Late Preconditioning against Myocardial Stunning

An Endogenous Protective Mechanism That Confers Resistance to Postischemic Dysfunction 24 h after Brief Ischemia 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 of stage I). The recovery of systolic wall thickening (WTh) after the 10th reperfusion was markedly improved on days 2 and 3 compared with day 1, indicating that the myocardium had become preconditioned against "stunning." 10 d after stage I, pigs underwent again a sequence of 10 2-min coronary occlusions for two consecutive days (days 1 and 2 of stage II). On day 1 of stage II, the recovery of WTh after the 10th reperfusion was similar to that noted on day 1 of stage I; on day 2 of stage II, however, the recovery of WTh was again markedly improved compared with day 1. Blockade of adenosine receptors with 8-*p*-sulfophenyl theophylline failed to prevent the development of preconditioning against stunning. Northern blot analysis demonstrated an increase in heat stress protein (HSP) 70 mRNA 2 h after the preconditioning ischemia; at this same time point, immunohistochemical analysis revealed a concentration of HSP70 in the nucleus and an overall increase in staining for HSP70. 24 h after the preconditioning ischemia, Western dot blot analysis demonstrated an increase in HSP70. This study indicates the existence of a new, previously unrecognized cardioprotective phenomenon. The results demonstrate that a brief ischemic stress induces a powerful, long-lasting (at least 48 h) adaptive response that renders the myocardium relatively resistant to stunning 24 h later (late preconditioning against stunning). This adaptive response disappears within 10 d after the last ischemic stress but can be reinduced by another ischemic stress. Unlike early and late preconditioning against infarction, late preconditioning against stunning is not blocked by adenosine receptor antagonists, and therefore appears to involve a mechanism different from that of other forms of preconditioning currently known. The increase in myocardial HSP70 is compatible with, but does not prove, a role of HSPs in the pathogenesis of this phenomenon. (*J. Clin. Invest.* 1995. 95:388–403.) Key words: heat stress proteins • adenosine receptors • 8-*p*-sulfophenyl theophylline • myocardial dysfunction • reperfusion

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Introduction

Ischemic preconditioning is the phenomenon whereby a brief episode of ischemia renders the myocardium resistant to a subsequent sustained period of ischemia (1–4). Ischemic preconditioning has been found to be remarkably effective in limiting infarct size (1, 5, 6) and in decreasing the incidence of ventricular arrhythmias associated with ischemia–reperfusion (7). In contrast, ischemic preconditioning has failed to attenuate postischemic dysfunction (or myocardial "stunning") in a number of studies (8–12) in which stunning was induced shortly after the preconditioning protocol.

In these studies (8–12), the question addressed was whether ischemic preconditioning confers immediate protection against postischemic dysfunction; to our knowledge, no information is available regarding the late effects of ischemic preconditioning on myocardial stunning. We hypothesized that the stress of sublethal ischemia may induce myocellular adaptations that protect against the development of stunning after subsequent exposure to ischemia, but that these adaptations develop slowly and therefore require several hours or perhaps days to become manifest. The present study was undertaken to test this hypothesis. The specific goals were to determine (*a*) whether exposure of the myocardium to a sequence of brief ischemic episodes results in a late preconditioning effect, whereby the heart becomes resistant to the stunning induced by a second identical sequence applied 24 h later; (*b*) whether this late preconditioning effect disappears after 10 d and, if so, whether it can be reinduced; (*c*) whether it is mediated by activation of adenosine A_1-receptors; and (*d*) whether it is associated with induction of heat shock proteins (HSPs).[1] A pig model was used to eliminate the variability in collateral flow and the spontaneous growth of collaterals that are associated with canine models. Meticulous attention was paid to the variables that govern myocardial stunning to ensure that any changes in postischemic recovery of contractility after preconditioning could not be ascribed to favorable modifications of the extrinsic determinants of postischemic myocardial dysfunction. The results demonstrate that, in the conscious pig, a sequence of brief coronary occlusions activates an unknown endogenous cardioprotective mechanism, not mediated by adenosine receptors, which increases the resistance of the myocardium to stunning 24 h later.

Methods

A total of 51 pigs were used for this investigation. The study was performed in accordance with the guidelines of the Committee on Ani-

1. *Abbreviations used in this paper:* CCPA, 2-chloro-N^6-cyclopentyladenosine; HSP, heat shock protein; LAD, left anterior descending; LV, left ventricular; SPT, 8-*p*-sulfophenyl theophylline; VF, ventricular fibrillation; WTh, wall thickening.

mals of Baylor College of Medicine and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, Publication No. [NIH] 86–23).

Experimental preparation

Domestic pigs of either sex (weight at surgery, 26.3±1.7 kg [range, 19–33 kg]; age, 3–4 mo) were premedicated with acepromazine maleate (1 mg/kg, i.m.) and atropine (0.02 mg/kg i.m.). 60 min later, anesthesia was induced with methohexital sodium (4–8 mg/kg i.v.), after which the animals were intubated and anesthesia was maintained with 0.5–1.0% methoxyflurane. A left thoracotomy was performed under sterile conditions at the level of the fifth intercostal space. Tygon catheters were placed in the left atrium and right ventricle and an additional catheter was introduced into the femoral artery and advanced to the thoracic aorta. A hydraulic occluder and a Doppler flow velocity probe were implanted around the mid left anterior descending coronary artery (LAD) and, in five pigs, a Konigsberg (P7) high-fidelity micromanometer was introduced into the LV cavity through the apex. Two insulated copper wires were sutured to the right ventricle to record the electrocardiogram. To measure left ventricular (LV) wall thickening (WTh), 10-MHz pulsed Doppler ultrasonic crystals (13) were sutured to the epicardial surface, two in the center of the region to be rendered ischemic and another in an area remote from it (posterior LV wall); each probe was sutured with four 6–0 prolene stitches penetrating 0.5–1.0 mm into the myocardium, thus producing minimal trauma. To avoid the "tethering" effect of nonischemic myocardium on adjacent ischemic-reperfused myocardium, the crystals were placed at least 1.0 cm inside the boundaries of the ischemic region, which were identified by occluding the LAD for 30 s. All wires and catheters were tunneled under the skin and exteriorized through small incisions on the back. 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, 13.1±1.0 d) after surgery and were trained for at least 6 d to lie quietly for 6 h in a specially designed cage. The cage is constructed of wood and can be adjusted (length, 90–115 cm; width, 55–73 cm) to match the size of the pig.

Pilot studies

Because the conscious pig is a relatively new model for studying myocardial stunning, and because of the high proclivity of this species to develop malignant arrhythmias, pilot studies were performed to identify a protocol of coronary occlusion and reperfusion that would cause significant postischemic dysfunction without inducing ventricular fibrillation (VF). Initially, we tested one 5-min coronary occlusion ($n = 1$); we found that the pig developed only mild myocardial stunning (thickening fraction recovered to 71% of preocclusion at 30 min of reperfusion, 79% at 1 h, and 88% at 2 h), indicating that a greater ischemic burden was necessary to induce more severe dysfunction. We therefore used two 5-min coronary occlusions separated by 10 min of reperfusion ($n = 2$); both pigs, however, developed VF upon the second reperfusion. To shorten the duration of each ischemic episode (and thereby reduce the likelihood of VF) while increasing the total ischemic burden, we then examined a protocol consisting of six 2.5-min coronary occlusions separated by 5 min of reperfusion ($n = 1$). This pig also developed VF (upon the fifth reperfusion). We therefore decided to shorten the duration of each ischemic episode further to 2 min (to minimize the probability of VF) and to increase the number of ischemic episodes to 10 (so as to increase the total ischemic burden even further); this led us to test a protocol consisting of 10 2-min coronary occlusions separated by 2 min of reperfusion. Using this protocol, we found that the occurrence of VF was rare and a significant and reproducible degree of myocardial stunning was present after the 10th reperfusion (see Results). Consequently, this protocol was chosen for the present study.

Experimental protocol

Throughout the experiment, pigs were studied while lying quietly in a cage in a quiet, dimly lit room. Aortic and left atrial pressures were measured with Statham P23 Db pressure transducers. The first derivative of LV pressure (LV dP/dt) was obtained by electronic differentiation. All measured variables (aortic pressure, LV pressure, LV dP/dt, left atrial pressure, LAD blood flow velocity, WTh, and the electrocardiogram) were recorded simultaneously on an eight-channel, direct writing oscillograph (Gould Brush System 200; Gould Inc., Valley View, OH). Pigs were assigned to the following groups.

Group I (demonstration of late preconditioning). In these animals, the experimental protocol included two stages (stages I and II), which consisted of 3 and 2 consecutive days of LAD occlusion, respectively (days 1, 2, and 3 of stage I and days 1 and 2 of stage II). Each stage was preceded by 3 d of sham studies, so that the pigs were studied for six consecutive days in stage I and for five consecutive days in stage II. 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 hemodynamics and WTh were monitored. The laboratory environment, the handling of the animal, and other experimental conditions were the same as on the days when the LAD was occluded. 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 of stage I), 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 of stage I). Thus, the difference between the 3 d of sham studies and the 3 d of stage I was the induction of myocardial ischemia and reperfusion. After day 3 of stage I, the pigs were allowed to recover for 10 d, and then subjected to stage II. The protocol of stage II was identical to that of stage I except that there were only 2 d of LAD occlusion (days 1 and 2) instead of 3. The purpose of performing sham studies for three consecutive days before stage I and stage II 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.

On each day of coronary occlusion, hemodynamic and WTh measurements were obtained before administration of diazepam (baseline), 14 min after administration of diazepam (preocclusion), 1 min into the 1st and 10th LAD occlusion, 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 (13) 30–60 s into the 5th LAD occlusion.

Group II (effect of adenosine receptor blockade on late preconditioning). These animals underwent the same protocol described above for stage I, except that on day 1 they received the adenosine receptor antagonist 8-*p*-sulfophenyl theophylline (SPT). SPT (Research Biomedicals, Inc., Natick, MA) was dissolved in normal saline (8 mg/ml) and administered as an i.v. bolus (10 mg/kg) 5 min before the 1st LAD occlusion followed by a continuous i.v. infusion (0.33 mg/kg/min) starting 4 min before the first occlusion and ending 5 min after the 10th occlusion (total dose: 26.2 mg/kg dissolved in 50 ml of normal saline). The solution was filtered through a 0.22-μm Millipore filter to ensure sterility (Millipore Corp., Bedford, MA). SPT was chosen despite its cost (~ $400/pig) because (*a*) it is a nonselective adenosine antagonist, having a K_i of 4.5, 6.3, and 10 μM for the A_1-, A_2-, and A_3-receptors, respectively (14) and thus should block all possible adenosine receptor-mediated pathways, and (*b*) the sulfophenyl group prevents the molecule

from entering the cells, so that the extracellular receptors are blocked with no effect on intracellular phosphodiesterase (6).

Group III (analysis of HSPs). These animals were instrumented as groups I and II, were allowed to recover for 2 wk, and then were subjected to 3 d of sham studies as in groups I and II. After the sham studies, the pigs were assigned to three subgroups. For Western blot analysis of HSP70 and Northern blot analysis of HSP70 mRNA, a subgroup was subjected to a sequence of 10 2-min LAD occlusions/2-min reperfusions (same protocol as day 1 of stage I in group I) and was sacrificed 24 h later (day 2). For immunohistochemical analysis of HSP70 and Northern blot analysis of HSP70 mRNA, a second subgroup was subjected to a sequence of 10 2-min LAD occlusions/2-min reperfusions and was sacrificed 2 h after the 10th reperfusion. A third subgroup (controls) was sacrificed without undergoing coronary occlusion. Thus, up to the time of sacrifice, the protocol used in group III was the same as that used in group I. The myocardial content of HSP70 and HSP70 mRNA was assessed as described below.

Postmortem tissue analysis

At the end of the study, the pigs were given heparin (5,000 U i.v.), after which they were anesthetized with pentobarbital sodium (35 mg/kg i.v.) and killed with a bolus of KCl. In groups I and II, the hearts were excised and the size of the occluded coronary vascular bed was determined by tying the LAD at the site of the previous occlusion and by perfusing the aortic root for 2 min with a 0.5% solution of Monastral blue dye in 6% dextran 70 in normal saline at a pressure of 100 mmHg. The rationale for using dextran in the perfusate was to prevent myocardial edema, which may hinder perfusion and cause underestimation of myocardial blood flow by the microsphere technique. 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. The portion of the left ventricle supplied by the previously occluded coronary artery (occluded bed) was identified by the absence of blue dye and separated from the rest of the left ventricle. Both components were weighed to determine occluded bed size as a percentage of total LV weight. Four transmural specimens (1–2 g) were then obtained from both the ischemic and the nonischemic regions (to avoid admixture of ischemic and nonischemic tissue, ischemic specimens were obtained at least 0.5 cm inside the boundaries of the occluded bed). Each specimen was divided into endocardial and epicardial halves, weighed, and placed in scintillation vials containing 10% neutral buffered formalin. Regional myocardial blood flow was calculated by standard methods (13).

Measurement of regional myocardial function

Regional myocardial function was assessed as systolic thickening fraction using a pulsed Doppler probe, as previously described (13, 15–17). In the five 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 (13). 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. (In the five 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 1.7±1.1 ms from the peak negative LV dP/dt; in these five 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 (13). 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 (15–17) (Fig. 1); the recovery phase was defined as the interval between the 10th reperfusion and the time

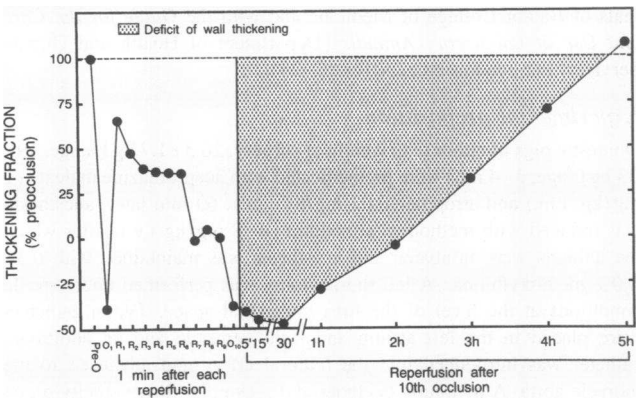

Figure 1. Total postischemic deficit of wall thickening in a pig in group I. This figure illustrates the measurements of thickening fraction in the ischemic-reperfused region at 1 min into the 1st occlusion (O_1), 1 min into each of the first nine reperfusions (R_1–R_9), 1 min into the 10th occlusion (O_{10}), and at selected time-points during the final 5-h reperfusion interval. Thickening fraction is expressed as a percentage of preocclusion values. The total deficit of wall thickening (*shaded area*) is the area comprised between the wall thickening 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 wall thickening 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.

when thickening fraction returned to values > 90% of preocclusion values. 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, two 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 more severe degree of myocardial stunning) after reperfusion.

RNA isolation and analysis (group III)

The heart was excised immediately after death and transmural samples (~ 1 g) were rapidly removed from the ischemic-reperfused region and the nonischemic region (posterior LV wall) and stored in liquid nitrogen until use. The samples were homogenized in guanidinium thiocyanate and the total RNA isolated as previously described (18). The samples were analyzed on 0.9% agarose gels and passively transferred overnight to a nylon membrane (Genescreen; NEN-DuPont, Boston, MA). cDNAs for HSP analysis: the 2.3-kb BamHI/HindIII fragment of pH 2.3 (human HSP70) (19) was used to probe for HSP70, whereas the 500-basepair HindIII/Xbal fragment of pUC 13 was used for glyceraldehyde-3-phosphate dehydrogenase (American Type Culture Collection, Rockville, MD) (20). Probes were synthesized by multiprime labeling (Amersham Corp., Arlington Heights, IL). Hybridization and wash conditions were as previously described (21). Blots were exposed to preflashed film (Amersham Corp.) overnight at −70°C.

Dot blot analysis was performed as previously described (18). Briefly, 2, 4, and 8 μg of total RNA were applied to a nylon membrane (Genescreen; NEN-Dupont) and hybridized as described (18). The blot was exposed to preflashed film (Amersham Corp.) for 48 h at −70°C. Subsequently, densitometric analysis was performed and the mean densitometric value determined for each sample.

Immunohistochemical analysis (group III)

In the subgroups of pigs used for RNA analysis (see above), transmural samples for immunohistochemistry were obtained from the ischemic-

reperfused region and from the nonischemic region (posterior LV wall). The samples were fixed for 18 to 24 h in zinc-buffered formalin and then processed and sectioned using standard histological methods for paraffin technique. Two different antibodies were used for immunohistochemistry: anti-HSP72, which binds to the inducible form of HSP70, and anti-HSP72/73, which binds both to the inducible and to the constitutive forms of HSP70 (SPA-810 and SPA-820, respectively, StressGen Biotechnologies Corp., Victoria, B.C., Canada). In mammalian cells both HSP73 (constitutive) and HSP72 are present in unstressed states, though HSP72 is present in low amounts. Although both antibodies showed localization to the nucleus after ischemia-reperfusion, the anti-HSP72 antibody gave more distinct results, and therefore was used for the work presented here. Alkaline phosphatase was used as the reporter system; endogenous alkaline phosphatase was quenched with levamisol before chromagen application. Nitro-blue tetrazolium was used to visualize the presence and distribution of HSP70; the sections were counterstained with an aqueous eosin solution and coverslipped using an aqueous mountant. For control, sections of ischemic-reperfused tissue were incubated with PBS alone (no antibody) and then developed as described.

Western blot analysis (group III)

The heart was excised immediately after death and the aorta was perfused with normal saline for 2 min at 100 mmHg to wash out all intravascular blood. Transmural samples ($\sim$ 1 g) were rapidly removed from the ischemic-reperfused region and the nonischemic region (posterior LV wall), snap frozen, and stored in liquid nitrogen until use. The samples were homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) in 10 mM Tris, 0.9% NaCl, 0.02% SDS, and 1.5 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 3,000 *g* to remove the radioactive microspheres, followed by a second centrifugation at 12,000 *g* to remove membranes and extracellular matrix. The supernatant was stored in aliquots at −70°C. Protein concentration was measured by the bicinchoninic acid assay (Pierce, Rockford, IL). On initial examination of the samples by 10% SDS-PAGE, we found that there was considerable variation in the distribution of proteins present: samples from hearts subjected to coronary occlusion had a much higher content of albumin than nonischemic hearts. Since we were interested in changes in the amount of intracellular HSP70 and not in the amount of total protein, we normalized our data to the actin content, which reflects intracellular protein, rather than to the total protein content, which reflects a combination of intracellular and extracellular proteins. This approach has been recently used by Marber et al. (22). HSP70 and actin levels were compared by a dot blot analysis as previously described (23). Simultaneous identical dot blots were prepared using 10, 20, and 50 μg of total protein for each sample. The anti-HSP72/73 antibody (SPA-820; StressGen), which binds both to constitutive and to inducible HSP70, was used for these studies (1:1,000 concentration) because we found this antibody to have a greater range for our samples than the anti-HSP72 antibody (SPA-810, StressGen). For actin detection, the second blot was incubated with a monoclonal anti-actin antibody (Amersham Corp.) at a 1:5,000 concentration. All blots were incubated with anti-mouse IgG-HRP at 1:1,000 and developed with the ECL system (Amersham Corp.) as previously described (23). Quantitation was done by laser densitometry. For each sample, the relative amount of HSP70 was normalized to actin as determined on the matching blot. This set of assays was repeated three times and the results of the three assays were averaged. Matching samples were used to normalize for differences in overall exposure.

Statistical analysis

Data are reported as means±SEM. 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 (24). The total deficits of WTh were analyzed by a one-way ANOVA with repeated measures; post hoc contrasts were performed with Student's *t* tests for paired data using the Bonferroni correction. Myocardial levels of HSP70 mRNA were analyzed with a one-way ANOVA followed by unpaired Student's *t* tests. Myocardial levels of HSP70 were analyzed with a two-way ANOVA (group and zone) followed by unpaired or paired Student's *t*-tests, as appropriate. All statistical analyses were performed using the SAS software system (25). Two-way ANOVA was performed using the procedure GLM (general linear models) (25).

Results

Exclusions and histochemical analysis

Of the 51 pigs entered into the study, 11 (22%) died because of technical problems during surgical instrumentation (i.e., laryngospasm during attempted intubation, death upon induction of anesthesia with pentobarbital, excessive dose of methoxyflurane, rupture of the subclavian artery during attempted cannulation, arterial hypotension secondary to manipulations of the heart, VF secondary to coronary artery spasm, atrial fibrillation degenerating into VF, rupture of the right atrium). The surgical mortality was particularly high at the beginning of this investigation (4 of the first 5 pigs died), but subsequently decreased as more experience was gained, so that only 2 of the last 20 pigs died. Two pigs died during the postoperative period. Of the 38 surviving pigs, 4 were used for the pilot studies; as detailed above, 3 of these 4 pigs died because of VF upon reperfusion, and one did not develop severe stunning. The remaining 34 pigs form the basis of the present study. They were assigned to group I (10 pigs), group II (9 pigs), or group III (15 pigs). Of the 10 pigs in group I, one developed arterial hypotension after injection of microspheres on day 2 of stage I and was therefore excluded from days 2 and 3 of stage I; this pig was allowed to recover and studied again 13 d later for stage II. Another pig died of VF during the 2nd reperfusion on day 3 of stage I. Therefore, a total of 10, 9, and 8 pigs completed days 1, 2, and 3 of stage I, respectively. In one pig, the balloon occluder broke during the 1st occlusion of stage II, whereas another pig died as a result of an allergic reaction to microspheres on day 1 of stage II; thus, only seven pigs completed day 1 of stage II. Of these, five were subjected to another sequence of 10 LAD occlusions on the next day (day 2 of stage II), whereas two were sacrificed on day 1 of stage II. Of the 9 pigs in group II, 1 died of VF during the 7th reperfusion on day 3. Therefore, 9 pigs completed days 1 and 2 and 8 pigs completed day 3. None of the pigs in group III was excluded.

Postmortem tissue analysis was performed in groups I and II. Tetrazolium staining demonstrated absence of infarction in every pig, indicating that the injury associated with the 10 cycles of 2-min occlusion/2-min reperfusion 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.

Group I: demonstration of late preconditioning

ARTERIAL BLOOD GASES, HEMATOCRIT, TEMPERATURE, AND DIAZEPAM DOSE

Arterial pH, PO_2, hematocrit, and rectal temperature were within physiological limits throughout stage I and stage II. For example, on day 1 of stage I arterial pH averaged 7.48±0.01, arterial PO_2 80.3±2.5 mmHg, hematocrit 37.1±1.4%, and temperature 39.1±0.1°C. Similar values were observed on days 2

and 3 of stage I and on days 1 and 2 of stage II. The doses of diazepam given to induce sedation were similar in the 3 d of stage I (2.03±0.13, 2.01±0.10, and 2.22±0.15 mg/kg on days 1, 2, and 3, respectively) and in the 2 d of stage II (2.15±0.17 and 2.47±0.29 mg/kg on days 1 and 2, respectively). The doses of diazepam subsequently given to maintain sedation were also similar: 1.00±0.18, 0.62±0.22, and 1.04±0.19 mg/kg on days 1, 2, and 3 of stage I, respectively, and 1.04±0.28 and 0.72±0.26 mg/kg on days 1 and 2 of stage II, respectively.

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) (the only exception was a slight [9.0%] decrease in heart rate on day 2 of stage I after administration of diazepam). 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 a transient increase in heart rate during the 1st occlusion on day 3 of stage I, and an increase in the rate-pressure product at 4 h of reperfusion on days 1 and 2 of stage II (Table I). In general, the hemodynamic variables were also similar among the different days of the protocol, with the exception of heart rate at 4 h of reperfusion, which was greater on day 3 of stage I compared with day 1 of stage I, and the rate–pressure product at 4 h of reperfusion, which was higher on days 2 and 3 of stage I compared with day 1 of stage I (Table I). Taken together, these results indicate that the assessment of myocardial contractility was performed in a preparation in which the hemodynamic determinants of postischemic dysfunction were stable throughout the experimental protocol.

OCCLUDED BED SIZE AND REGIONAL MYOCARDIAL BLOOD FLOW

The LV weight averaged 92.7±6.2 g, and the mean size of the occluded vascular bed was 21.5±1.8 g (23.7±1.8% of LV weight). On day 1 of stage I, blood flow to the ischemic region (measured during the 5th LAD occlusion) was virtually zero in both the subepicardial and subendocardial layers of the LV wall (Table II). No increase in flow during coronary occlusion was observed on days 2 and 3 of stage I and on days 1 and 2 of stage II (Table II). Thus, even after 30 or 40 episodes of LAD occlusion, ischemic zone flow remained essentially nil, which is consistent with the notion that coronary collaterals are absent in the porcine heart and develop very slowly in response to ischemia (26–29). There were no statistically significant differences among the various days of the experimental protocol with respect to epicardial, endocardial, or mean transmural flow to the nonischemic zone (Table II).

REGIONAL MYOCARDIAL FUNCTION

The administration of diazepam had no significant effect on WTh on any day of the protocol, either in the region to be rendered ischemic (Table I; see Figs. 2 and 4) 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 various days of the protocol (Table I).

The baseline (pre-diazepam) systolic thickening fraction in the region to be rendered ischemic was similar on days 1, 2, and 3 of stage I and on days 1 and 2 of stage II (Table I). Likewise, after administration of diazepam (preocclusion measurements), the values of thickening fraction were similar on days 1, 2, and 3 of stage I and on days 1 and 2 of stage II (Table I). We shall describe stages I and II separately.

Stage I. In stage I, 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 (−36.6±7.4 vs. −29.8±6.0% of preocclusion on day 1, −37.9±8.6 vs. −30.7±7.5% on day 2, −43.3±12.0 vs. −44.0±13.1% on day 3) (Fig. 2). The extent of systolic thinning during the 1st or 10th coronary occlusion was similar on days 1, 2, and 3 (Fig. 2).

On day 1, the first occlusion–reperfusion cycle was associated with an average 15.8% decrease in thickening fraction vs. preocclusion (Fig. 2). Each of the subsequent four occlusion–reperfusion cycles caused an additional loss of function of roughly comparable magnitude (7–14% of preocclusion values), so that thickening fraction exhibited a progressive deterioration, reaching 42.5±8.9% of preocclusion values during the 5th reflow (Fig. 2). After the 5th reperfusion, thickening fraction remained relatively stable at ~ 40% of preocclusion values until the 9th reperfusion but then decreased further to 6.9±9.4% of preocclusion values at 5 min after the 10th reflow (Fig. 2). After the 10th reflow, a delayed recovery of contractile function was observed in the reperfused myocardium, with thickening fraction averaging 20.6±10.2% of preocclusion values at 1 h ($P < 0.01$ vs. preocclusion values), 49.7±8.6% at 2 h ($P < 0.01$), and 71.4±6.6% at 3 h ($P < 0.05$) (Fig. 2). Thickening fraction returned to values not significantly different from preocclusion at 4 h (Fig. 2). Thus, the sequence of 10 2-min occlusions resulted in severe myocardial stunning, which lasted, on average, 3 h.

On day 2, the recovery of WTh after the 10 2-min occlusions was markedly improved compared with day 1 (Fig. 2). Statistical analysis demonstrated that the measurements of thickening fraction were significantly greater than those on day 1 throughout the first 3 h of reperfusion (63.9±6.6 vs. 24.5±14.0% at 30 min, $P < 0.05$; 55.9±11.5 vs. 20.6±10.2% at 1 h, $P < 0.01$; 77.8±4.4 vs. 49.7±8.6% at 2 h, $P < 0.01$; 96.7±3.4 vs. 71.4±6.6% at 3 h, $P < 0.05$). The total deficit of WTh after the 10th reperfusion (an integrative assessment of postischemic dysfunction) was 53% less on day 2 compared with day 1 ($P < 0.01$) (Fig. 3). On day 3, thickening fraction was significantly greater than on day 1 during the 4th, 5th, and 6th reperfusion; however, it did not differ significantly from day 1 after the 7th, 8th, and 9th reperfusion (Fig. 2). After the 10 2-min occlusions, the recovery of WTh was enhanced compared with day 1 and similar to that observed on day 2 (Fig. 2). Statistical analysis demonstrated that the measurements of thickening fraction on day 3 were significantly greater than those on day 1 throughout the first 3 h of reperfusion (66.9±3.8 vs. 20.6±10.2% at 1 h, $P < 0.01$; 74.5±4.5 vs. 49.7±8.6% at 2 h, $P < 0.05$; 88.4±3.1 vs. 71.4±6.6% at 3 h, $P < 0.05$). The total deficit of WTh after the 10th reperfusion was 48% less on day 3 compared with day 1 ($P < 0.01$) (Fig. 3). Thus, myocar-

Table I. Hemodynamic Variables in Group I

						Reperfusion					
		Baseline	Preocclusion	First occlusion	Fourth reperfusion	5 min	30 min	1 h	2 h	3 h	4 h
Heart rate (beats/min)											
Stage I	Day 1	140±7	134±9	135±9	134±8	129±7	132±9	133±10	133±10	139±8	135±8
	Day 2	144±7	131±7*	138±8	134±8	131±7	129±8	133±8	132±8	133±8	138±9
	Day 3	133±9	124±12	136±12‡	124±10	126±9	124±10	131±10	134±9	135±8	142±9§
Stage II	Day 1	144±8	135±5	141±6	134±4	130±6	138±6	139±5	146±3	145±4	147±4
	Day 2	134±11	138±7	143±9	138±7	131±8	141±12	141±11	145±11	150±10	159±13
Systolic arterial pressure (mmHg)											
Stage I	Day 1	122±6	126±8	117±8	118±14	125±5	125±5	124±6	124±5	124±6	121±5
	Day 2	130±5	124±7	120±8	124±11	128±7	127±4	126±5	127±4	126±5	127±5
	Day 3	131±8	125±9	121±7	137±10	124±6	125±7	123±5	123±4	121±5	121±4
Stage II	Day 1	120±12	110±8	106±10	135±5	115±9	113±8	122±7	119±7	120±7	122±8
	Day 2	106±13	114±14	112±12	113±12	113±10	110±11	115±14	117±13	119±13	121±11
Rate–pressure product (heart rate × systolic blood pressure/1,000)											
Stage I	Day 1	16.9±1.0	16.4±0.8	16.7±1.1	17.1±2.5	16.1±0.8	16.6±1.3	16.9±1.1	17.0±1.3	17.6±1.1	16.1±0.9
	Day 2	19.1±0.8	16.6±1.0	17.5±1.5	16.6±1.0	17.1±1.5	16.7±1.0	17.1±0.9	17.0±0.8	17.2±0.5	17.5±1.2‖
	Day 3	17.0±1.3	14.9±0.8	17.2±1.2	15.9±1.2	15.5±1.1	15.2±0.9	15.9±1.0	16.4±1.2	16.1±0.9	17.1±1.1‖
Stage II	Day 1	17.2±1.6	14.8±0.6	15.3±1.6	17.2±1.3	14.7±1.3	15.7±1.3	17.3±1.2	17.6±1.3	17.6±1.1	17.8±1.1‡
	Day 2	15.7±2.9	16.3±2.3	17.3±2.1	17.5±1.3	15.5±1.6	16.9±2.3	17.5±2.8	18.3±2.7	18.1±2.8	19.5±3.1‡
Mean left atrial pressure (mmHg)											
Stage I	Day 1	5.7±1.5	7.6±1.2	6.4±1.3	—	6.7±1.4	6.5±1.5	7.6±1.5	6.4±1.6	6.4±1.4	6.4±1.1
	Day 2	9.0±2.1	7.4±1.6	5.5±1.7	—	7.3±1.7	7.4±1.3	8.3±1.2	6.8±1.2	6.5±1.3	6.3±1.6
	Day 3	8.8±4.1	8.4±1.8	6.8±2.5	—	7.5±1.7	6.8±1.6	6.6±1.9	6.6±1.5	5.1±1.8	6.2±2.0
Stage II	Day 1	10.0±4.0	5.0±1.5	5.5±1.5	—	8.0±4.0	4.8±1.3	7.5±1.5	5.0±1.0	5.0±1.0	6.5±2.5
	Day 2	8.0±4.0	8.0±3.5	5.0±1.0	—	7.2±2.2	6.3±2.3	6.7±1.8	5.7±2.2	7.3±2.4	6.3±2.8
LAD Flow (ml/min)											
Stage I	Day 1	18.9±3.3	19.2±2.7	0	—	20.6±3.3	20.3±2.8	15.7±2.6	17.3±2.9	17.6±2.4	16.5±2.7
	Day 2	18.9±3.0	20.1±4.0	0	—	18.7±3.5	14.5±2.6	14.2±2.1	15.8±2.5	16.3±2.4	16.8±2.7
	Day 3	16.1±2.8	16.7±3.2	0	—	18.3±3.4	15.0±2.8	13.5±2.7	14.4±3.1	15.9±2.9	16.3±3.3
Stage II	Day 1	19.9±2.7	20.3±3.0	0	—	17.9±3.7	16.1±2.8	15.7±2.5	16.3±2.5	17.2±3.0	18.8±3.2
	Day 2	19.3±3.7	19.2±4.3	0	—	19.8±5.1	15.7±3.8	15.6±2.6	16.2±2.8	16.0±2.6	16.6±4.1
Percent thickening fraction (ischemic zone)											
Stage I	Day 1	29.8±2.9	30.0±2.7	−8.0±1.1	16.8±3.4	3.9±3.0	9.7±4.3	7.8±3.3	16.5±3.6	22.3±3.4	26.6±2.8
	Day 2	27.5±3.5	29.3±3.3	−7.7±1.1	20.1±2.3	9.6±3.1	18.9±2.9	17.8±3.9	23.6±3.5	28.3±3.3	30.1±3.0
	Day 3	28.6±3.4	29.9±3.1	−12.2±3.4	24.5±3.0	8.5±3.9	16.7±4.3	20.4±2.8	22.9±3.4	26.9±3.5	27.9±2.7
Stage II	Day 1	23.9±3.7	25.9±4.1	−11.5±1.1	14.0±4.6	−0.4±3.7	0.0±3.5	6.3±4.5	12.9±4.7	17.9±6.5	23.3±4.6
	Day 2	23.4±5.4	23.3±6.6	−14.4±2.1	15.8±4.1	4.0±5.1	6.4±4.8	11.3±5.5	20.0±4.2	23.5±5.5	22.5±5.1
Percent thickening fraction (nonischemic [control] zone)											
Stage I	Day 1	28.5±5.2	26.8±4.8	24.2±5.5	—	26.1±4.2	25.5±3.9	25.5±3.7	27.1±3.8	24.6±4.3	26.7±4.3
	Day 2	28.0±3.9	26.4±4.3	21.8±5.9	—	26.4±3.7	28.2±4.1	26.5±3.3	31.8±4.1	26.8±3.7	29.1±4.3
	Day 3	27.6±3.6	27.0±3.8	21.2±5.0	—	30.5±3.6	27.6±3.5	27.1±3.6	27.5±3.4	27.6±3.6	28.5±3.8
Stage II	Day 1	30.8±6.3	26.5±6.7	25.6±6.0	—	25.6±5.5	24.3±6.7	23.3±7.0	27.8±6.8	26.5±5.3	23.9±5.8
	Day 2	24.0±4.8	22.8±6.5	23.1±6.9	—	23.3±7.5	22.8±5.8	25.5±8.3	28.7±8.0	28.4±3.6	28.3±0.5

Data are means±SEM. 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. baseline; ‡ $P < 0.05$ vs. preocclusion; ‖ $P < 0.05$; § $P < 0.01$ vs. day 1 of stage I. n = 10, 9, 8, 7, and 5 on days 1, 2, and 3 of stage I and days 1 and 2 of stage II, respectively.

Table II. Regional Myocardial Blood Flow

	Ischemic Zone Flow (ml/min per g)			Nonischemic Zone Flow (ml/min per g)			IZF/NZF ×100
	Epi	Endo	Mean	Epi	Endo	Mean	
Group I							
Stage I							
Day 1	0.04±0.01	0.04±0.01	0.04±0.01	1.53±0.19	1.79±0.23	1.66±0.21	3.0±0.6
Day 2	0.04±0.02	0.03±0.01	0.04±0.02	1.61±0.24	1.82±0.27	1.72±0.25	2.3±0.8
Day 3	0.04±0.02	0.03±0.01	0.04±0.01	1.07±0.11	1.22±0.15	1.14±0.12	3.2±1.1
Stage II							
Day 1	0.02±0.01	0.03±0.02	0.03±0.01	1.14±0.18	1.36±0.20	1.25±0.19	1.8±0.9
Day 2	0.04±0.02	0.05±0.03	0.04±0.02	1.37±0.22	1.60±0.20	1.49±0.21	2.8±1.4
Group II							
Day 1	0.06±0.02	0.06±0.02	0.06±0.01	0.94±0.08	0.98±0.05	0.96±0.06	5.8±1.3
Day 2	0.07±0.02	0.05±0.01	0.06±0.01	1.29±0.11	1.72±0.20	1.51±0.15	4.3±0.9
Day 3	0.08±0.03	0.06±0.02	0.07±0.02	1.42±0.12	1.45±0.11	1.44±0.08	4.9±1.5
Group III							
Day 1	0.05±0.01	0.04±0.01	0.04±0.01	1.25±0.13	1.47±0.16	1.36±0.14	3.3±0.6

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

dial stunning was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1.

Stage II. As in stage I, the extent of systolic thinning during subsequent coronary occlusions did not change significantly in stage II, so that during the 10th occlusion it was similar to that measured during the 1st occlusion (−53.9±9.1 vs. −52.2±8.7% of preocclusion on day 1 and −85.9±26.9 vs. −82.5±20.0% on day 2) (Fig. 4). Furthermore, systolic thinning during the 1st or 10th occlusion did not differ significantly between day 1 and day 2 of stage I, or between day 1 of stage II and day 1 of stage I (Fig. 4).

On day 1 of stage II, which was 10 days after the end of stage I, both the decline of WTh during the ten occlusion-reperfusion cycles and its subsequent recovery during the final reperfusion period were similar to those noted on day 1 of stage I (Fig. 4). This indicates that the myocardium had returned to its original (nonpreconditioned) state. On day 2 of stage II, however, the recovery of WTh was again markedly improved compared with day 1 of stage II (Fig. 4). Statistical analysis demonstrated that thickening fraction was significantly greater on day 2 than on day 1 at 1 h (43.9±15.2% vs. 16.8±12.9% of preocclusion, $P < 0.01$) and 2 h (95.5±13.3% vs. 43.3±11.8%, $P < 0.05$) of reperfusion. The total deficit of WTh after the last reperfusion was 45% less on day 2 compared with day 1 ($P < 0.05$) (Fig. 3). Thus, the attenuation of myocardial stunning noted on days 2 and 3 of stage I disappeared 10 days later (on day 1 of stage II) but appeared again on day 2 of stage II.

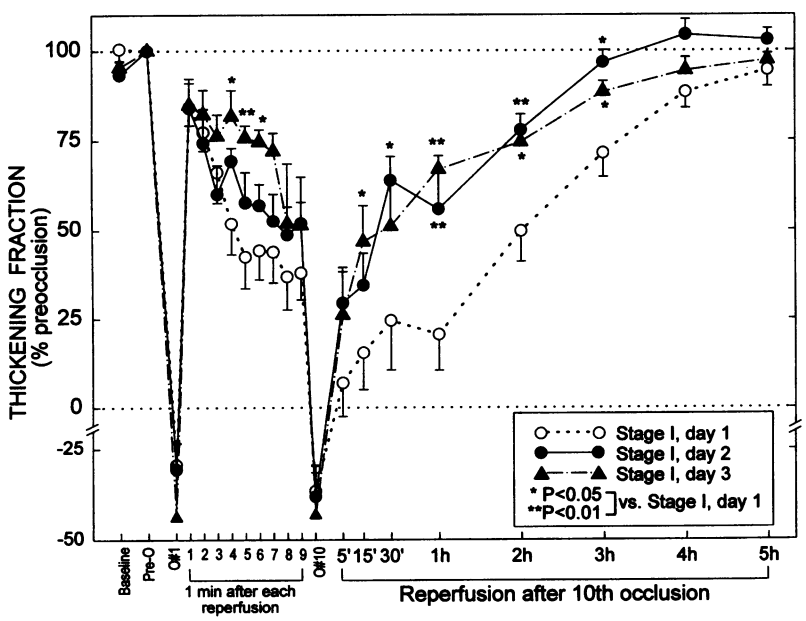

Figure 2. Systolic thickening fraction in the ischemic-reperfused region during stage I in group I. Shown are the measurements of thickening fraction obtained before administration of diazepam (baseline), 14 min after the initial dose of diazepam (immediately before the 1st occlusion) (preocclusion, *Pre-O*), 1 min into the 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 ($n = 10$); (—●—) measurements taken on day 2 ($n = 9$); and (– ·–▲– ·–) measurements taken on day 3 ($n = 8$). Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

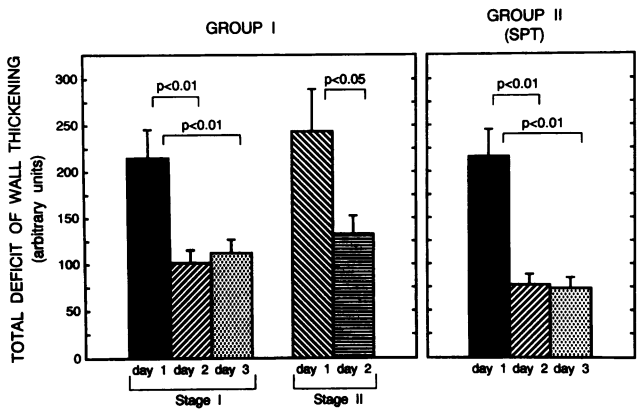

Figure 3. Total deficit of wall thickening after the 10th reperfusion in group I, which did not receive SPT, and in group II, which received SPT on day 1. The total deficit of wall thickening was measured in arbitrary units as illustrated in Fig. 1. This parameter represents an integrative assessment of postischemic dysfunction and facilitates comparison of myocardial stunning among different days. SPT, 8-*p*-sulfophenyl theophylline. Data are means±SEM.

Group II: effect of adenosine receptor blockade

DOCUMENTATION OF ADENOSINE RECEPTOR BLOCKADE

The dose of SPT selected for this study was greater than or equal to the doses previously shown to block early (6) and late (30) preconditioning against infarction. To verify effective blockade of adenosine A_1-receptors, the heart rate and P-R interval responses to 2-chloro-N^6-cyclopentyladenosine (CCPA), a selective A_1-receptor agonist, were observed in the absence or presence of SPT in a chronically instrumented pig. On the first day, the pig was given CCPA (5 μg/kg i.v. bolus) and the heart rate and P-R interval were recorded every 5 min for 70 min (higher doses of CCPA were found to cause asystole in two other pigs, and thus were not examined). This same protocol was repeated on the next day except that the pig was given SPT (10 mg/kg i.v. bolus 5 min before CCPA followed by a continuous i.v. infusion of 0.33 mg/kg/min for 49 min, total dose: 26.2 mg/kg; this is the same dose that was used in group II). The effect of SPT was tested on a different day to avoid possible cumulative effects of two consecutive injections of CCPA. On the first day, CCPA produced a 25–35% decrease in heart rate (from 140 beats/min to 90–105 beats/min) and a 20–25% prolongation of the P-R interval (from 10.5 ms to 12.5–13.0 ms), both of which lasted for at least 40 min after the bolus. In the presence of SPT, however, these effects were completely prevented, both immediately after administration of SPT and 45 min later (i.e., 40 min after CCPA). These results indicate that the dose of SPT used in this study effectively blocks myocardial A_1-receptors for at least 45 min (an interval that exceeds the 40-min interval during which endogenous adenosine is released during the sequence of ten 2-min occlusion/2-min reperfusion cycles).

ARTERIAL BLOOD GASES, HEMATOCRIT, TEMPERATURE, AND DIAZEPAM DOSE

Arterial pH, PO_2, hematocrit, and rectal temperature were within normal limits throughout the 3 d of LAD occlusion. For example, on day 1 arterial pH averaged 7.48±0.01, arterial PO_2 83.0±3.2 mmHg, hematocrit 37.3±1.0%, and temperature 39.2±0.1°C. The values measured on days 2 and 3 were similar. The doses of diazepam were similar on days 1, 2, and 3 and were also similar to the doses given during stage I in group I (see above): the initial doses averaged 1.83±0.19, 1.82±0.15, and 1.90±0.18 mg/kg on days 1, 2, and 3, respectively, and the maintenance doses 0.52±0.10, 0.55±0.09, and 0.56±0.11 mg/kg, respectively.

HEMODYNAMIC VARIABLES

(For the sake of brevity, the entire set of hemodynamic data is not shown.) At baseline (before diazepam), heart rate averaged

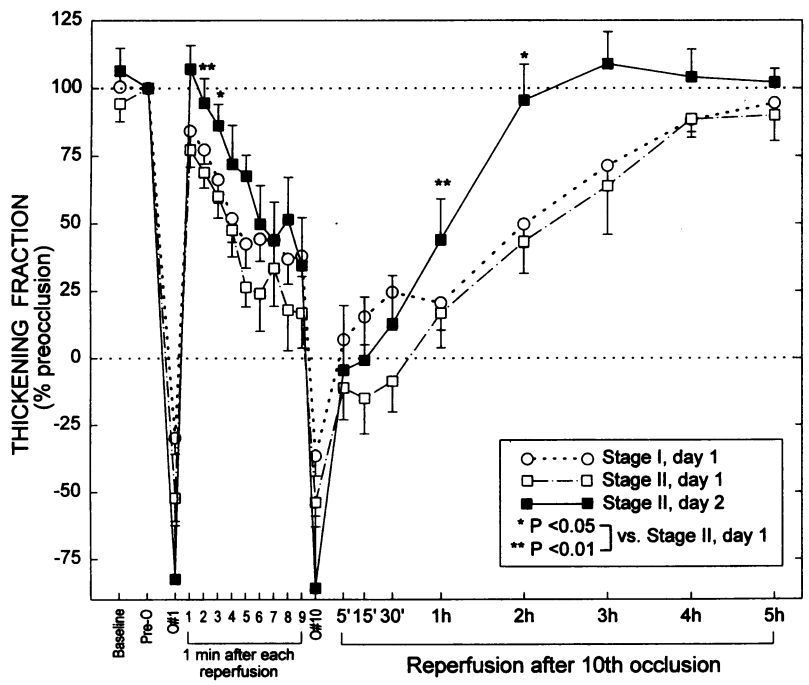

Figure 4. Systolic thickening fraction in the ischemic-reperfused region during stage II in group I. Shown are the measurements of thickening fraction obtained before administration of diazepam (*baseline*), 14 min after the initial dose of diazepam (immediately before the 1st occlusion) (preocclusion, *pre-O*), 1 min into the 1st LAD occlusion (*O#1*), 1 min into each of the first nine reperfusions, 1 min into the 10th LAD occlusion (*O#10*), and at selected times during the 5-h reperfusion interval following the 10th coronary occlusion. (–·–□–·–) Measurements taken on day 1 (n = 7); and (—■—) measurements taken on day 2 (n = 5). Measurements obtained on day 1 of stage I (···○···) (n = 10) are also included for comparison. Day 1 of stage II was 10 d after the end of stage I. Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

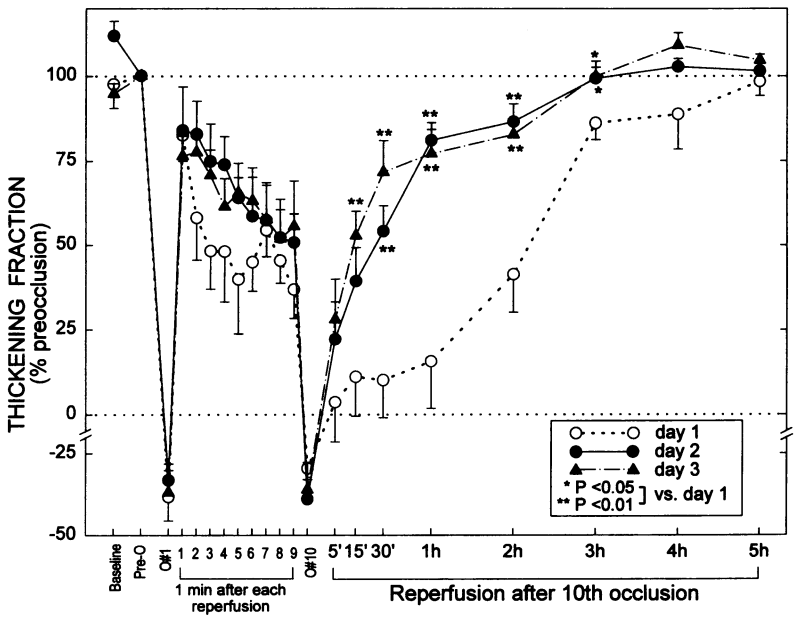

Figure 5. Systolic thickening fraction in the ischemic-reperfused region in group II, which received SPT on day 1. Shown are the measurements of thickening fraction obtained before administration of diazepam (*baseline*), 14 min after the initial dose of diazepam, i.e., 4 min after the bolus of SPT (preocclusion, *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 LAD occlusion (*O#10*), and at selected times during the 5-h reperfusion interval following the 10th coronary occlusion. (···○···) Measurements taken on day 1 are (n = 9); (—●—) measurements taken on day 2 (n = 9); and (– ·–▲–·–) measurements taken on day 3 (n = 8). Thickening fraction is expressed as a percentage of preocclusion values. Data are means±SEM.

136±6, 136±6, and 134±5 beats/min on days 1, 2, and 3, respectively; systolic arterial pressure 122±3, 125±4, and 122±5 mmHg; rate-pressure product 16,800±600, 17,100±900, and 16,200±400; left atrial pressure 7.1±1.2, 6.5±0.9, and 7.0±1.1 mmHg; and LAD blood flow 19.2±2.7, 18.3±3.6, and 19.5±2.8 ml/min. As in group I, in group II diazepam did not produce significant hemodynamic alterations: after diazepam (preocclusion measurements), heart rate was 138±5, 136±6, and 137±5 beats/min on days 1, 2, and 3, respectively; systolic arterial pressure 127±4, 124±4, and 124±5 mmHg; rate-pressure product 17,500±800, 16,700±800, and 16,900±600; and LAD flow 17.6±0.3, 18.3±3.5, and 19.1±3.6 ml/min. No hemodynamic changes were noted after administration of SPT. All measured variables (heart rate, systolic arterial pressure, rate-pressure product, left atrial pressure, and LAD flow) remained stable within each day of the protocol, i.e., they deviated from preocclusion values by < 10% (except for a transient decrease in LAD flow at 30 min [−15%], 1 h [−12%], and 2 h [−14%] of reperfusion on day 2 and at 30 min [−17%], 1 h [−30%], and 2 h [−20%] on day 3 [P = NS at all time points]). These variables were also similar among days 1, 2, and 3, with no significant differences noted at any time point.

OCCLUDED BED SIZE AND REGIONAL MYOCARDIAL BLOOD FLOW

The LV weight averaged 123.2±6.9 g and the size of the occluded vascular bed 25.3±4.0 g (20.0±2.6% of LV weight). As in group I, in group II there was virtually no blood flow to the ischemic region during coronary occlusion on days 1, 2, or 3 (Table II).

REGIONAL MYOCARDIAL FUNCTION

As in group I, in group II diazepam had no significant effect on WTh, either in the region to be rendered ischemic (Fig. 5) or in the nonischemic (control) region (data not shown). Systolic thickening fraction in the nonischemic region remained stable within each day of the protocol, and was also similar among different days (data not shown for the sake of brevity), except at 1 h of reperfusion, when it was higher on day 2 than on day 1 (26.6±2.0% vs. 20.6±2.0%, $P < 0.05$). Baseline (pre-diazepam) thickening fraction in the region to be rendered ischemic was 22.3±1.5%, 25.5±2.7%, and 21.7±2.7% on days 1, 2, and 3, respectively. After diazepam (preocclusion measurements), the values of thickening fraction averaged 24.3±2.9%, 23.0±2.5%, and 22.8±2.6% on days 1, 2, and 3, respectively. There were no significant differences among the three baseline measurements or among the three preocclusion measurements. Administration of SPT had no appreciable effect on WTh either in the soon to be ischemic region (Fig. 5) or in the control region. The extent of paradoxical systolic thinning during coronary occlusion was similar on days 1, 2, and 3 (Fig. 5). On day 1 (when SPT was administered), the first five occlusion-reperfusion cycles caused a progressive decrease in thickening fraction, which reached 39.3±16.2% of preocclusion values during the 5th reflow (Fig. 5). After the 5th reflow, thickening fraction remained relatively stable at ~ 45% of preocclusion values until the 9th reperfusion but fell to 2.8±11.8% of preocclusion values 5 min after the 10th reflow (Fig. 5). The decrease in thickening fraction during the sequence of ten occlusion–reperfusion cycles was similar to that observed on day 1 in group I (Fig. 2).

After the 10th reperfusion, thickening fraction was significantly depressed at 1, 2, and 3 h and returned to values not significantly different from preocclusion values at 4 h (Fig. 5). There were no significant differences between the measurements of thickening fraction after the 10th reperfusion and the corresponding measurements on day 1 of stage I in group I (Fig. 5). The total deficit of WTh after the 10th reperfusion was also similar to that observed on day 1 of stage I in group I (Fig. 3). Thus, unlike the results obtained in rabbits subjected to multiple 5-min coronary occlusions (31), blockade of adenosine receptors had no effect on myocardial stunning in this porcine model of repetitive ischemia.

On day 2, the recovery of WTh after the 10 2-min occlusions was considerably faster than on day 1 (Fig. 5). Statistical analysis demonstrated that throughout the first 3 h of reperfusion, the measurements of thickening fraction were significantly

greater than those recorded on day 1 (53.7±7.6 vs. 9.3±11.1% at 30 min, $P < 0.01$; 80.8±5.2 vs. 14.9±13.9% at 1 h, $P < 0.01$; 86.3±5.3 vs. 40.8±11.3% at 2 h, $P < 0.01$; 99.2±3.3 vs. 84.7±4.2% at 3 h, $P < 0.05$). The total deficit of WTh after the 10th reperfusion was 64% less on day 2 compared with day 1 ($P < 0.01$) (Fig. 3). On day 3, the recovery of WTh was also improved compared with day 1, and was similar to that observed on day 2 (Fig. 5). The values of thickening fraction on day 3 were significantly greater than those on day 1 throughout the first 3 h of reperfusion (71.4±9.3 vs. 9.3±11.1% at 30 min, $P < 0.01$; 76.9±7.1 vs. 14.9±13.9% at 1 h, $P < 0.01$; 82.5±4.1 vs. 40.8±11.3% at 2 h, $P < 0.01$; 99.8±4.5 vs. 84.7±4.2% at 3 h, $P < 0.05$), and the total deficit of WTh was 66% less on day 3 compared with day 1 ($P < 0.01$) (Fig. 3). Thus, blockade of adenosine receptors on day 1 did not prevent a marked attenuation of myocardial stunning on days 2 and 3 similar to that previously observed in group I (Fig. 3).

Group III: analysis of HSPs

On the day of LAD occlusion, arterial pH averaged 7.49±0.01, arterial PO_2 83.5±2.7 mmHg, hematocrit 39.0±1.2%, and rectal temperature 39.0±0.2°C. The initial and maintenance doses of diazepam were 1.86±0.12 and 0.41±0.16 mg/kg, respectively.

NORTHERN BLOT ANALYSIS OF HSP70

A subset of four pigs underwent a sequence of 10 2-min LAD occlusions, each separated by 2 min of reperfusion, and was sacrificed 2 h after the 10th reperfusion. In these animals, the HSP70 mRNA in the ischemic-reperfused region was increased compared with the anterior LV wall of six control pigs that did not undergo coronary occlusion-reperfusion (Fig. 6). Densitometric analysis showed a 60% increase in the message (348±43 vs. 218±19 densitometric units, $P < 0.02$) (Fig. 6). In five pigs sacrificed 24 h after the sequence of 10 2-min LAD occlusions, the levels of HSP70 mRNA had returned to values similar to those in the control group (Fig. 6).

IMMUNOHISTOCHEMICAL ANALYSIS OF HSP70

In two pigs sacrificed 2 h after the sequence of 10 2-min LAD occlusions, immunohistochemical analysis demonstrated a concentration of HSP70 in the nuclei (particularly in the nucleoli) as well as an overall increase in HSP70 staining in the myocytes within the ischemic-reperfused region compared with the LAD-dependent region of a control pig that did not undergo ischemia–reperfusion (Fig. 7). In the nonischemic region of the two pigs subjected to ten LAD occlusions, myocytes also exhibited an overall increase in HSP70 staining, but to a lesser degree than myocytes within the ischemic-reperfused region (not shown).

WESTERN BLOT ANALYSIS OF HSP70

A subset of five pigs underwent a sequence of 10 2-min LAD occlusions, each separated by 2 min of reperfusion (day 1) and was sacrificed 24 h later (day 2). In these animals, the HSP70/actin ratio in the ischemic-reperfused region was significantly increased not only when compared to the nonischemic region of the same animals (6.49±1.27 vs. 2.44±0.47, $P < 0.05$), but also when compared to the anterior LV wall of four control pigs that did not undergo coronary occlusion–reperfusion (6.49±1.27 vs. 1.84±0.64, $P < 0.05$) (Fig. 8). In contrast to the animals subjected to ischemia, in the control pigs the myocardial levels of HSP70 were virtually identical in the anterior LV wall (LAD-dependent region) and in the posterior LV wall (Fig. 8).

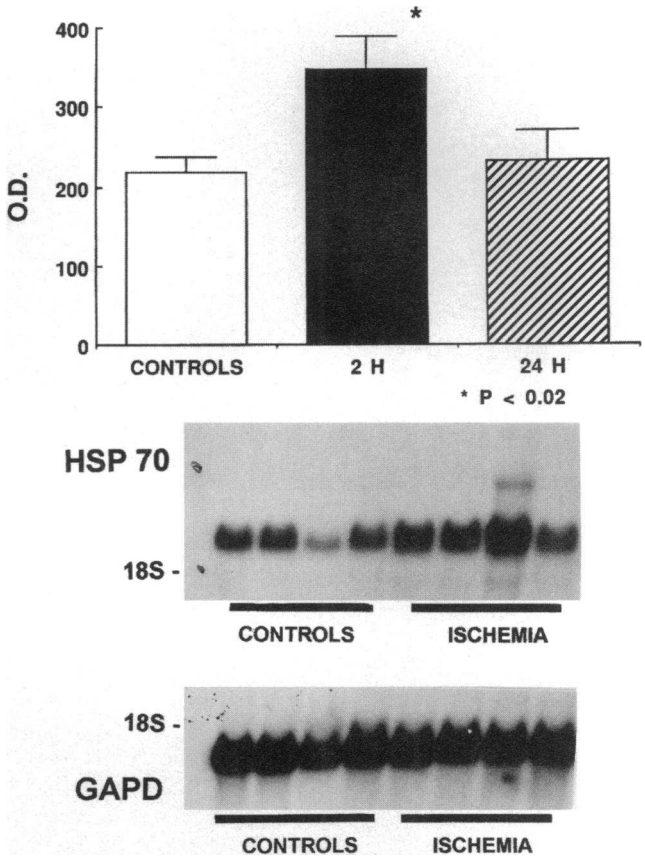

Figure 6. (Upper section) Densitometric analysis (using dot blot) of the relative amounts of HSP70 mRNA in myocardial samples obtained from pigs that did not undergo coronary occlusion-reperfusion (*CONTROLS, n* = 6) and from pigs that underwent a sequence of 10 2-min occlusions/2-min reperfusions and were sacrificed 2 h later (*2 H, n* = 4) or 24 h later (*24 H, n* = 5). *$P < 0.02$ vs. controls. Data are means±SEM. (*Lower section*) Representative Northern blot for HSP70 comparing four control pigs and four pigs sacrificed 2 h after repetitive ischemia. The lower panel shows the same blot probed for glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) for comparison. Note the increase in HSP70 mRNA in postischemic samples compared with control samples.

Discussion

This study demonstrates that brief, sublethal ischemia induces a powerful protective response that renders the myocardium relatively resistant to stunning 24 h later—a phenomenon that can be described as late ischemic preconditioning against stunning. Our salient findings can be summarized as follows: (*a*) in conscious pigs subjected to two identical ischemic insults (10 2-min coronary occlusions) at a distance of 24 h, the severity of the stunning observed after the second insult is ~ 50% less than that observed after the first; thus, a brief ischemic stress triggers delayed and/or long-lasting adaptations that result in a strikingly different response to the same ischemic stress; (*b*) the same attenuation of stunning is still present 24 h after the second ischemic stress, indicating that the preconditioned state can be maintained for at least 48 h; (*c*) the preconditioning effect disappears within 10 d after the last ischemic stress; (*d*) once disappeared, the preconditioning effect can be reinduced in the same manner by another ischemic stress; (*e*) the development

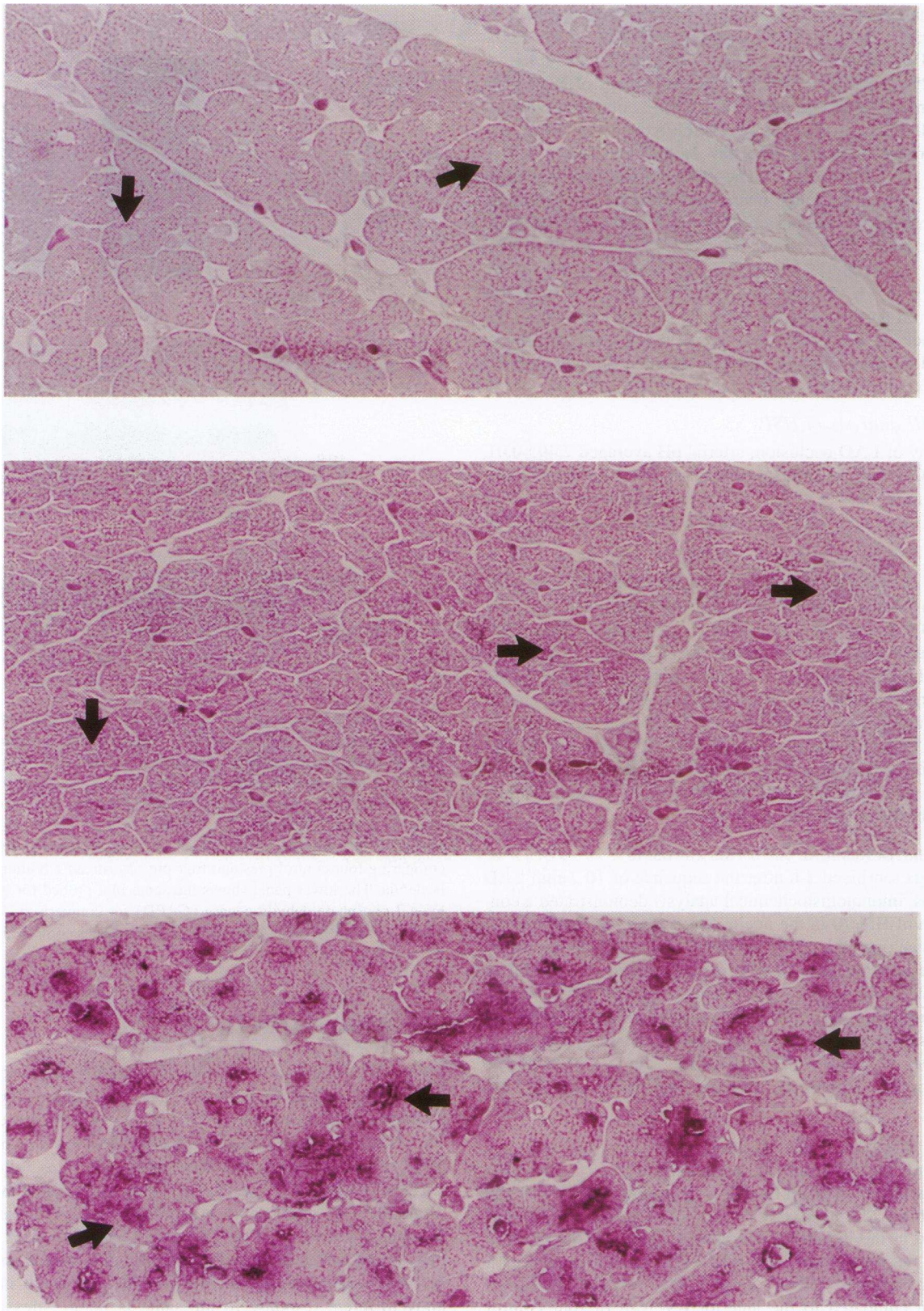

Figure 7. Immunohistochemical analysis of HSP70 in a pig subjected to 10 2-min LAD occlusions/2-min reperfusions and sacrificed 2 h after the 10th reperfusion (*upper* and *lower panels*) and in a control pig sacrificed without coronary occlusion-reperfusion (*middle panel*). In the pig subjected to ten LAD occlusions, samples of ischemic-reperfused myocardium were incubated with a monoclonal antibody against the inducible form of HSP70 (*lower panel*) or with PBS alone (*upper panel*). In the control pig, samples of anterior LV wall were incubated with the same antibody (*middle panel*). Alkaline phosphatase was used as the reporter system. Nitro-blue tetrazolium was used to visualize HSP70 and the sections were counterstained with an aqueous eosin solution. There is no staining for HSP70 2 h after ischemia–reperfusion in the absence of the antibody (*upper*

of this type of preconditioning is not blocked by adenosine receptor antagonists, and therefore its mechanism of action appears to be different from that of other forms of preconditioning presently known; and (*f*) the induction of protection against stunning is associated with a typical early stress response and with a subsequent increase in the myocardial levels of HSP70, which is compatible with a role of HSPs in the pathogenesis of this phenomenon. Ischemic preconditioning has recently been the focus of intense investigation, and considerable insights into its pathogenesis and pathophysiology have been gained. However, to our knowledge, this is the first report describing the phenomenon of late preconditioning against myocardial stunning. Furthermore, this is the first demonstration that ischemia can precondition the myocardium at a distance of 24 h in a conscious animal preparation.

Methodological considerations. The choice of a pig model for this study was dictated by several considerations. First, the paucity of collateral vessels in the porcine heart (26–29) eliminates the variability in collateral flow that is inherent in canine models. Since collateral flow is the major determinant of the severity of myocardial stunning (13), the elimination of this variable ought to result in more reproducible postischemic dysfunction among different animals. Our results support this concept. For example, on day 1 of stage I in group I, collateral flow was essentially nil (< 0.08 ml/min per g) in all pigs and the postischemic depression of thickening fraction was reasonably uniform, with a standard deviation of 32.4% at 1 h, 27.2% at 2 h, 20.9% at 3 h, and 14.4% at 4 h. Comparable results were obtained in group II. This differs from the conscious dog model, in which the effects of a 15-min coronary occlusion on thickening fraction vary widely (depending on the collateral flow), so that the standard deviations are relatively large (38 to 57% at 1 h of reperfusion, 33 to 52% at 2 h, 34 to 50% at 3 h, and 31 to 50% at 4 h [13, 15–17]). Second, the growth of collateral vessels in response to ischemia is known to be slow in the porcine heart (28). Indeed, our results demonstrate that even after 40 LAD occlusions (day 2 of stage II in group I), collateral flow was still negligible (0.04±0.02 ml/min per g). Thus, a highly reproducible degree of ischemia was observed over a period of 3 wk, which is an indispensable requisite for longitudinal studies comparing the response of the heart to coronary occlusions repeated on different days. Such a result probably could not have been obtained in the dog, in which new collaterals develop rapidly in response to cardiac instrumentation as well as brief (2-min) coronary occlusions (32). Finally, the coronary circulation of the pig is anatomically and physiologically similar to that of humans (26, 29) and like the human heart (33), the porcine heart has minimal xanthine oxidase activity (34).

Sedation of the pigs was necessary to ensure stable hemodynamic conditions within each day of occlusion and between subsequent days. We found that even after multiple days of training in the cage, many pigs continued to exhibit episodes of restlessness or excitement over the 7-h duration of the protocol, which resulted not only in marked variations in heart rate, arterial pressure, and WTh but also, probably, in increased risk of VF during ischemia or reperfusion. In contrast, after administration of diazepam, the animals remained calm and the hemodynamics were stable throughout the protocol, as documented by our measurements (see Results and Table I). Administration of diazepam did not have appreciable inotropic or hemodynamic effects (see Results, Table I, and Figs. 2, 4, and 5). The usefulness of our sedation protocol is further supported by the observations of Pinto et al. (35), who found that pretreatment with diazepam (1 mg/kg) increased VF latency and prevented heart rate increases in conscious pigs undergoing a permanent coronary occlusion.

The protocol of 10 2-min coronary occlusions was developed in order to subject the heart to an ischemic burden sufficient to cause stunning while minimizing the risk of VF. We felt it was important to limit the duration of each occlusion because the porcine heart has a striking proclivity to arrhythmias, and because the probability of reperfusion VF increases as the duration of ischemia increases from 0 to 20 min (36). (As detailed in Methods, in our pilot studies both a 5-min and a 2.5-min coronary occlusion caused VF upon reperfusion.) With our final protocol of 10 2-min occlusions, every pig developed significant myocardial stunning but the overall incidence of VF was very low: of a total of 76 sequences of 10 2-min occlusions, only 2 (3%) were associated with VF. On the other hand, the pathophysiology of myocardial stunning after repetitive ischemia is a clinically relevant problem, because many patients with coronary artery disease experience multiple recurrent episodes of myocardial ischemia (painful or painless) in the same territory as a result of recurrent spasm and/or thrombosis (37, 38). This is the main reason why several previous investigators have used models of myocardial stunning induced by multiple brief ischemic episodes (39).

The longitudinal design of this study obviously required a conscious animal model. Thus far, the phenomenon of late preconditioning has been described only in open-chest animals; that is, there is no published report demonstrating that ischemia produced in the conscious state preconditions the myocardium 24 h later. The present study expands our knowledge regarding late preconditioning by demonstrating that this phenomenon occurs in the conscious state, at least as far as protection against stunning is concerned.

The heart rates observed in our pigs (range of average values: 123–144 beats/min) are comparable with those reported in previous studies in conscious standard domestic pigs (range of average values: 115–150 beats/min) (35, 40–42), but are faster than those generally reported in conscious miniswine (usual range of average resting values: 90–110 beats/min) (43–47). The reasons for these differences are unknown. The heart rates observed in our pigs during stages I and II were similar to those measured in the same animals before instrumentation, and therefore cannot be ascribed to surgery or other factors related to our manipulations.

The measurements of systolic thickening fraction obtained at baseline in this study (range of average values: 22–31%) are

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panel). A slight amount of HSP70 is present in the control pig under unstressed conditions (*middle panel*). Note the typical early localization of HSP70 to the nucleus after the ischemic stress (*lower panel*). Some localization of antibody to the striations of the cardiomyocytes is also seen at 2 h after ischemia–reperfusion (*lower panel*). Also note the presence of interstitial edema in the ischemic-reperfused samples (*upper* and *lower panels*). Arrows indicate representative nuclei. ×82.

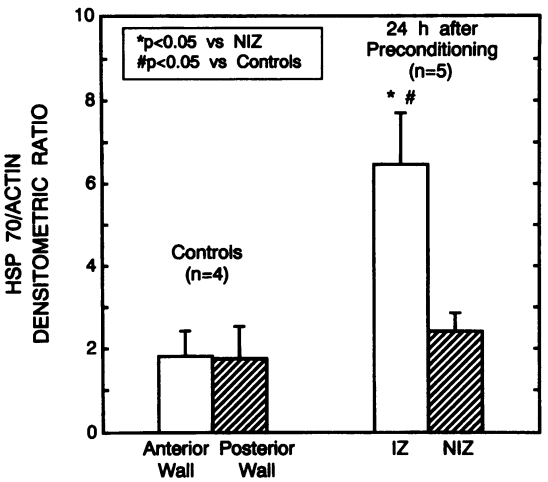

Figure 8. Densitometric assessment of HSP70 in myocardial samples obtained from pigs that did not undergo coronary occlusion-reperfusion (*Controls*, $n = 4$) and pigs that underwent a sequence of 10 2-min occlusions/2-min reperfusions and were sacrificed 24 h later ($n = 5$). In each sample, the relative amounts of HSP70, assessed by dot blot analysis, were normalized to the relative amounts of actin, as determined by a second dot blot done simultaneously. *IZ*, ischemic zone; *NIZ*, nonischemic zone. Data are means±SEM.

comparable with those reported in previous studies in conscious standard domestic pigs (range of average values: 23–32%) (40, 48, 49), but are lower than those reported in conscious miniswine (range of average values: 30–60%) (43–47). We do not have a definite explanation for these differences. It is possible that the differences depend in part on the orientation of the myocardial fibers, which varies at different base-to-apex distances within the heart. In any case, since the purpose of this investigation was to compare the recovery of WTh in the same region on subsequent days, the important point is that the baseline values of thickening fraction in our animals were stable and extremely reproducible from one day to the next, both in the LAD-dependent and in the circumflex-dependent territories (see Results and Table I) and that, at the end of each day, thickening fraction in the LAD-dependent territory always returned to values similar to baseline (Figs. 2, 4, and 5). These observations indicate that the effects of repetitive coronary occlusions were evaluated in a preparation in which regional myocardial function was otherwise stable.

Possible extrinsic influences on stunning. Before ascribing the improved recovery on days 2 and 3 to a myocardial adaptive response, the possibility must be considered that it could have been caused by favorable modifications of the extrinsic variables that modulate the performance of the stunned myocardium. As indicated in Results, arterial blood gases, hematocrit, and body temperature were within normal limits on each day of coronary occlusion, and the doses of diazepam were similar among all days. As a result of the relatively long training period and the 3 d of sham studies preceding day 1, the animals were in a stable state when the coronary occlusions were performed. Indeed, the hemodynamic variables that might influence myocardial stunning (heart rate, systolic arterial pressure, rate–pressure product, left atrial pressure, LAD blood flow) did not differ between day 1 and the subsequent days in either stage I or stage II (see Results and Table I). The preocclusion measurements of thickening fraction in the LAD-dependent region (to which all subsequent measurements were normalized) were virtually identical in stage I and closely similar in stage II (Table I). The degree of paradoxical wall thinning during coronary occlusion was not significantly different on different days in stage I or in stage II (Figs. 2, 4, and 5). The close similarity of thickening fraction in the nonischemic region on different days (see Results and Table I) further corroborates the concept that regional myocardial function was stable throughout stages I and II. As mentioned above, collateral perfusion was virtually absent throughout the protocol (Table II). In summary, none of the extrinsic variables known to affect myocardial stunning can account for the marked improvement in recovery of WTh on days 2 and 3 compared with day 1. We therefore conclude that this improvement reflects a change intrinsic to the myocardium itself, whereby the heart becomes preconditioned against stunning.

The exact time-course of this preconditioning phenomenon remains to be defined. Our results demonstrate that it is present 24 h after the first sequence of LAD occlusions, that it can be extended for an additional 24 h by a repeated sequence of occlusions, and that it dissipates within 10 d after the last exposure to ischemia. Studies using different intervals between the first and the second ischemic challenge (days 1 and 2 in our protocol) will be necessary to elucidate this issue. It seems likely, however, that the preconditioning effect persists for at least several hours, perhaps even days, and thus affords a sustained protection against myocardial stunning.

Previous studies of preconditioning against infarction. It is well established that ischemic preconditioning affords an immediate protection against infarction, which lasts ~ 1 h (for reviews see references 2–4). Recent data, however, suggest the existence of a second, more prolonged window of protection. Using open-chest rabbits, Marber et al. (22) reported that preconditioning with four 5-min occlusions significantly decreased infarct size after a 30-min occlusion performed 24 h after the preconditioning protocol; this effect was associated with an increase in myocardial HSP70 and HSP60. Interestingly, the protective effect was abolished by the administration of the adenosine receptor antagonist SPT during the preconditioning protocol and, conversely, was reproduced by pretreatment with the selective adenosine A_1-agonist CCPA 24 h before the 30-min occlusion, indicating that it was mediated by an adenosine A_1-receptor–coupled pathway (30). Using open-chest dogs, Kuzuya et al. (50) demonstrated that the size of the infarcts produced by a sustained 90-min occlusion was significantly reduced when the animals were preconditioned with four 5-min occlusions 24 h earlier.

The present study found a temporally similar protection against a completely different end point, i.e., myocardial stunning. Thus, our present results, taken together with these prior studies (22, 30, 50), indicate that sublethal ischemia induces a late preconditioning effect that protects not only against irreversible injury (cell death) but also against reversible postischemic dysfunction (stunning). At present, however, we do not know whether the protocol of ten 2-min LAD occlusions, which we found to precondition against stunning, also induces late preconditioning against infarction in this conscious pig model.

Previous studies of preconditioning against stunning. Our finding that ischemic preconditioning attenuates myocardial stunning in conscious pigs differs from previous results in acute animal models. In the "classic" setting of a completely reversible ischemic insult induced by a 15-min coronary occlusion in

open-chest dogs, Ovize et al. (10) demonstrated that when a 5- or 2.5-min occlusion preceded the 15-min occlusion, the recovery of contractile function was not enhanced. Similar results were obtained by Miyamae et al. (12) in open-chest pigs. In the setting of a partly reversible ischemic insult (subendocardial infarction), Cohen et al. (9) found that preconditioning with one 5-min occlusion resulted not only in smaller infarctions but also in improved segment shortening after a sustained 20-min occlusion in rabbits; however, the enhanced recovery of function could be explained entirely by the reduction in infarct size. Ovize et al. (11) demonstrated in open-chest dogs that preconditioning with either four 3-min occlusions or four 5-min occlusions failed to attenuate myocardial stunning in the peri-infarct tissue after a 1-h occlusion. Similar findings were reported by Schott et al. (8) in open-chest pigs. Thus, the available evidence indicates that ischemic preconditioning does not alleviate myocardial stunning after either a brief (< 20 min) occlusion unassociated with cell death or a longer occlusion associated with subendocardial infarction. These results have led to the conclusion that ischemia does not precondition the myocardium against stunning.

In all of the studies performed thus far (8–12), however, stunning was induced immediately after the preconditioning protocol; the long-term sequelae of ischemic preconditioning on postischemic dysfunction have not been previously examined. The present investigation significantly changes our understanding of the effect of ischemia on stunning by demonstrating that although ischemia does not protect immediately after reflow, it does induce a preconditioning effect that becomes manifest 24 h later and can be maintained for at least 48 h.

Mechanism of late preconditioning against stunning. It is well established that the mechanism of early preconditioning against infarction involves the activation of adenosine A_1-receptors as a result of the release of adenosine during the preconditioning ischemia (2–4, 6). Recent data indicate that this mechanism is involved in late preconditioning against infarction as well (30). The ability of adenosine receptor antagonists to block preconditioning against infarction in pigs has been documented (51). Accordingly, we tested the hypothesis that an adenosine receptor-coupled pathway could also be responsible for the late preconditioning against stunning. Our results demonstrate that this is not the case. Administration of SPT during the preconditioning ischemia on day 1 failed to block or attenuate the development of preconditioning on day 2, despite the fact that the dose of SPT was sufficient to inhibit the chronotropic and dromotropic effects of CCPA. Our dose of SPT was greater than or equal to the doses of SPT previously shown to block early (6) and late (30) preconditioning against infarction. Thus, we conclude that with our 10 2-min occlusion protocol, the mechanism of late preconditioning against stunning does not involve activation of adenosine receptors. Further studies will be necessary to determine whether late preconditioning against stunning is mediated by protein kinase C, which appears to be the final common pathway of early preconditioning against infarction and which can be activated by a variety of stimuli other than adenosine (4).

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 cardioprotective proteins. Our results demonstrate that the presence of protection 24 h after the first ischemic stress was associated with an increase in the myocardial levels of HSP70—a finding consonant with previous observations in other models of reversible ischemia (18, 22). Furthermore, we observed the two other major components of the stress response (52): (*a*) a rapid translocation of HSP70 to the nucleus, as demonstrated by the immunohistochemical analysis performed 2 h after the preconditioning ischemia, and (*b*) an increase in steady-state levels of HSP70 mRNA within 2 h from the preconditioning ischemia with a subsequent return to control levels by 24 h. On the basis of these results, it is attractive to speculate that the late preconditioning against stunning may be mediated by the synthesis of HSPs. Numerous studies have demonstrated that induction of HSPs with heat stress results in subsequent protection against ischemia–reperfusion injury in isolated hearts subjected to global ischemia (53–55). More recent experiments in models of regional ischemia in vivo suggest that the presence of protection depends on the duration of coronary occlusion (i.e., on the severity of injury), with a reduction in infarct size reported after a 30–35-min occlusion (22, 56, 57) but not after longer occlusions (56–58). This suggests that heat stress can delay the development of injury during relatively brief periods of ischemia, although it does not prevent the injury associated with a sustained ischemic insult. Since the duration of ischemia that induces stunning is shorter than that which induces infarction, these results appear to be compatible with the hypothesis that HSPs may confer protection against stunning.

The precise mechanism whereby an elevation of HSPs could protect against myocardial stunning is unknown. HSPs may maintain the integrity of the contractile machinery by protecting key cellular proteins from denaturization and/or by reactivating inactivated enzymes during ischemia and reperfusion (52, 59). HSPs may also exert an antioxidant effect, either directly or by preserving the activity of enzymes involved in free radical removal (for reviews see references 60 and 61). It must be stressed, however, that ischemia is likely to induce a complex cascade of cellular responses, some of which may be as important as, or more important than, HSP synthesis in mediating the protection. Additional investigations will be necessary to establish whether HSPs play a causal role in late preconditioning against stunning and whether other proteins (e.g., antioxidant enzymes, calcium regulatory proteins) are involved in this phenomenon.

Conclusions and implications. In this study we have described a new, previously unrecognized cardioprotective phenomenon. We found that a brief ischemic stress triggers a powerful, long-lasting (at least 48 h) adaptive response that renders the heart partially resistant to the same ischemic stress applied 24 h later. Unlike early (2–4, 6) and late (30) preconditioning against infarction, this late preconditioning against stunning is not mediated by stimulation of adenosine receptors, and thus appears to involve a mechanism different from that of other forms of preconditioning currently known. Hence, some unknown intrinsic cardioprotective mechanism is activated by brief coronary occlusions in conscious pigs. Although further studies will be necessary to identify the nature of this mechanism, our observations are compatible with the hypothesis that it is mediated by HSPs and possibly other cardioprotective proteins.

Late preconditioning against stunning may be an important (and thus far not considered) determinant of LV dysfunction in patients with coronary artery disease who experience recurrent episodes of myocardial ischemia in the same territory. In these patients, the development of late preconditioning may mitigate

the severity of postischemic contractile abnormalities and protect against their cumulative effect on LV performance. In addition, the presence of late preconditioning in some patients but not in others could explain in part the variability and inconsistency in the degree of postischemic LV dysfunction observed in clinical settings such as unstable angina, exertional angina, acute infarction with early reperfusion, and cardiac surgery (38). Elucidation of the mechanism responsible for this endogenous protective response should be useful not only for enhancing our understanding of the pathogenesis of myocardial stunning, but also because the same mechanism could be exploited to develop effective clinical therapies aimed at preventing postischemic dysfunction.

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