st occlusion (Fig. 3). The extent of paradoxical systolic thinning during the 1st or 10th coronary occlusion was similar on days 1, 2, and 3 (Fig. 3).

On day 1, regional myocardial function exhibited a progressive deterioration with subsequent occlusion-reperfusion cycles, such that 5 min after the 10th reflow, thickening fraction averaged 13.5±9.0% of preocclusion values (Fig. 3). (The values at 1 min after the 10th reflow are not shown; at this time-point, thickening fraction was similar to that recorded at 1 min after the 9th reflow, but then deteriorated over the ensuing 4 min.) The recovery of contractile function after the sequence of ten occlusion-reperfusion cycles was delayed, with thickening fraction averaging 25.1±8.6% of preocclusion values at 1 h ($P < 0.01$ vs. preocclusion values), 54.0±7.5% at 2 h ($P < 0.01$), 76.5±5.1% at 3 h ($P < 0.01$), and 82.7±6.0% at 4 h ($P < 0.05$) (Fig. 3). Thus, the sequence of ten 2-min occlusions resulted in severe myocardial stunning, which lasted, on average, 4 h.

On day 2, the recovery of WTh after the ten 2-min occlusions was markedly improved compared with day 1 (Fig. 3). Statistical analysis demonstrated that the measurements of thickening fraction were significantly greater than those on day 1 at 30 min ($P < 0.05$), 1 h ($P < 0.01$), 2 h ($P < 0.01$), 3 h ($P < 0.01$), and 4 h ($P < 0.05$) of reperfusion. The total deficit of WTh after the 10th reperfusion (an integrative assessment of postischemic dysfunction) decreased in 8 of the 9 pigs and, on average, was 53% less on day 2 compared with day 1 ($P < 0.01$) (Fig. 7). On day 3, the recovery of WTh after the ten 2-min occlusions was again enhanced compared with day 1 and similar to that observed on day 2 (Fig. 3). The measurements of thickening fraction on day 3 were significantly greater than those on day 1 at 15 min ($P < 0.01$), 30 min ($P < 0.01$), 1 h ($P < 0.01$), 2 h ($P < 0.01$), and 3 h ($P < 0.01$) of reperfusion. The total deficit of WTh after the 10th reperfusion was 56% less on day 3 compared with day 1 ($P < 0.01$) (Fig. 7). Thus, myocardial stunning was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1.

Antioxidant-treated group (group II). As in the control group, in the antioxidant-treated group the extent of systolic thinning during coronary occlusion was similar on days 1, 2, and 3 (Figs. 2 and 4). Systolic thinning during occlusion was also similar between the control and the antioxidant-treated groups (Figs. 3, 4, and 5).

On day 1, regional myocardial function declined progressively during the sequence of ten occlusion-reperfusion cycles, reaching a nadir of 40.6±7.1% of preocclusion values at 5 min after the 10th reflow (Figs. 4 and 5). The values of thickening fraction measured after each of the first nine reperfusions were higher than the corresponding values in control pigs, and the differences achieved statistical significance after the 3rd, 6th, 8th, and 9th reperfusion ($P < 0.05$ at all time points) (Fig. 5). After the 10th reperfusion, the recovery of WTh was considerably faster than in the control group (Fig. 5). Statistical analy-

Table I. Hemodynamic Variables

	Baseline	Pre-occlusion	First occlusion	Fourth reperfusion	Reperfusion 5 min	30 min	1 h	2 h	3 h	4 h
HR (beats/min)										
Control										
Day 1	136±4	133±5	134±8	135±7	132±7	133±6	130±4	132±6	134±5	135±7
Day 2	141±4	133±4	138±5	143±8	138±8	130±7	135±7	130±7	135±7	137±7
Day 3	130±5	123±6	129±8	124±7	125±9	123±8	121±8	130±7	133±8	138±5
Antioxidant-treated										
Day 1	138±6	123±5	126±4	124±4	126±4	134±4*	133±6	152±6‡‖	147±6‡‖	146±6‡
Day 2	138±4	135±4	137±5	133±6	133±6	127±6	126±6	124±6§	139±5	132±5
Day 3	143±5	141±5‖	143±5	137±4	135±6	136±4	138±3	141±5	138±4	139±4
Nisoldipine-treated										
Day 1	148±7	149±7	158±9	150±11	149±16	154±10‖	141±6	135±9	145±2	144±7
Day 2	147±5	139±6	139±6	137±2	136±2	141±7	144±7	132±4	139±6	135±6
Day 3	144±6	140±5	136±7	139±8	131±8	136±9	139±6	132±5	134±7	140±8
SAP (mmHg)										
Control										
Day 1	128±5	127±5	121±2	123±10	124±4	124±4	124±5	122±5	128±5	124±5
Day 2	130±4	127±5	125±7	121±8	132±7	129±4	127±4	130±4	126±5	127±4
Day 3	129±5	126±6	130±3	139±5	124±4	125±5	128±4	120±5	121±4	123±4
Antioxidant-treated										
Day 1	126±4	119±5	125±3	119±6	122±6	119±5	121±4	129±4	134±4*	132±6
Day 2	125±4	129±4	128±6	140±3§	132±6	131±6	131±5	126±4	124±4	126±3
Day 3	125±4	129±5	129±6	128±4	124±2	122±3	121±3	125±4	127±4	126±6
Nisoldipine-treated										
Day 1	126±7	130±8	129±8	136±10	131±4	131±7	135±5	132±4	130±3	133±4
Day 2	131±3	131±2	137±5	130±6	131±3	130±2	129±2	128±5	133±3	132±1
Day 3	133±2	132±5	131±8	128±6	130±5	132±5	133±4	131±4	130±2	134±4
RPP										
Control										
Day 1	17.4±0.9	16.8±0.8	17.7±1.1	17.5±2.0	16.7±1.1	16.6±1.1	16.3±0.7	16.3±1.0	17.2±1.0	16.7±0.9
Day 2	18.3±0.9	16.9±1.0	17.2±1.3	17.3±1.2	18.3±1.9	16.8±1.0	17.1±1.0	16.9±0.9	17.4±0.5	17.4±1.0
Day 3	16.8±0.9	15.4±0.8	16.0±1.2	16.7±1.0	15.7±1.3	15.3±1.1	15.4±1.0	15.7±1.1	16.0±1.0	16.9±0.8
Antioxidant-treated										
Day 1	17.4±0.6	14.6±0.4	15.3±0.5	14.6±0.6	15.3±0.6	15.8±0.6	16.0±0.6	19.4±0.7‡‖	19.7±0.5‡‖	19.1±0.8‡
Day 2	17.3±0.5	17.4±0.5§	17.2±0.9	18.5±0.7§	17.5±0.8	16.4±0.9	16.3±0.6	15.0±0.6§	17.3±0.6	16.5±0.5
Day 3	17.8±0.6	18.1±0.7§‖	17.6±0.7	17.2±0.6	16.6±0.6	16.5±0.5	16.7±0.7	17.7±0.8	17.5±0.7	17.5±0.9
Nisoldipine-treated										
Day 1	18.3±1.7	19.0±1.9	19.9±1.7	19.3±2.3	19.2±1.9	20.2±2.2	19.0±1.6	18.0±1.6	19.0±0.5	19.0±1.7
Day 2	19.3±1.1	18.3±1.0	19.3±1.4	17.6±2.0	18.0±0.6	18.5±1.1	18.4±0.9	16.6±0.7	18.5±0.8	17.7±0.8
Day 3	19.2±1.1	18.4±1.5	17.7±2.2	17.4±1.3	17.1±1.8	18.0±1.9	18.4±1.1	17.4±1.0	17.3±0.9	18.6±1.5
LAP (mmHg)										
Control										
Day 1	5.1±1.0	6.2±0.8	6.6±0.8	—	6.7±1.1	4.8±1.0	6.0±1.3	4.8±1.2	5.8±1.1	5.4±0.7
Day 2	7.0±1.5	6.0±0.8	5.3±1.7	—	5.0±1.4	5.5±0.8	7.3±0.7	5.9±0.8	5.6±1.0	4.9±1.1
Day 3	6.4±0.7	6.2±1.1	6.4±1.3	—	7.2±0.8	6.7±1.1	7.3±0.7	6.3±1.4	5.1±1.1	4.9±1.1
Antioxidant-treated										
Day 1	5.0±1.5	4.6±0.9	5.3±0.9	—	4.5±1.3	3.4±1.0	5.5±1.4	6.6±1.1	5.8±1.3	5.8±0.9
Day 2	5.6±0.9	5.7±0.6	5.8±0.9	—	6.0±1.0	6.1±1.3	6.3±1.1	6.0±0.6	6.6±1.0	5.0±0.6
Day 3	3.7±0.6	5.6±1.0	6.0±1.9	—	4.1±0.7	4.9±0.7	4.8±0.8	6.0±1.0	6.5±1.3	5.7±0.7
Nisoldipine-treated										
Day 1	5.2±1.7	5.6±1.1	6.0±1.2	—	5.0±0.7	4.4±1.2	4.0±0.9	6.0±1.4	4.5±1.0	3.6±0.5
Day 2	6.4±1.0	5.0±1.0	4.3±1.5	—	4.2±1.1	4.3±0.7	4.0±1.2	3.2±0.5	4.0±1.0	3.0±1.0
Day 3	4.3±0.8	5.0±1.0	5.0±1.0	—	5.0±1.2	3.5±1.5	5.3±0.3	4.7±0.3	4.8±1.0	5.3±0.3

Table I. Continued

	Baseline	Pre-occlusion	1st occlusion	4th reperfusion	Reperfusion 5 min	30 min	1 h	2 h	3 h	4 h
LAD flow (ml/min)										
Control										
Day 1	19.1±3.1	19.4±3.2	0	—	19.6±4.8	19.8±3.4	14.8±3.1	16.2±3.4	16.9±3.2	16.4±3.2
Day 2	17.6±3.2	15.7±3.1	0	—	18.2±4.3	13.9±2.7	14.0±2.3	14.7±2.4	15.6±2.9	16.6±3.3
Day 3	16.6±2.5	16.8±3.2	0	—	17.6±2.8	14.8±2.6	13.3±2.4	14.6±2.9	16.6±2.6	17.3±3.0
Antioxidant-treated										
Day 1	20.7±4.1	20.2±3.6	0	—	18.1±2.6	18.5±3.0	16.9±2.3	20.3±3.1	22.4±5.1	23.1±4.4
Day 2	24.7±3.7	27.4±4.9	0	—	30.0±5.6	23.6±4.3	20.4±4.3	21.5±4.4	20.4±4.9	22.4±4.3
Day 3	23.6±3.9	23.8±6.0	0	—	21.3±4.1	22.1±5.6	21.4±4.0	22.4±5.0	19.9±3.5	19.0±2.9
Nisoldipine-treated										
Day 1	14.8±3.1	14.6±3.1	0	—	22.4±5.6	17.8±4.3	13.2±2.5	14.4±2.9	15.0±3.4	14.6±2.9
Day 2	14.4±2.8	15.8±3.7	0	—	21.4±5.4	14.6±3.1	13.6±2.8	13.8±3.1	17.4±4.3	16.2±4.3
Day 3	13.0±2.2	15.8±4.1	0	—	20.0±6.4	14.0±3.3	13.1±2.9	13.8±2.9	14.6±3.2	16.0±3.8
% ThF (NIZ)										
Control										
Day 1	29.1±4.3	24.2±3.9	23.2±4.3	—	24.7±4.7	26.2±3.6	23.3±3.7	24.1±3.6	27.1±3.6	25.0±4.2
Day 2	26.0±3.8	24.8±4.0	23.2±4.3	—	24.0±3.4	26.4±3.5	25.6±2.7	27.9±4.3	25.6±3.1	25.6±3.5
Day 3	25.9±3.1	25.6±3.0	23.2±3.7	—	28.6±3.4	26.9±3.4	25.0±3.5	27.1±3.9	26.4±3.9	26.3±4.0
Antioxidant-treated										
Day 1	27.9±1.9	25.5±3.2	27.1±3.1	—	25.4±2.9	28.5±3.8	24.6±3.4	24.4±3.6	25.7±2.8	23.7±2.5
Day 2	22.5±3.2	22.0±3.0	25.9±4.8	—	23.0±3.3	24.5±3.4	24.7±3.4	25.1±3.2	24.9±2.7	22.4±1.9
Day 3	28.2±3.3	24.2±3.2	23.6±3.0	—	22.7±3.7	25.7±3.7	24.5±2.7	25.7±3.5	24.9±2.7	26.3±3.0
Nisoldipine-treated										
Day 1	23.6±3.5	19.1±4.5	19.6±5.0	—	22.0±3.9	20.2±4.6	20.1±4.5	22.8±3.9	20.0±2.9	22.5±4.5
Day 2	24.6±4.4	22.2±2.6	20.5±4.1	—	27.5±5.2	23.0±4.1	27.2±3.6	24.1±2.1	23.2±4.2	23.5±4.1
Day 3	25.0±4.6	22.8±3.9	23.8±4.2	—	26.2±4.8	24.7±5.2	25.3±2.5	23.3±3.2	24.1±4.3	21.9±3.6

The antioxidant-treated group ($n = 9$) received a combination of antioxidant therapy on day 1: *N*-2-mercaptopropionyl glycine from 60-min preocclusion to 60 min after the 10th reperfusion, and superoxide dismutase and catalase from 5-min preocclusion to 5 min after the last reperfusion. The nisoldipine-treated group ($n = 6$) received nisoldipine on day 1 from 15-min preocclusion to 30 min after the last reperfusion. The control group ($n = 9$) received vehicle (normal saline) on day 1 at the same rate and with the same timing as the solutions of antioxidants. Data are means±SEM. *HR*, heart rate, *SAP*, systolic arterial pressure; *RPP*, rate-pressure product (heart rate × systolic blood pressure/1,000); *LAP*, mean left atrial pressure; *LAD flow*, blood flow in the left anterior descending coronary artery; *%ThF(NIZ)*, % thickening fraction in the nonischemic (control) zone. Baseline measurements were taken before administration of diazepam (~ 70 min before occlusion); preocclusion measurements were taken ~ 10 min after the initial dose of diazepam, immediately before occlusion. $^{*}P < 0.05$ vs. preocclusion; $^{\ddagger}P < 0.01$ vs. preocclusion; $^{\S}P < 0.05$ vs. day 1; $^{\|}P < 0.05$ vs. control group.

sis demonstrated that thickening fraction was significantly greater than in control pigs at 5 min ($P < 0.05$), 1 h ($P < 0.01$), 2 h ($P < 0.05$), and 3 h ($P < 0.01$) after the 10th reperfusion. The total deficit of WTh after the 10th reperfusion was 54% less in the antioxidant-treated group compared with the control group ($P < 0.01$) (Fig. 7). Similar results were obtained when all 11 pigs studied on day 1 in the two groups were compared (data not shown for the sake of brevity). Thus, the combined administration of MPG, SOD, and catalase prevented approximately half of the myocardial stunning observed in this porcine model.

On day 2, the recovery of WTh during the final reperfusion period was impaired compared with day 1 (Figs. 2 and 4) and similar to that observed on day 1 in the control group (Fig. 3). The total deficit of WTh after the 10th reperfusion increased consistently on day 2 in all of the pigs treated with MPG, SOD, and catalase; on average, it was 85% greater than on day 1 (Fig. 7). These results indicate that administration of antioxidant therapy on day 1 prevented the development of preconditioning on day 2. On day 3, however, the recovery of WTh in treated pig was markedly improved compared with day 2 (Fig. 4) and similar to that noted on day 2 in the control group (Fig. 3). The total deficit of WTh after the 10th reperfusion decreased consistently on day 3 in all of the nine antioxidant-treated pigs; on average, it was 58% less than that noted on day 2 in the same animals and was comparable to that noted on day 2 in control pigs (Fig. 7). Thus, the sequence of ten coronary occlusions and reperfusions performed on day 1 failed to precondition the antioxidant-treated pigs against stunning on day 2, but the same sequence performed on day 2 did precondition these animals against stunning on day 3. Of note, the pattern of change between days 1 and 2 was exactly the opposite in the control and antioxidant-treated groups. In the former, the severity of stunning decreased (by approximately one-half), whereas in the latter it increased (approximately twofold) (Fig. 7). These changes were consistent within each group (Fig. 7).

Nisoldipine-treated group (group III). In the nisoldipine-treated group the magnitude of systolic thinning during coronary occlusion was similar on days 1, 2, and 3 (Fig. 6) and

Table II. Regional Myocardial Blood Flow

Group	Ischemic Zone Flow (ml/min/g)			Nonischemic Zone Flow (ml/min/g)			IZF/NZF × 100
	Epi	Endo	Mean	Epi	Endo	Mean	
Control							
Day 1	0.07±0.02	0.04±0.01	0.05±0.01	1.57±0.24	1.74±0.33	1.66±0.29	4.0±1.2
Day 2	0.04±0.02	0.03±0.02	0.04±0.02	1.36±0.24	1.68±0.29	1.52±0.26	2.9±1.0
Day 3	0.07±0.02	0.05±0.01	0.06±0.02	1.30±0.12	1.41±0.12	1.36±0.11	4.9±1.2
Antioxidant-treated							
Day 1	0.06±0.02	0.04±0.01	0.05±0.01	1.26±0.18	1.34±0.28	1.30±0.22	4.0±0.7
Day 2	0.05±0.02	0.05±0.01	0.05±0.01	1.37±0.14	1.64±0.19	1.51±0.16	3.6±0.8
Day 3	0.03±0.01	0.04±0.01	0.04±0.01	1.18±0.11	1.40±0.10	1.29±0.10	2.8±0.4
Nisoldipine-treated							
Day 1	0.07±0.01	0.06±0.01	0.06±0.01	1.27±0.10	1.63±0.18	1.45±0.13	4.2±0.5
Day 2	0.05±0.01	0.06±0.00	0.05±0.01	1.02±0.11	1.38±0.23	1.20±0.17	4.8±0.6
Day 3	0.05±0.01	0.06±0.01	0.05±0.01	1.09±0.08	1.41±0.12	1.25±0.09	4.3±0.3

Values are means±SEM. *Epi*, epicardial flow; *Endo*, endocardial flow; *Mean*, mean transmural flow; *IZF*, ischemic zone flow; *NZF*, nonischemic zone flow; *IZF/NZF*, ratio of transmural ischemic zone flow to simultaneous transmural nonischemic zone flow.

was also similar to that noted in the control group (Figs. 3 and 6).

On day 1, systolic WTh after each of the ten reperfusions was similar in nisoldipine-treated and control pigs (Fig. 5). However, the recovery of WTh during the final 5-h reperfusion interval was markedly improved compared with the control pigs (Fig. 5), so that the total deficit of WTh after the 10th reperfusion was 53% less than that noted in controls ($P < 0.01$) (Fig. 7). Similar results were obtained when all 11 pigs studied on day 1 in the control group were used (data not shown for the sake of brevity). Thus, nisoldipine attenuated myocardial stunning, and the magnitude of this effect was similar to that observed after administration of antioxidant therapy in group II (Fig. 7).

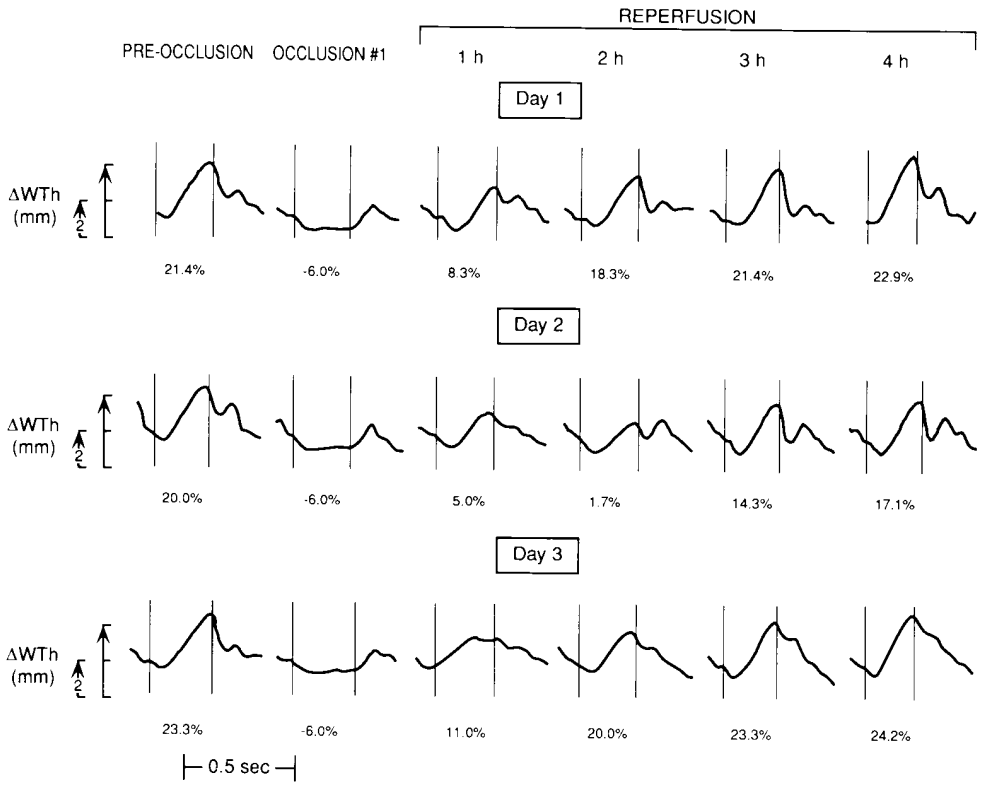

Figure 2. Original recordings from an antoxidant-treated pig illustrating the changes in systolic WTh in the ischemic-reperfused region during coronary occlusion and subsequent reperfusion. On day 1, the systolic WTh present at preocclusion was replaced by holosystolic thinning during the first coronary occlusion. During the final reperfusion interval following the 10th coronary occlusion, WTh was depressed at 1 h but recovered almost completely by 2 h (a recovery considerably faster than that seen in control pigs). On day 2, WTh was similar to day 1 at preocclusion and during coronary occlusion; however, during the final reperfusion interval, WTh was markedly depressed at 1, 2, and 3 h, and still slightly decreased at 4 h, indicating that myocardial stunning was more severe than on day 1. This example illustrates our finding that antioxidant therapy not only attenuated myocardial stunning on day 1 but also inhibited the development of preconditioning on day 2. ΔWTh indicates the change in wall thickness in the ischemic-reperfused region. Vertical lines indicate the beginning and end of systole.

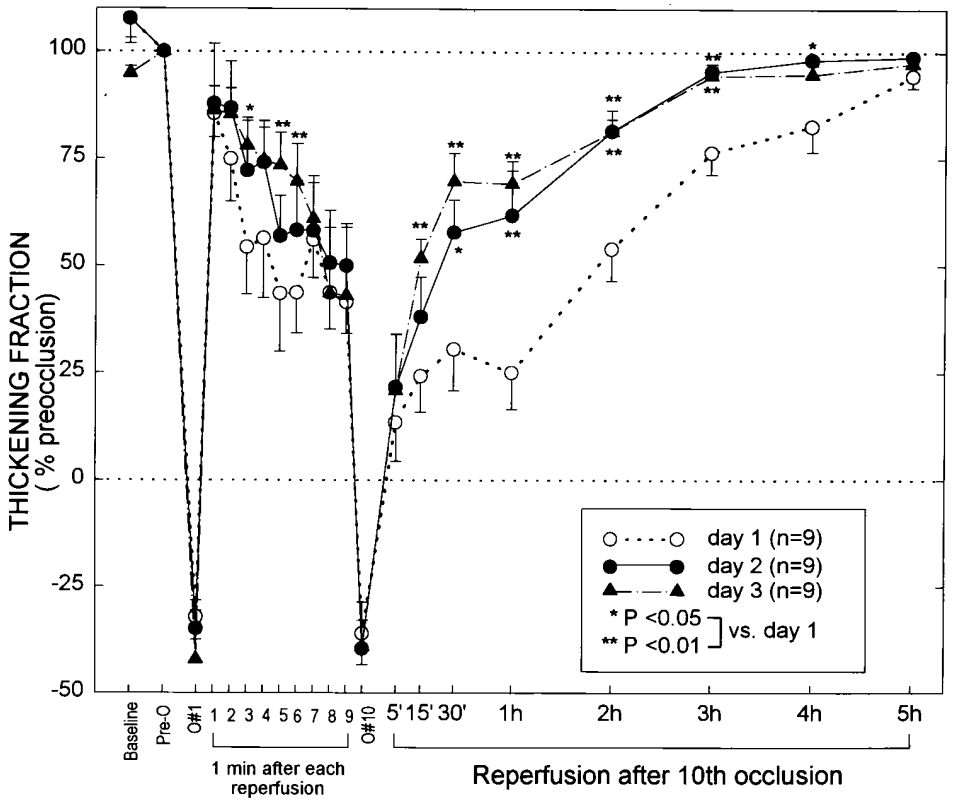

Figure 3. Systolic thickening fraction in the ischemic-reperfused region in the control group (group I) before administration of diazepam (baseline), 14 min after the initial dose of diazepam (immediately before the first occlusion) (preocclusion [*pre-O*]), 1 min into the 1st LAD occlusion (*O#1*), 1 min into each of the first nine reperfusions, 1 min into the 10th occlusion (*O#10*), and at selected times during the 5-h reperfusion interval following the 10th coronary occlusion. Measurements taken on day 1 are represented by the dashed line with open circles ($n = 9$), measurements taken on day 2 are represented by the continuous line with solid circles ($n = 9$), and measurements taken on day 3 are represented by the interrupted line with solid triangles ($n = 9$). Only the nine pigs that completed all 3 d of the protocol are included in this figure. Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

In contrast to group II, however, in group III the recovery of WTh after the ten 2-min occlusions on day 2 was not slower than that noted on day 1 and was similar to that observed on day 2 in the control group (Figs. 3 and 6). On day 3, the recovery of WTh after the ten 2-min occlusions was similar to that observed on day 2 (Fig. 6). Compared with day 1 in the control group, the total deficit of WTh after the 10th reperfusion in the nisoldipine-treated group was 64% less on day 2 and 67% less on day 3 (Fig. 7); these changes were similar to those noted on days 2 and 3 in the control group (Fig. 7), indicating that a full

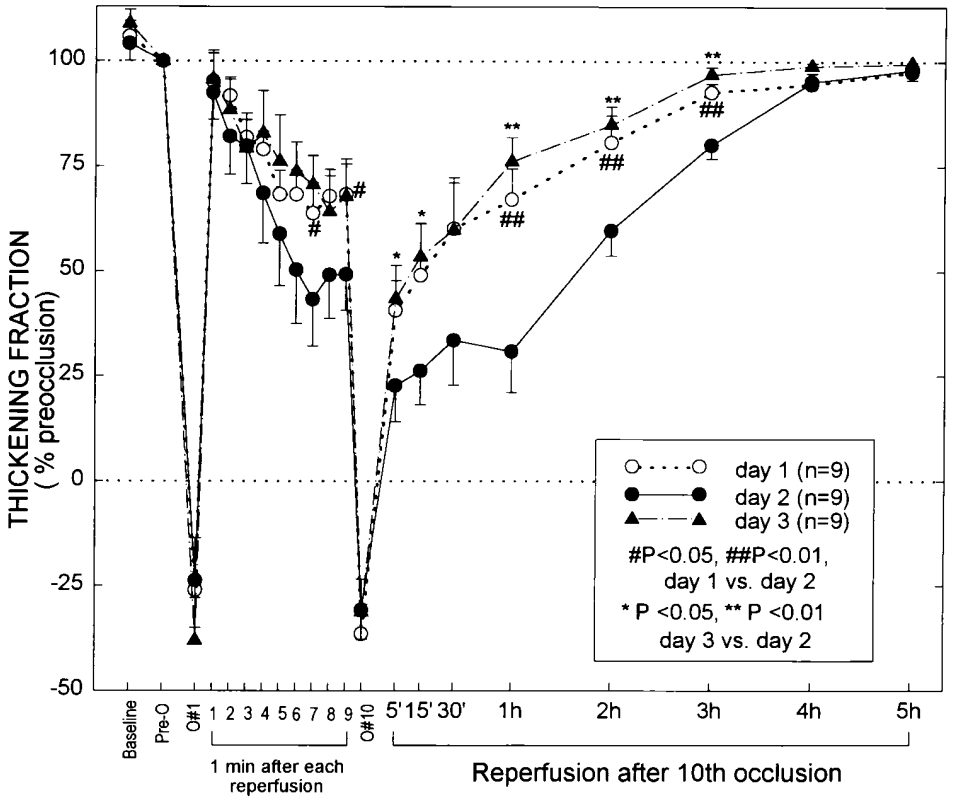

Figure 4. Systolic thickening fraction in the ischemic-reperfused region in the antioxidant-treated group (group II) before administration of diazepam (baseline), 14 min after the initial dose of diazepam (immediately before the first occlusion) (preocclusion [*pre-O*]), 1 min into the first LAD occlusion (*O#1*), 1 min into each of the first nine reperfusions, 1 min into the 10th occlusion (*O#10*), and at selected times during the 5-h reperfusion interval following the 10th coronary occlusion. Measurements taken on day 1 are represented by the dashed line with open circles ($n = 9$), measurements taken on day 2 are represented by the continuous line with solid circles ($n = 9$), and measurements taken on day 3 are represented by the interrupted line with solid triangles ($n = 9$). Only the nine pigs that completed all 3 d of the protocol are included in this figure. Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

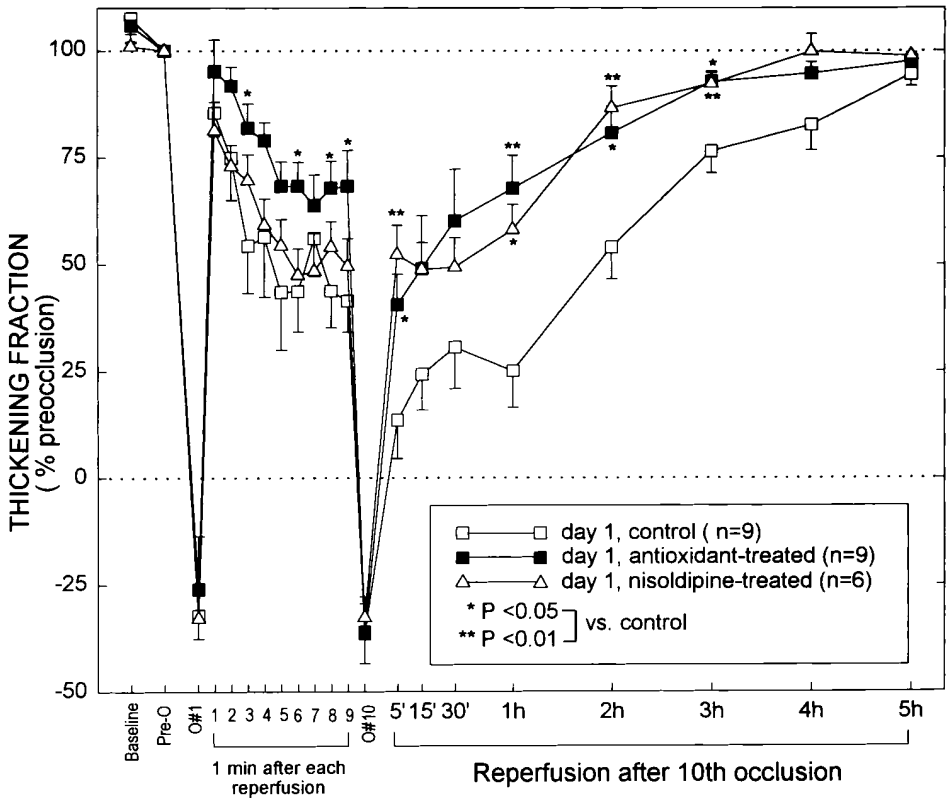

Figure 5. Comparison of systolic thickening fraction on day 1 between control and treated groups. Illustrated are measurements of thickening fraction in the ischemic-reperfused region taken before administration of diazepam (baseline), 14 min after the initial dose of diazepam (immediately before the 1st occlusion) (preocclusion [*pre-O*]), 1 min into the first LAD occlusion (*O#1*), 1 min into each of the first nine reperfusions, 1 min into the 10th occlusion (*O#10*), and at selected times during the 5-h reperfusion interval following the 10th coronary occlusion. Measurements taken in the control group are represented by open squares ($n = 9$), measurements taken in the antioxidant-treated group are represented by solid squares ($n = 9$), and measurements taken in the nisoldipine-treated group are represented by open triangles ($n = 6$). The control and antioxidant-treated pigs included in this figure are those that completed all 3 days of the protocol (the results were similar when all of the 11 pigs studied on day 1 in the control and antioxidant-treated groups were compared [data not shown for the sake of brevity]). Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

preconditioning effect took place in nisoldipine-treated pigs. Thus, both antioxidant therapy and nisoldipine produced a marked attenuation of stunning on day 1; however, antioxidant therapy blocked the development of preconditioning on day 2, whereas nisoldipine had no effect on preconditioning.

Discussion

The major findings of this study can be summarized as follows: (*a*) In the conscious pig, antioxidant therapy administered on day 1 completely prevents the development of late preconditioning against stunning, indicating that the production of reactive oxygen species on day 1 is the mechanism whereby ischemia induces the protective response observed on day 2; (*b*) antioxidant therapy markedly attenuates myocardial stunning on day 1, indicating that reactive oxygen species play an important pathogenetic role in postischemic dysfunction in the porcine heart; and (*c*) although the administration of a calcium channel antagonist is as effective as antioxidant therapy in attenuating myocardial stunning on day 1, it has no effect on late preconditioning on day 2, indicating that the ability of antioxidants to block late preconditioning is not a nonspecific result of the mitigation of myocardial stunning on day 1. To our knowledge, this is the first evidence that the oxidative stress incurred during brief ischemia and reperfusion can trigger a protective response that makes the myocardium resistant to stunning 24 h later. Furthermore, this is the first evidence that despite the absence of xanthine oxidase in the pig heart, oxygen radicals contribute to the pathogenesis of myocardial stunning in this species. Generation of oxyradicals during reperfusion is generally viewed as a deleterious process. Our finding that oxyradicals contribute to the genesis of myocardial stunning but, at the same time, trigger the development of late preconditioning against stunning sheds new light on the significance of oxyradical generation during brief myocardial ischemia and reperfusion in awake animals. A complex pathophysiological paradigm emerges, in which reactive oxygen species play an injurious role in the short term (as mediators of stunning) and a useful function in the long term (as mediators of subsequent preconditioning).

Mechanism of late preconditioning against stunning. Besides the fact that myocardial HSP70 is increased (14), virtually nothing is known regarding the mechanism of late preconditioning against stunning. The present study provides significant new insights by demonstrating that oxygen radicals play a major role in this phenomenon. When oxygen radicals were scavenged by antioxidant therapy on day 1, the development of late preconditioning on day 2 was virtually abolished; in contrast, when oxygen radicals were not scavenged (day 2), a marked preconditioning effect became apparent 24 h later (day 3), similar to that observed in control pigs on day 2 (Fig. 7). As indicated under Results, this pattern cannot be explained by nonspecific factors, such as occluded bed size, collateral flow, systemic hemodynamics, arterial blood gases, hematocrit, body temperature, and dose of diazepam. Furthermore, the failure of antioxidant-treated pigs to develop pre-

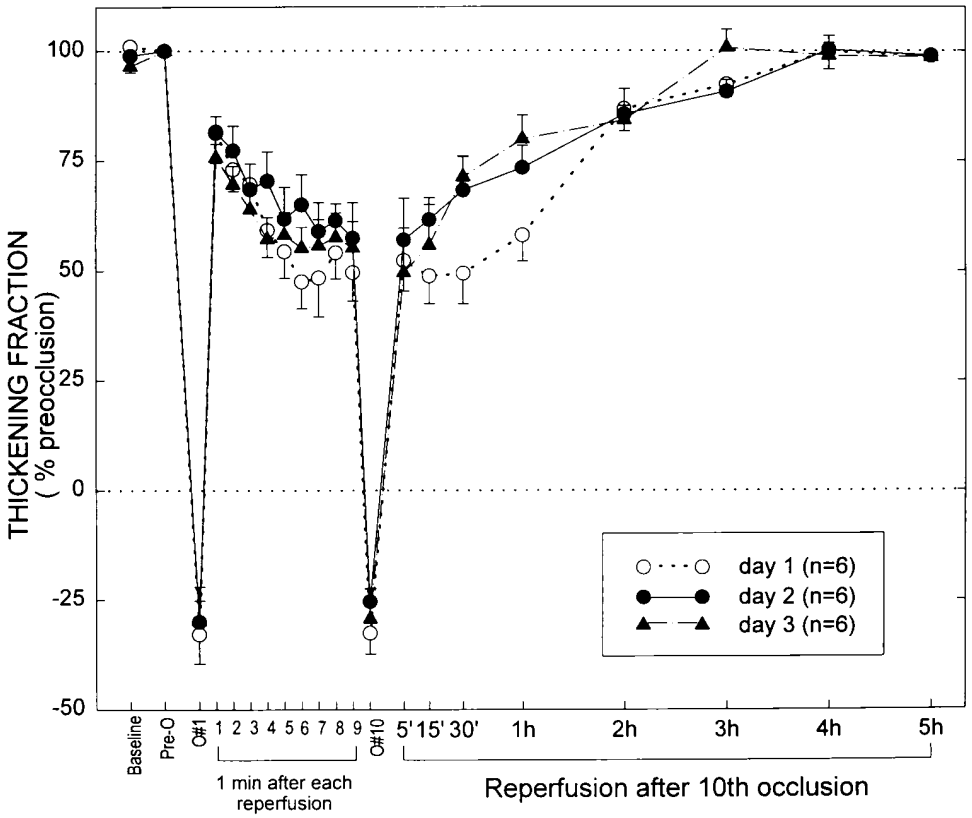

Figure 6. Systolic thickening fraction in the ischemic-reperfused region in the nisoldipine-treated group (group III) before administration of diazepam (baseline), 14 min after the initial dose of diazepam (immediately before the first occlusion) (preocclusion [*pre-O*]), 1 min into the 1st LAD occlusion (*O#1*), 1 min into each of the first nine reperfusions, 1 min into the 10th occlusion (*O#10*), and at selected times during the 5-h reperfusion interval following the 10th coronary occlusion. Measurements taken on day 1 are represented by the dashed line with open circles ($n = 6$), measurements taken on day 2 are represented by the continuous line with solid circles ($n = 6$), and measurements taken on day 3 are represented by the interrupted line with solid triangles ($n = 6$). Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

conditioning on day 2 was not due to an inherent inability of the myocardium to become preconditioned, because a marked protective effect was observed in these animals on day 3 (Fig. 7). On the basis of these observations, we conclude that the oxidative stress associated with the ten occlusion-reperfusion cycles is necessary for late preconditioning against stunning to occur; that is, the formation of reactive oxygen species represents an obligatory step in the development of the protective response.

Further studies will be necessary to identify the precise mechanism whereby the exposure to oxygen radicals during the preconditioning ischemia leads to the development of late preconditioning against stunning. The fact that late preconditioning is manifest at a distance of 24 h from the initial ischemic stress suggests that it is caused by a relatively sustained adaptive response, such as the synthesis of new proteins. The two classes of proteins most likely to mediate the protection appear to be HSPs and antioxidant enzymes. It is well established that exposure to an oxidative stress can induce HSPs (31–33, 35; reviewed in reference 34) and antioxidant enzymes, such as catalase and Mn-SOD (24–30). In this regard, we have found that the presence of late preconditioning against stunning in our model is associated with increased myocardial levels of HSP70 (14). Others have reported that a sequence of four 5-min coronary occlusions results in an increase, 24 h later, in myocardial HSP70 and HSP60 levels in rabbits (47) and in myocardial Mn-SOD activity in dogs (48, 49), and that these changes are associated with increased resistance against cell death (late preconditioning against infarction) (47, 49). Besides antioxidant enzymes and HSPs, it is possible that other proteins may also be induced by oxidative stress in our model, since a large number of genes have been identified which are regulated by reactive oxygen species (reviewed in reference 50).

Taken together, the above considerations are compatible with the hypothesis that the oxygen radicals generated during the first ischemic challenge induce cardioprotective proteins, which then render the heart resistant to subsequent ischemic challenges. How exactly oxygen metabolites activate gene transcription is not clear; this may occur via activation of protein kinase C (5, 51) or via a cis-acting regulatory element (antioxidant responsive element) that enables cells to sense and respond to oxidative stress (52).

Free radical production was not measured in this study because of the considerable technical difficulties and costs involved in performing such measurements in our conscious swine model. However, previous studies using spin-trapping techniques have demonstrated that free radicals are generated in porcine myocardium reperfused after 15 min of regional ischemia (19) as well as after 90 min of global hypothermic ischemia (53). Free radical generation after brief ischemia followed by reperfusion has also been documented in the rabbit heart (21, 54–56), which, like the porcine heart, lacks xanthine oxidase (57). There are a number of pathways that could lead to formation of reactive oxygen species in xanthine oxidase-deficient tissues, including activation of the arachidonate cascade, autoxidation of catecholamines and other compounds, accumulation of reducing equivalents, and mitochondrial respiration (36).

Previous studies of the role of oxygen radicals in preconditioning. There are no published data regarding the role of reactive oxygen species in late preconditioning, either against stunning or against infarction. With regard to early preconditioning against infarction, there is some evidence that oxyradi-

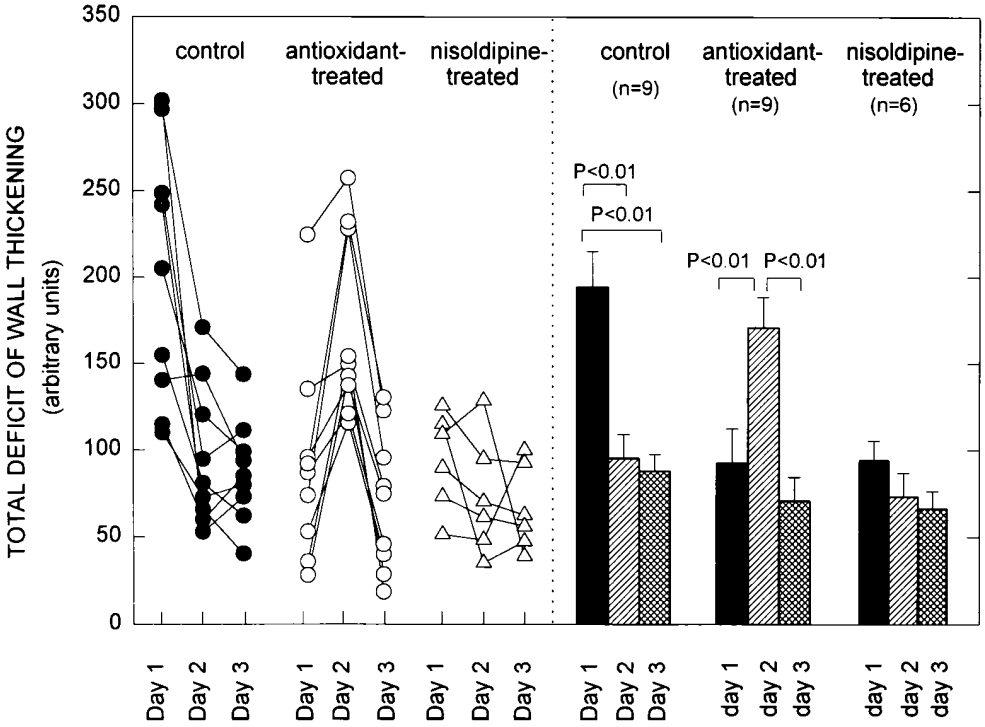

Figure 7. Total deficit of WTh after the 10th reperfusion on days 1, 2, and 3 in the control ($n = 9$), antioxidant-treated ($n = 9$), and nisoldipine-treated ($n = 6$) groups. The control and antioxidant-treated pigs included in this figure are those that completed all 3 d of the protocol. The total deficit of WTh is the area comprised between the WTh-vs.-time line and the baseline (100% line) during the recovery phase (the recovery phase was defined as the interval between the 10th reperfusion and the time when the thickening fraction returned to values > 90% of preocclusion values [see text]). The total deficit of WTh is an integrated measure of the magnitude and duration of postischemic dysfunction; its use facilitates comparisons of the severity of postischemic dysfunction among different days. The values of total deficit of WTh in individual pigs are illustrated in the left panel; the mean (±SEM) values of total deficit of WTh in each group are depicted in the right panel.

cals may contribute to this phenomenon, but the data are conflicting. Murry et al. (58) reported that the protection against infarction was partially lost when preconditioning was performed during infusion of SOD and catalase in open-chest dogs. Similarly, Tanaka et al. (59) found that MPG prevented preconditioning against infarction in open-chest rabbits. In keeping with these results, Tritto and coworkers (60) noted that a brief (5-min) exposure of isolated rabbit hearts to a free radical-generating solution resulted in subsequent reduction of infarct size. In contrast, Iwamoto et al. (61) were unable to abolish the protective effects of ischemic preconditioning against infarction with either SOD alone or SOD and catalase in open-chest rabbits. Richard et al. (62) were also unable to block preconditioning against infarction with MPG in open-chest rats. Because of the differences between the type of preconditioning studied (early vs. late), between the animal models used, and between the end-points examined (cell death vs. stunning), it is not possible to compare our present results with these previous data (58–62).

Is the prevention of preconditioning by antioxidants due to attenuation of stunning? Theoretically, antioxidants could block preconditioning either by interfering with free radical reactions or by diminishing the severity and/or duration of postischemic dysfunction. Perhaps it is myocardial stunning, rather than oxidative stress, that induces late preconditioning; perhaps the stimulus that triggers this protective response is the mechanical stimulation (stretch) associated with dyskinesis/hypokinesis, which is known to induce HSPs (63) as well as early preconditioning against infarction (64). Since antioxidant therapy attenuated myocardial stunning on day 1 (Fig. 5), it could be argued that the absence of preconditioning on day 2 was simply the consequence of the lesser degree of dysfunction incurred after the first ischemic challenge rather than a specific result of free radical scavenging. If this were the case, then any agent that alleviates stunning, irrespective of its mechanism of action, should block preconditioning. To evaluate this possibility, we examined the effects of nisoldipine, a calcium antagonist that has been previously shown to attenuate myocardial stunning in open-chest dogs (44). We found that nisoldipine was as effective as the combination of antioxidants in enhancing the recovery of function on day 1 but nevertheless failed to prevent preconditioning on day 2 (Figs. 6 and 7). Thus, despite similar effects on postischemic dysfunction, antioxidant therapy and calcium antagonist therapy had divergent effects on late preconditioning, indicating that the prevention of late preconditioning by MPG, SOD, and catalase cannot be ascribed to attenuation of myocardial stunning.

Previous studies of antioxidant therapy against stunning in xanthine oxidase-deficient species. Most of the studies supporting the concept that oxyradicals contribute to the pathogenesis of myocardial stunning have been performed in the dog, a species in which the heart contains xanthine oxidase activity. Since the xanthine oxidase reaction appears to be an important source of reactive oxygen species in stunned canine myocardium (37), it is uncertain whether the oxyradical hypothesis of stunning, which was developed in dog models, is also applicable to species that are deficient in myocardial xanthine oxidase. Studies in rabbits (a species in which the heart lacks xanthine oxidase (57)) indicate that various antioxidants (SOD plus catalase, MPG, probucol, and polyethylene glycol-conjugated SOD) improve the postischemic recovery of function after 15 min (65) or 20 min (66) of ischemia; however, it is unclear whether this is due to attenuation of stunning because a 10-min coronary occlusion is sufficient to cause some degree of necrosis in the rabbit heart (67).

The studies reported thus far in porcine models have con-

cluded that free radicals do not contribute to myocardial stunning in this species. Buchwald et al. (68) found that intracoronary infusion of SOD at the time of reperfusion failed to improve the recovery of systolic shortening after an 8-min coronary occlusion in open-chest pigs, and Rohmann et al. (69) reported that SOD plus catalase did not improve the recovery of wall thickening 30 min after reperfusion following two 10-min coronary occlusions in open-chest swine. There are several possible explanations for the apparent discrepancies between these previous results (68, 69) and our present findings. First, we administered a combination of MPG, SOD, and catalase, whereas Buchwald et al. (68) infused only SOD and Rohmann et al. (69) infused only SOD and catalase. Studies in dogs (43) have shown that SOD alone does not attenuate myocardial stunning. Although SOD plus catalase do attenuate stunning in dogs (36), it is possible that scavenging of ·OH by MPG is important to demonstrate a beneficial effect of antioxidant therapy in our porcine model. Second, Rohmann et al. infused SOD and catalase without bolus administration, at a rate of 400 U/kg per minute and 667 U/kg per minute, respectively. This protocol is similar to our pilot studies, in which SOD and catalase were infused without bolus at a rate of 270 U/kg per minute and 2,125 U/kg per minute, respectively, and myocardial stunning was not attenuated. Attenuation of myocardial stunning was observed, however, in our final protocol, in which two boluses of SOD and catalase were given, suggesting that the higher plasma levels of enzymes obtained with this protocol were important for the protection. Third, the aforementioned studies examined the stunning induced by an 8-min coronary occlusion (68) or by two 10-min occlusions (69), whereas we evaluated the stunning induced by 10 2-min occlusions. Fourth, Rohmann et al. (69) followed the recovery of function for only 30 min after the second reperfusion, an interval which may have been too short to rule out a beneficial effect (36). Finally, these previous studies (68, 69) used open-chest swine whereas we used conscious pigs. Since the conditions associated with open-chest preparations markedly exaggerate the severity of stunning and the magnitude of free radical generation in dogs (22), it is possible that a protective effect of antioxidant therapy may be more difficult to demonstrate in open-chest swine. To our knowledge, the present study is the first to examine the influence of antioxidant therapy on myocardial stunning in the conscious pig.

Since the porcine heart lacks xanthine oxidase activity (38, 70), our results imply that oxygen metabolites derived from sources other than xanthine oxidase play an important role in the genesis of myocardial stunning in the pig and that the oxyradical hypothesis of myocardial stunning is applicable to species that are deficient in myocardial xanthine oxidase.

Conclusions. Since the generation of reactive oxygen species during myocardial reperfusion is generally viewed as a deleterious process, considerable efforts have been made to identify therapies that can effectively antagonize the oxidant stress inflicted by these species upon restoration of blood flow. The present study supports a new paradigm regarding the pathophysiological role of oxygen metabolites in myocardial ischemia-reperfusion injury. Our results indicate that, in the conscious pig, oxygen radicals contribute to the genesis of myocardial stunning but, at the same time, mediate the development of the late preconditioning against stunning observed 24 h later, suggesting that the radical species generated after a brief ischemic episode play both a harmful role (as mediators of the immediate injury) and a beneficial role (as triggers of a delayed, powerful, and probably long-lasting protective response). This dual function implies that free radicals do not invariably have a detrimental effect: After a mild ischemic insult, free radical generation could serve as a "warning" signal (primordial in evolutionary terms) that activates a protective response designed to minimize further injury through the upregulation of a family of redox-sensitive genes (antioxidant enzyme genes, HSP genes, and possibly other genes). In this manner, the sublethal oxidative stress associated with brief ischemic episodes could act as a transduction pathway signaling an imminent threat and the need to develop cellular defenses against it. The present findings raise the provocative question of whether antioxidant therapies may, in some cases, be detrimental by removing the physiological stimuli that induce protective adaptations in the heart.

Elucidation of the molecular mechanisms whereby oxygen radicals trigger late preconditioning could yield important new insights regarding the pathogenesis of myocardial stunning and the regulation of redox-sensitive cardiac genes. Furthermore, the concept that late preconditioning is mediated by oxygen radicals has potential therapeutic implications; for example, a sustained state of resistance to ischemia-reperfusion injury could be induced by pharmacological manipulations that activate the redox-sensitive regulatory elements responsible for the changes in gene expression after brief ischemia.

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