

Increased protein synthesis is necessary for the development of late preconditioning against myocardial stunning

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Increased protein synthesis is necessary for the development of late preconditioning against myocardial stunning. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H874–H884, 1999.—In *phase I* of this study, the rate of protein synthesis was measured by the incorporation of [³H]leucine into the protein pool in the heart of conscious rabbits. At 2 h after ischemic preconditioning (PC) with six 4-min occlusion/4-min reperfusion (O/R) cycles (*group II*), the [³H]leucine content in the ischemic-reperfused region was increased by 82% compared with that in controls (*group I*), indicating increased protein synthesis. This increase was completely abrogated by pretreatment with cycloheximide (CH; *group III*). In *phase II*, rabbits underwent six O/R cycles for three consecutive days (*days 1–3*). Controls (*group IV*) exhibited late PC against myocardial stunning on *days 2* and *3*. In *group V*, which received CH 30 min before the 1st O/R cycle on *day 1* (same dose as *group III*), late PC against stunning on *day 2* was completely abrogated. In *group VI*, pretreatment with CH 24 h before the 1st sequence of O/R cycles had no effect on myocardial stunning on *day 1*, indicating that the absence of late PC on *day 2* in *group V* cannot be ascribed to delayed toxicity of CH. Taken together, these results demonstrate that, in the conscious rabbit, ischemic PC causes a rapid increase in myocardial protein synthesis and that this increased protein synthesis (or at least a fraction of it) is necessary for the development of the protection against myocardial stunning 24 h later. The late phase of ischemic PC is therefore dependent on the formation of new proteins in intact animals.

cycloheximide; myocardial ischemia; myocardial reperfusion; [³H]leucine; systolic wall thickening

ISCHEMIC PRECONDITIONING (PC) is the phenomenon whereby brief episodes of ischemia increase the resistance of the myocardium to subsequent ischemic insults (12, 17, 30, 34). Initially, PC was described as a protective effect that develops almost immediately and disappears within 2–4 h (“early phase” of PC) (1, 11–13, 17, 34). Recently, however, it has become apparent that PC induces a second or late phase of protection, which begins 12–24 h after the ischemic stimulus and disappears within 4–5 days (2–4, 7, 8, 27, 29, 37, 40, 41, 46–50, 57, 58). This late phase has been found to be associated with protection against myocardial infarction in dogs (27) and rabbits (2–4, 29, 30, 37, 41, 48, 49,

57, 58). In addition, the late phase of PC confers powerful protection against myocardial stunning in conscious pigs (46, 47, 50) and rabbits (7, 8, 47, 40, 49).

The mechanism responsible for late PC is the focus of much current investigation. One plausible hypothesis is that this phenomenon is the result of the appearance of new gene products that are induced by sublethal ischemia as part of the cellular stress response to the PC stimulus. Indeed, a number of reports have shown an association between ischemic PC and the formation of proteins such as antioxidant enzymes (21, 60) and heat shock proteins (HSPs) (29, 30). However, to date, there is no conclusive evidence that the late phase of ischemic PC *in vivo* is mediated by the synthesis of new proteins. It is entirely plausible, for example, that this delayed cytoprotective phenomenon may be mediated instead by activation (e.g., phosphorylation) of preexisting proteins. Moreover, the notion that ischemic PC induces expression of antioxidant enzymes or HSPs is controversial. Neither upregulation of HSP70 nor increased protein content of superoxide dismutase (SOD) has been observed consistently after ischemic PC (20), and we have been unable to document an increase in antioxidant enzymes 24 h after ischemic PC in conscious pigs (51) and rabbits (unpublished observations). Perhaps more importantly, a cause-and-effect relationship between increased protein synthesis and cardioprotection has not been established. Even when increased expression of certain proteins [e.g., HSP70 (43)] is observed, it remains unclear whether such proteins are necessary for the protection to develop or are merely an epiphenomenon (43). For a cause-and-effect relationship to be proven, one would have to show that inhibiting protein synthesis results in loss of PC protection.

The goal of the present investigation was to test the hypothesis that augmented protein synthesis is required for the development of the late phase of ischemic PC in conscious animals. To this end, in *phase I* of the study we investigated whether the PC stimulus is associated with accelerated synthesis of proteins in conscious rabbits. Having found an increase in protein synthesis, in *phase II* we examined whether blocking this phenomenon with cycloheximide, a translational inhibitor (24), would abolish protection against myocardial stunning 24 h later in the same model. A PC protocol was employed (six 4-min coronary occlusions) that has previously been established to induce a potent and reproducible late PC effect against both myocardial stunning (7, 8, 37, 40, 49, 58) and infarction (37, 41, 48, 49, 58). The study was conducted in conscious rabbits to obviate the confounding effects of factors associated with open-chest preparations, such as anesthesia, sur-

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gical trauma, fluctuations in temperature, elevated catecholamines, excessive free radical formation, release of cytokines, etc., which can interfere with myocardial stunning (6, 28, 54) and/or with ischemic PC (11, 13, 19, 45) and can also, in themselves, induce protein synthesis (21, 23).

METHODS

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 86-23].

Experimental Preparation

The experimental preparation and techniques have previously been described in detail (7, 8, 37, 40, 41, 48, 49, 58). Briefly, New Zealand White male rabbits (weight 2.0–3.4 kg; age 3–4 mo) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed-Doppler ultrasonic crystal in the center of the region to be rendered ischemic, and bipolar electrocardiogram (ECG) leads on the chest wall. The chest wound was closed in layers, and a small tube was left in the thorax for 3 days to evacuate air and fluids postoperatively. Gentamicin was administered before surgery and on the first and second postoperative days (0.7 mg/kg im each day). Rabbits were allowed to recover for a minimum of 10 days after surgery.

General Experimental Protocol

Throughout the experiments, the rabbits were kept in a cage in a quiet, dimly lit room. Left ventricular (LV) systolic wall thickening, the range gate depth, and the ECG were continuously recorded on a thermal array chart recorder (Gould TA6000, Valley View, OH). No sedative or antiarrhythmic agents were given at any time. The general experimental protocol consisted of a sequence of six 4-min coronary occlusions interspersed with 4 min of reperfusion (Fig. 1). The performance of successful coronary occlusions was verified by observing the development of S-T segment elevation and changes in the QRS complex on the ECG and the appearance of paradoxical systolic wall thinning on the ultrasonic crystal recordings. The study consisted of two subsequent phases (*phases I and II*).

Phase I: Measurement of Protein Synthesis

Pilot studies. Although we anticipated that interfering with protein synthesis in a conscious animal would inevitably lead to some untoward effects, pilot studies were carried out in five rabbits to identify a dose of cycloheximide (Sigma Chemical, St. Louis, MO) that would effectively block protein synthesis without causing severe toxicity. In one rabbit, a dose of 16 mg/kg of cycloheximide administered intraperitoneally caused death 24 h later. A lower dose of 12 mg/kg was administered intraperitoneally in two rabbits: one died 24 h later, whereas the other one survived, but with obvious toxic effects. In another rabbit, a dose of 8 mg/kg intraperitoneally produced severe toxicity (lethargy, absence of spontaneous movements, and inability to feed) without causing death. Finally, in a fifth rabbit, a dose of 2 mg/kg intraperitoneally was tolerated well with no obvious side effects. Consequently, this dose was chosen for the present experiments. The results obtained in *phases I and II* confirmed that this dose of cycloheximide did not produce severe toxicity.

Experimental protocol. Having established that 2 mg/kg intraperitoneally is the highest dose (or one of the highest doses) of cycloheximide that can be given to rabbits without severe side effects, we next determined whether this dose blocks protein synthesis *in vivo*. To this end, we measured the incorporation of tritium-labeled leucine ($[^3\text{H}]$ leucine) into the protein pool of the heart, liver, and skeletal muscle. Rabbits were assigned to three groups: *group I*, a nonischemic control group; *group II*, an ischemic control group; and *group III*, a cycloheximide-treated group (Fig. 1A). *Group I* received $[^3\text{H}]$ leucine (50 $\mu\text{Ci/kg}$ iv) without undergoing coronary occlusion-reperfusion. In *group II*, the rabbits underwent the six coronary occlusion-reperfusion cycles without administration of cycloheximide, whereas in *group III*, cycloheximide was administered 30 min before the sequence of six coronary occlusion-reperfusion cycles. In both *groups II and III*, $[^3\text{H}]$ leucine was given at the onset of the sixth reperfusion (50 $\mu\text{Ci/kg}$ iv). Two hours after the administration of $[^3\text{H}]$ leucine, the rabbits were euthanized and tissue samples (~1 g) were obtained from the ischemic-reperfused LV region (or the anterior LV wall), the nonischemic LV region (or the posterior LV wall), the liver, and the quadriceps muscle. Each sample was homogenized in 10 ml of ice-cold sodium phosphate buffer (pH 7.4) using a Polytron homogenizer. We precipitated proteins in 5 ml of the homogenate by adding 5 ml of 20% trichloroacetic acid. The rest of the homogenate was saved for Bio-Rad protein analysis. The precipitated homogenate was centrifuged at 3,000 rpm for 15 min at 10°C. The supernatant was discarded, and the pellet was washed with 10 ml of 95% ethanol and centrifuged again (3,000 rpm for 15 min). The supernatant was again discarded. The pellet was solubilized by boiling in 2 ml of 1 M NaOH, and the radioactivity was counted with a liquid scintillation counter. Data are expressed as disintegration counts per minute (cpm) per milligram of protein.

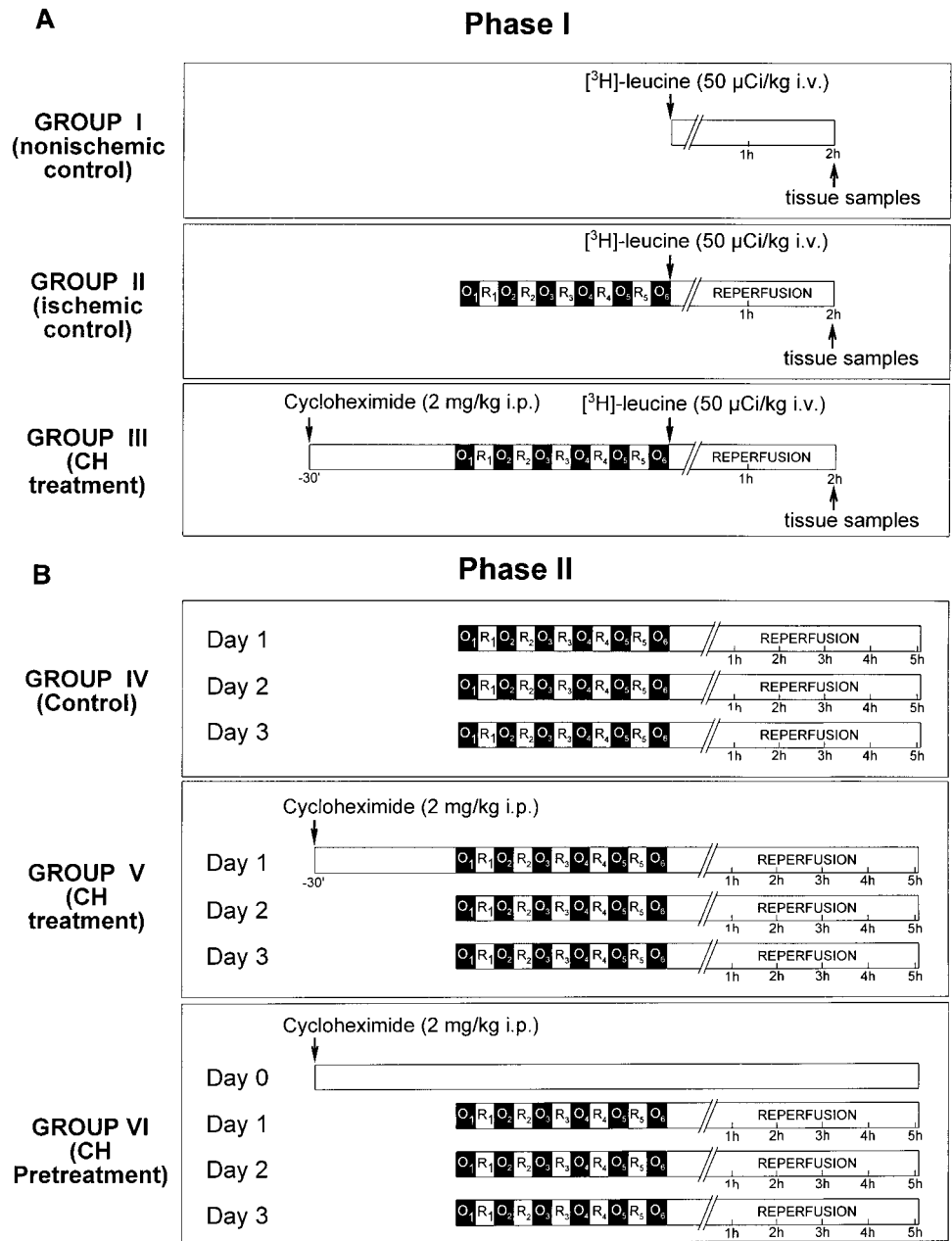
The rationale for measuring the rate of protein synthesis in the first 2 h after the ischemic PC stimulus was that, in this rabbit model, the mobilization of the cellular kinases and transcription factors involved in late PC occurs very rapidly [within 30 min from the stimulus (38, 40, 57)], suggesting that the synthesis of the cytoprotective protein(s) that mediate(s) late PC may start soon after the six occlusion-reperfusion cycles. If this were the case, measuring $[^3\text{H}]$ leucine incorporation into proteins at later time points might fail to detect an early inhibitory effect of cycloheximide.

Phase II: Studies of Late PC

Experimental protocol. All rabbits underwent the six coronary occlusion-reperfusion cycles for three consecutive days (*days 1–3*) (Fig. 1B). Rabbits were assigned to three groups: *group IV*, a control group; *group V*, a cycloheximide-treated group; and *group VI*, a cycloheximide-pretreated group (Fig. 1B). *Group IV* was subjected to the coronary occlusion-reperfusion protocols without any treatment. On *day 1*, rabbits in *group V* received cycloheximide 30 min before the first coronary occlusion at a dose of 2 mg/kg intraperitoneally; on *days 2 and 3*, the rabbits underwent the same coronary occlusion-reperfusion protocol without any treatment. Cycloheximide was dissolved in normal saline (3.5 mg/ml), and the solution was filtered through a 0.2- μm Millipore filter to ensure sterility. In *group VI*, rabbits were pretreated with the same dose of cycloheximide 24 h before the first sequence of coronary occlusion-reperfusion cycles (*day 0*); the animals were then subjected to the coronary occlusion-reperfusion protocol on *days 1–3*.

Measurement of regional myocardial function. Regional myocardial function was assessed as systolic thickening

Fig. 1. Experimental protocol for the studies of protein synthesis (*phase I; A*) and for the studies of late preconditioning (PC) (*phase II; B*). *A*: in *phase I*, 3 groups of rabbits were studied. *Group I* ($n = 6$, nonischemic control group) received tritium-labeled leucine ($[^3\text{H}]$ leucine, $50 \mu\text{Ci/kg}$ iv) without undergoing coronary occlusion-reperfusion; rabbits were euthanized 2 h later, and tissue samples were obtained for measurement of $[^3\text{H}]$ leucine incorporation into the protein pool. *Group II* ($n = 6$, ischemic control group) underwent a sequence of six 4-min coronary occlusion (O)/4-min reperfusion (R) cycles and received $[^3\text{H}]$ leucine ($50 \mu\text{Ci/kg}$ iv) at the onset of the 6th reperfusion. Rabbits were euthanized 2 h later, and tissue samples were obtained. *Group III* [$n = 6$, cycloheximide (CH)-treated group] received CH 30 min before the six 4-min coronary occlusion-reperfusion cycles (2 mg/kg ip) and then $[^3\text{H}]$ leucine ($50 \mu\text{Ci/kg}$ iv) at the onset of the 6th reperfusion. Rabbits were euthanized 2 h later, and tissue samples were obtained. *B*: in *phase II*, 3 groups of rabbits were studied. All groups underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles followed by a 5-h observation period for three consecutive days (*days 1-3*). Rabbits in *group IV* ($n = 8$, control group) received no treatment. Rabbits in *group V* ($n = 8$, CH-treated group) received CH (2 mg/kg ip) 30 min before the 1st coronary occlusion on *day 1*. Rabbits in *group VI* ($n = 5$, CH-pretreated group) were pretreated with CH (2 mg/kg ip) 24 h before the 1st coronary occlusion on *day 1*.



fraction using the pulsed-Doppler probe, as previously described (9). Percent systolic thickening fraction was calculated as the ratio of net systolic thickening to end-diastolic wall thickness, multiplied by 100 (9). The total deficit of systolic wall thickening after reperfusion (an integrative assessment of the overall severity of myocardial stunning after the 6th reperfusion) was calculated by measuring the area comprised between the systolic wall thickening-versus-time line and the baseline (100% line) during the 5-h recovery phase after the sixth reperfusion (7, 8, 37, 40, 49, 58). In all animals, measurements were averaged from at least 10 beats at baseline and from at least 5 beats at all subsequent time points.

Postmortem tissue analysis. At the conclusion of the study, the rabbits were given heparin (1,000 U iv), after which they were anesthetized with pentobarbital sodium (50 mg/kg iv) and euthanized with an intravenous bolus of KCl. The heart was excised, and the size of the occluded-reperfused coronary vascular bed was determined by tying the coronary artery at

the site of the previous occlusion and perfusing the aortic root for 2 min with a 5% solution of phthalo blue dye in normal saline at a pressure of 70 mmHg using a Langendorff apparatus (8). The heart was then cut into 2-mm-thick transverse slices, which were incubated for 15 min at 37°C in a 1% solution of triphenyltetrazolium chloride in phosphate buffer (pH 7.4) to verify the absence of infarction (8). The portion of the LV supplied by the previously occluded coronary artery (occluded bed) was identified by the absence of blue dye and separated from the rest of the LV. Both components were weighed to determine the occluded bed size as a percentage of total LV weight.

Statistical Analysis

Data are reported as means \pm SE. For intragroup comparisons, hemodynamic variables and wall thickening 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 (55). For intergroup comparisons, continuous variables were analyzed by either a one-way or a two-way repeated-measures (time and group) ANOVA, followed by unpaired Student's *t*-tests with the Bonferroni correction (55). The [³H]leucine content was compared using a one-way ANOVA followed by paired or unpaired Student's *t*-tests, as appropriate, with the Bonferroni correction. All statistical analyses were performed using the SAS software system (44). ANOVA was performed using the General Linear Models (44) procedure.

RESULTS

A total of 44 conscious rabbits were used in this investigation: 5 for the pilot studies, 18 for *phase I*, and 21 for *phase II*.

Phase I: Measurement of Protein Synthesis

Of the 18 rabbits instrumented for *phase I*, 6 were assigned to the nonischemic control group (*group I*), 6 to the ischemic control group (*group II*), and 6 to the cycloheximide-treated group (*group III*). There were no exclusions in any group.

Group I: Control group. Rabbits in the control group received [³H]leucine without undergoing coronary occlusion-reperfusion; 2 h after the administration of [³H]leucine, tissue from the LV, liver, and skeletal muscle was harvested to determine [³H]leucine incorporation into the protein pool (Fig. 1A). The amount of [³H]leucine detected in the anterior LV wall (corresponding to the ischemic-reperfused region in *groups II* and *III*) and the amount detected in the posterior LV wall (corresponding to the nonischemic region in *groups II* and *III*) were similar (546 ± 102 and 583 ± 92 cpm/mg, respectively) (Fig. 2), indicating that under control conditions the

rate of protein synthesis in the conscious rabbit is uniform in the two regions of the LV. The rate of [³H]leucine incorporation was somewhat higher in the liver (694 ± 134 cpm/mg) and lower in skeletal muscle (371 ± 74 cpm/mg) (Fig. 2).

Group II: Ischemic control group. *Group II* underwent a sequence of six 4-min occlusion-reperfusion cycles and received [³H]leucine at the onset of the sixth reperfusion; the rabbits were euthanized 2 h later (Fig. 1A). Compared with *group I*, the incorporation of [³H]leucine was significantly increased in both the ischemic-reperfused LV region (+82%, *P* < 0.05) and in the nonischemic LV region (+53%, *P* < 0.05) (Fig. 2). The rate of [³H]leucine incorporation did not differ statistically between the two regions (991 ± 134 vs. 894 ± 104 cpm/mg) (Fig. 2). Thus the PC protocol employed in this study caused an increase in the rate of protein synthesis not only in the previously ischemic myocardium but also in the control nonischemic myocardium. The rate of [³H]leucine incorporation in the liver and skeletal muscle did not differ from that measured in the nonischemic control group (*group I*) (Fig. 2).

Group III: Cycloheximide-treated group. *Group III* underwent the same protocol as *group II* except that cycloheximide was given 30 min before the six 4-min occlusion-reperfusion cycles (Fig. 1A). Compared with *group II*, the incorporation of [³H]leucine was markedly reduced in all tissues examined: -79% in the ischemic-reperfused LV region (*P* < 0.05), -76% in the nonischemic LV region (*P* < 0.05), -50% in the liver (*P* < 0.05), and -57% in the skeletal muscle (*P* < 0.05) (Fig. 2). Thus the dose of cycloheximide used in this study completely abrogated the increased synthesis of proteins associated with ischemic PC.

Phase II: Studies of Late PC

Of the 21 rabbits instrumented for *phase II*, 8 were assigned to the control group (*group IV*), 8 to the

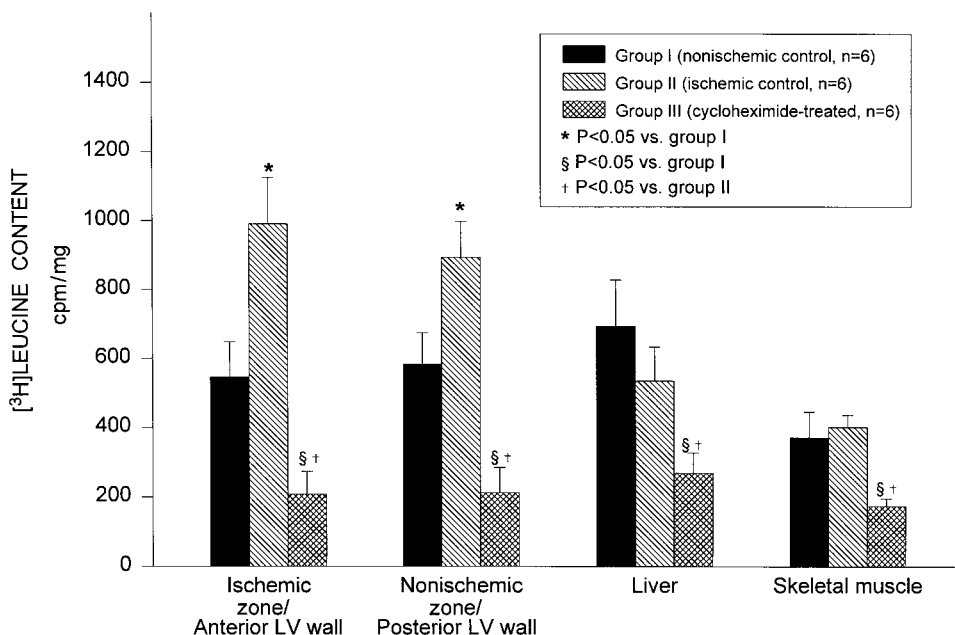


Fig. 2. Tissue content of [³H]leucine in the ischemic-reperfused left ventricular (LV) region (or anterior LV wall), nonischemic LV region (or posterior LV wall), liver, and skeletal muscle in *groups I-III*. *Group I* (nonischemic control, *n* = 6) received [³H]leucine without undergoing coronary occlusion-reperfusion. *Group II* (ischemic control, *n* = 6) underwent a sequence of six 4-min coronary occlusion-reperfusion cycles and received [³H]leucine at the onset of the 6th reperfusion. *Group III* (CH-treated group, *n* = 6) underwent the same protocol as *group II* except that it received CH 30 min before the 1st coronary occlusion. The tissue content of [³H]leucine is expressed as counts per min (cpm)/mg protein. Data are means ± SE.

cycloheximide-treated group (*group V*), and 5 to the cycloheximide-pretreated group (*group VI*). All of the rabbits assigned to *groups IV* and *V* completed the protocol. One of the five rabbits assigned to *group VI* died on *day 3* (because of ventricular fibrillation during the 4th occlusion); therefore, in *group VI* five rabbits completed *days 1* and *2* and four completed *day 3*.

Postmortem analysis. The size of the occluded-reperfused vascular bed was similar in the three groups: 1.19 ± 0.10 g ($23.2 \pm 2.2\%$ of LV weight) in *group IV*, 1.04 ± 0.22 g ($17.2 \pm 0.9\%$ of LV weight) in *group V*, and 0.98 ± 0.18 g ($20.0 \pm 3.0\%$ of LV weight) in *group VI*. Tissue staining with triphenyltetrazolium chloride demonstrated absence of infarction in all of the rabbits included in the final analysis, confirming that the injury associated with the six 4-min reperfusion cycles was completely reversible.

Hemodynamic variables. As shown in Table 1, in the control group (*group IV*) heart rate remained stable during each of the 3 days of the protocol. In *group V* (cycloheximide-treated group), on *day 1* heart rate was similar to that observed in *group IV* at baseline (before cycloheximide) and at preocclusion (25 min after cycloheximide); however, an increase in heart rate was observed at subsequent time points on *day 1* throughout the 5-h reperfusion period (Table 1). Compared with preocclusion values, in *group V* heart rate on *day 1* was significantly higher at 30 min and 1, 2, 3, 4, and 5 h ($P < 0.05$ at all time points); furthermore, heart rate was significantly higher in *group V* compared with *group IV* at 30 min ($P < 0.05$) and 1 ($P < 0.01$), 2 ($P < 0.01$), 3 ($P < 0.01$), 4 ($P < 0.01$), and 5 h ($P < 0.01$) (Table 1). In *group VI* (cycloheximide-pretreated group), heart rate did not differ significantly from *group IV* at any time point on *day 1* (Table 1). On *days 2* and *3*, heart rate in *groups V* and *VI* was similar to that in *group IV* (Table 1). In three rabbits assigned to *group V* arterial pressure was measured by cannulating the ear dorsal artery with a 24-gauge angiocatheter under local anes-

thesia (benzocaine). The catheter was connected to a fluid-filled high-sensitivity pressure transducer, which was connected to a pressure analyzer (model BPA-109, Micro-Med, Louisville, KY). The results demonstrated that in *group V* arterial blood pressure did not change significantly in the first 2.5 h after the administration of cycloheximide (Table 2).

Regional myocardial function. Baseline systolic thickening fraction in the region to be rendered ischemic was 36.0 ± 3.4 , 35.6 ± 2.9 , and $36.7 \pm 2.9\%$ on *days 1*, *2*, and *3*, respectively, in *group IV*; 39.8 ± 3.6 , 43.5 ± 4.4 , and $41.1 \pm 3.3\%$, respectively, in *group V*; and 39.9 ± 3.6 , 38.6 ± 3.4 , and $37.6 \pm 2.8\%$, respectively, in *group VI*. There were no significant differences among the three groups on the same day or among different days within the same group. In *group V*, the administration of cycloheximide had no immediate effect on thickening fraction, which averaged $39.8 \pm 3.6\%$ at baseline and $40.8 \pm 3.6\%$ 25 min after treatment (preocclusion) ($P =$ not significant, see Fig. 4). The results obtained in *group VI*, which received cycloheximide 24 h before the first coronary occlusion on *day 1*, further corroborate the conclusion that this agent had no significant effect on regional myocardial function. In the three rabbits in *group VI*, in which thickening fraction was measured before and after treatment, the values averaged $44.3 \pm 1.9\%$ before cycloheximide (baseline) and did not change appreciably after cycloheximide ($44.6 \pm 2.4\%$ at 1 h, $48.5 \pm 3.3\%$ at 2 h, $45.4 \pm 2.9\%$ at 3 h, $46.3 \pm 1.5\%$ at 4 h, and $44.6 \pm 1.4\%$ at 5 h).

Figures 3, 4, and 5 demonstrate the serial measurements of thickening fraction during the six occlusion-reperfusion cycles and the 5-h recovery phase, expressed as a percentage of preocclusion measurements, in *groups IV*, *V*, and *VI*, respectively. We first describe the results in the control group and then those in the cycloheximide-treated and pretreated groups.

Group IV: Control group. On *day 1*, regional contractile function remained significantly depressed for 3 h

Table 1. Heart rate during coronary occlusion and reperfusion

	Baseline	Preocclusion	1st Occlusion	6th Occlusion	Reperfusion					
					30 min	1 h	2 h	3 h	4 h	5 h
Group IV (control)										
Day 1	249 ± 16	251 ± 9	259 ± 11	251 ± 16	240 ± 13	242 ± 10	244 ± 7	242 ± 9	230 ± 8	235 ± 10
Day 2	245 ± 9	256 ± 10	263 ± 13	253 ± 9	249 ± 8	245 ± 8	246 ± 6	232 ± 7	239 ± 7	251 ± 4
Day 3	254 ± 7	259 ± 7	262 ± 10	241 ± 10	264 ± 11	257 ± 9	258 ± 10	256 ± 7	243 ± 9	239 ± 7
Group V (CH treatment)										
Day 1	263 ± 9	258 ± 10	270 ± 8	281 ± 10	285 ± 7*†	294 ± 7*‡	295 ± 10*‡	291 ± 9*‡	292 ± 7*‡	286 ± 8*‡
Day 2	251 ± 9	250 ± 6	253 ± 8	250 ± 7	236 ± 7	239 ± 8	242 ± 8	229 ± 7	236 ± 12	248 ± 9
Day 3	264 ± 10	260 ± 10	268 ± 9	258 ± 13	256 ± 8	242 ± 11	243 ± 10	253 ± 7	238 ± 10	240 ± 12
Group VI (CH pretreatment)										
Day 1	246 ± 12	251 ± 8	265 ± 10	251 ± 15	233 ± 10	242 ± 14	241 ± 8	231 ± 12	231 ± 10	226 ± 13
Day 2	253 ± 12	252 ± 10	246 ± 10	238 ± 13	246 ± 13	244 ± 11	246 ± 13	242 ± 13	240 ± 13	230 ± 13
Day 3	239 ± 8	238 ± 16	245 ± 17	232 ± 9	237 ± 15	239 ± 13	247 ± 16	225 ± 13	226 ± 13	213 ± 13

Data are means ± SE (beats/min). Rabbits were subjected to a sequence of 6 cycles of 4-min coronary occlusion/4-min reperfusion followed by a 5-h observation period for 3 consecutive days (*days 1–3*). On *day 1*, rabbits in *group IV* (control group, $n = 8$) received the six 4-min coronary occlusion-reperfusion protocol without any treatment. On *day 1*, rabbits in *group V* [cycloheximide (CH)-treated group, $n = 8$] received CH (2 mg/kg ip) 30 min before the 1st occlusion. Rabbits in *group VI* (CH-pretreated group, $n = 5$) received CH (2 mg/kg ip) 24 h before the 1st occlusion on *day 1*. Heart rate was measured before treatment (baseline), 25 min after treatment (preocclusion), at 3 min into the 1st occlusion, at 3 min into the 6th occlusion, and at selected times after the 6th reperfusion. * $P < 0.05$ vs. preocclusion; † $P < 0.05$ vs. *group IV*; ‡ $P < 0.01$ vs. *group IV*.

Table 2. Arterial blood pressure before and after cycloheximide in group V (day 1)

	Baseline	Preocclusion	Occlusion		Reperfusion		
			1st	6th	15 min	30 min	1 h
SAP	79 ± 5	78 ± 4	85 ± 3	81 ± 2	74 ± 5	72 ± 3	71 ± 1
DAP	70 ± 4	69 ± 4	76 ± 4	75 ± 3	70 ± 5	67 ± 4	66 ± 2
MAP	73 ± 4	72 ± 4	79 ± 3	77 ± 2	71 ± 5	68 ± 3	68 ± 2

Data are means ± SE (mmHg). Rabbits were subjected to a sequence of 6 cycles of 4-min coronary occlusion-reperfusion followed by a 5-h observation period for 3 consecutive days (days 1-3). On day 1, rabbits received CH (2 mg/kg ip) 30 min before the 1st occlusion. In 3 rabbits, arterial pressure was measured before treatment (baseline), 25 min after treatment (preocclusion), at 3 min into the 1st coronary occlusion, at 3 min into the 6th occlusion, and at selected times after the 6th reperfusion. SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MAP, mean arterial pressure.

after the sixth reperfusion, with thickening fraction averaging 55.9 ± 5.4% of preocclusion values at 30 min ($P < 0.01$ vs. preocclusion values), 67.2 ± 2.5% at 1 h ($P < 0.01$), 74.2 ± 2.4% at 2 h ($P < 0.01$), and 82.5 ± 1.9% at 3 h ($P < 0.01$) (Fig. 3). Therefore, in accordance with previous studies (7, 8, 37, 40, 49, 58), the sequence of six 4-min occlusions resulted in significant myocardial stunning that required, on average, 4 h to resolve.

As expected (7, 8, 37, 40, 49, 58), on day 2 the recovery of wall thickening after the six 4-min occlusions was markedly improved compared with that on day 1 (Fig. 3). Statistical analysis demonstrated that the measurements of thickening fraction were significantly greater than those on day 1 at 5 min ($P < 0.05$), 15 min ($P < 0.05$), 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 wall thickening after the sixth reperfusion was 56% less on day 2 than on day 1 ($P < 0.01$) (Fig. 6). On day 3, the recovery of wall thickening was again enhanced compared with day 1 and similar to that seen

on day 2 (Fig. 3). The total deficit of wall thickening was 53% less on day 3 than on day 1 ($P < 0.01$) (Fig. 6). Thus, in accordance with previous results in this model (7, 8, 37, 40, 49, 58), myocardial stunning was attenuated significantly on days 2 and 3 compared with that on day 1, indicating the development of a late PC effect.

Group V: Cycloheximide-treated group. On day 1, the recovery of wall thickening during the first 4 h of reperfusion was similar to that observed in the control group (Fig. 4). At 5 h of reperfusion, however, the values of wall thickening in group V were significantly lower than those in controls (Fig. 4). The measurements of wall thickening in group V returned to values >95% of preocclusion by 6 h of reperfusion (data not shown). Despite the transient deterioration of wall thickening noted at 5 h, the total deficit of wall thickening during the first 5 h of reperfusion in group V was virtually indistinguishable from that observed in the control group (Fig. 6), indicating that cycloheximide did not exert an overall detrimental effect on myocardial stunning.

On day 2, the results were different from those obtained in the control group. Unlike the pattern seen in controls, in the cycloheximide-treated group the recovery of wall thickening was not improved compared with day 1 (Fig. 4) so that the total deficit of wall thickening observed on day 2 was not significantly different from that observed on day 1 (Fig. 6). The total deficit of wall thickening on day 2 was 92% greater than the corresponding value in control rabbits ($P < 0.01$) and was similar to that observed in control rabbits on day 1 (Fig. 6). Thus the administration of cycloheximide before the six occlusion-reperfusion cycles on day 1 prevented the development of PC against stunning on day 2. On day 3, however, the recovery of wall thickening was markedly improved compared with day 2 (Fig. 4) and was similar to that noted on day 2 in the control group (Fig. 3). The total deficit of wall thickening on

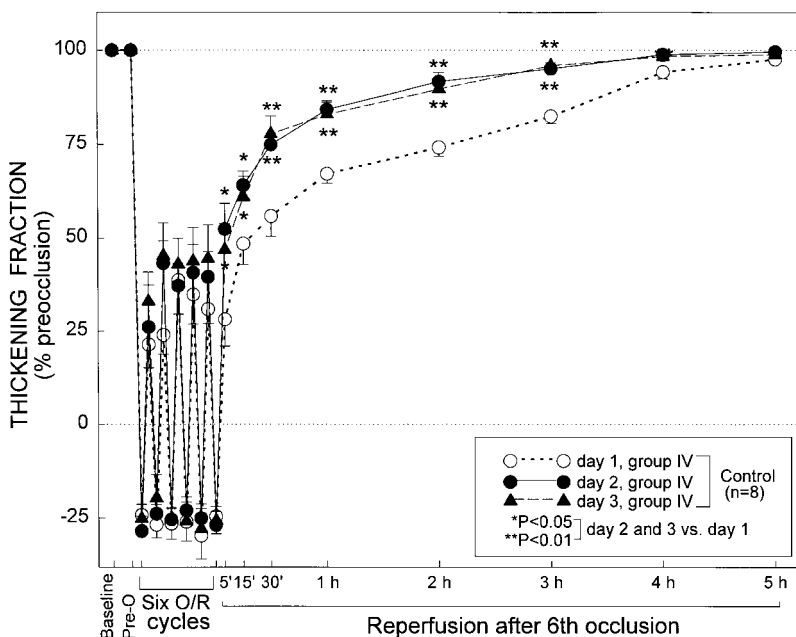
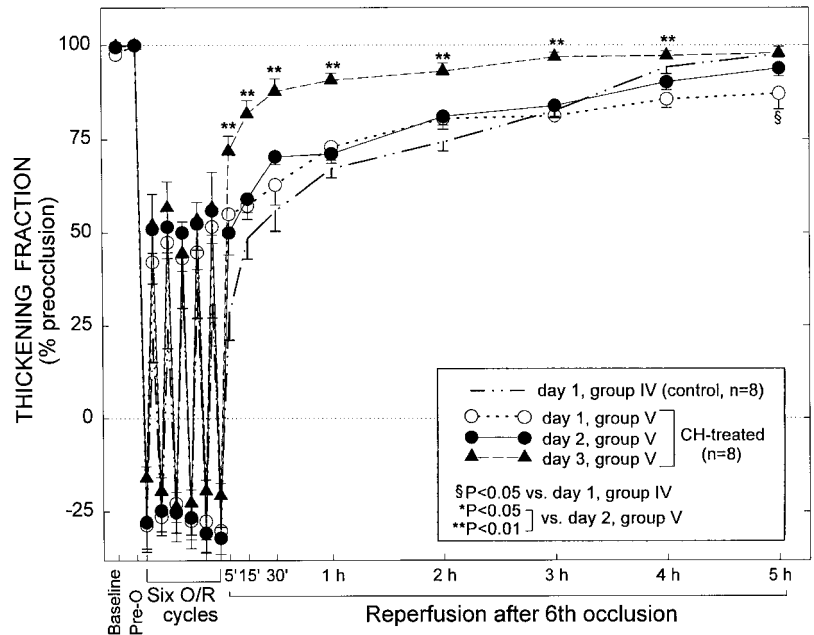


Fig. 3. Systolic thickening fraction in the ischemic-reperfused region in group IV (control group) at baseline, 1 min before the 1st coronary occlusion [preocclusion (Pre-O)], 3 min into each coronary occlusion (O), 3 min into each reperfusion (R), and at selected times during the 5-h reperfusion interval after the 6th occlusion. Thickening fraction is expressed as a percentage of preocclusion values. Data are means ± SE.

Fig. 4. Systolic thickening fraction in the ischemic-reperfused region in *group V* (CH-treated group) before administration of CH (baseline), 1 min before the 1st coronary occlusion (Pre-O), 3 min into each coronary occlusion (O), 3 min into each reperfusion (R), and at selected times during the 5-h reperfusion interval after the 6th occlusion. To facilitate comparisons, the data pertaining to *day 1* of *group IV* (control group) are also shown ($n = 8$). Thickening fraction is expressed as a percentage of preocclusion values. Data are means \pm SE.

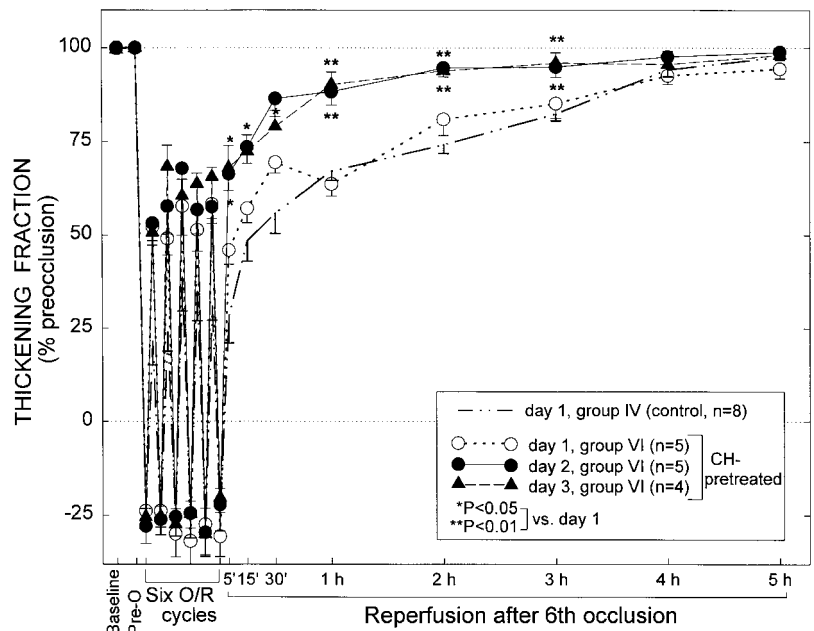


day 3 was 66% less than that noted on *day 2* ($P < 0.01$) and was comparable to that noted on *days 2* and *3* in control rabbits (Fig. 6). Thus in cycloheximide-treated rabbits the sequence of six coronary occlusion-reperfusion cycles performed on *day 1* failed to precondition against myocardial stunning on *day 2*, but the same sequence performed on *day 2* did precondition against stunning on *day 3*.

Group VI: Cycloheximide-pretreated group. *Group VI* was studied to rule out the possibility that the absence of late PC against stunning observed on *day 2* in *group V* may have been caused by a delayed adverse effect on myocardial contractility occurring as a result of the administration of cycloheximide on *day 1*. Twenty-four hours before the first sequence of coronary occlusion-reperfusion cycles, rabbits received cycloheximide in

the absence of ischemia and were then subjected to the sequence of six coronary occlusion-reperfusion cycles on *days 1-3*. On *day 1*, the recovery of wall thickening during the 5-h reperfusion period was similar to that observed on *day 1* in the control group (Fig. 5) so that the total deficit of wall thickening did not differ significantly from that observed in control rabbits on *day 1* (Fig. 6). Thus the administration of cycloheximide did not exacerbate the severity of myocardial stunning resulting from a sequence of six 4-min occlusion-reperfusion cycles performed 24 h later. On *days 2* and *3*, the recovery of wall thickening was significantly faster compared with that on *day 1* (Fig. 5) and the total deficit of wall thickening was markedly reduced, to an extent comparable to that seen in control rabbits (Fig. 6), indicating that the expected PC effect had fully

Fig. 5. Systolic thickening fraction in the ischemic-reperfused region in *group VI* (CH-pretreated group) at baseline, 1 min before the 1st coronary occlusion (Pre-O), 3 min into each coronary occlusion (O), 3 min into each reperfusion (R), and at selected times during the 5-h reperfusion interval after the 6th occlusion. These rabbits received CH 24 h before the sequence of six 4-min coronary occlusion-reperfusion cycles on *day 1*. Only 4 of the 5 rabbits were studied on *day 3* because 1 rabbit died of ventricular fibrillation during the 4th occlusion on *day 3*. To facilitate comparisons, the data pertaining to *day 1* of *group IV* (control group) are also shown ($n = 8$). Thickening fraction is expressed as a percentage of preocclusion values. Data are means \pm SE.



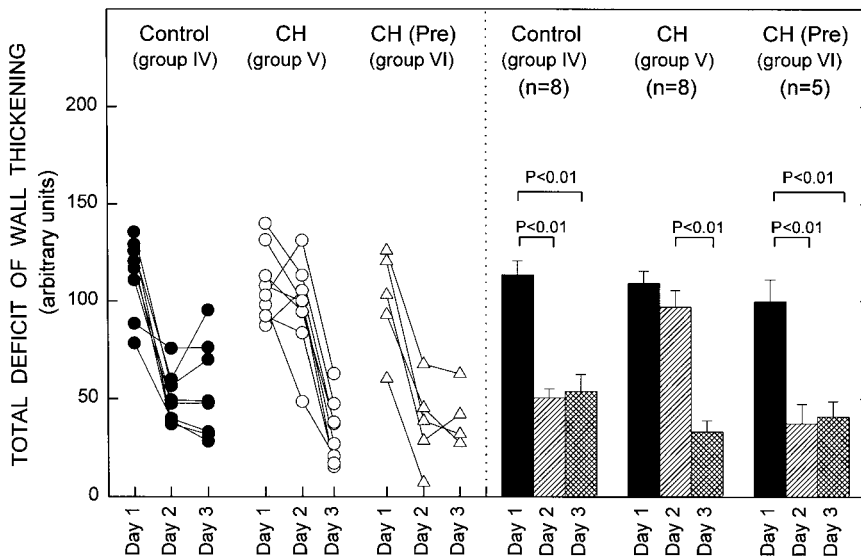


Fig. 6. Total deficit of wall thickening after the 6th reperfusion on days 1-3 in the control ($n = 8$), CH-treated ($n = 8$), and CH-pretreated ($n = 5$) groups (groups IV, V, and VI, respectively). In group VI, only 4 of the 5 rabbits were studied on day 3 (see text). Values of total deficit of wall thickening in individual rabbits are shown at left; mean \pm SE values of total deficit of wall thickening are depicted at right. The total deficit of wall thickening was measured in arbitrary units (see text).

developed. In view of these results, the absence of late PC against stunning observed in group V on day 2 cannot be ascribed to a delayed deleterious action of cycloheximide.

DISCUSSION

Although the late phase of ischemic PC has been proposed to be mediated by the formation of new proteins, it is also plausible that it could be caused by posttranslational modification of preexisting proteins. We addressed this issue in our conscious rabbit model of late PC. Despite its cost- and labor-intensiveness, a conscious animal model was felt to be particularly important for this investigation not only because the conditions associated with open-chest models can affect myocardial stunning (6, 28, 54) and ischemic PC (11, 13, 19, 45) but also because they can stimulate the synthesis of new proteins (21, 23). For example, a thoracotomy has been found to upregulate the myocardial protein content of Mn SOD 24 h later irrespective of ischemic PC (21), possibly as a result of the release of cytokines in the initial hours after surgery. The use of conscious rabbits eliminated these potential artifacts.

There are two major findings in this study. First, ischemic PC causes a rapid and substantial increase (almost a doubling) in the rate of myocardial protein synthesis (phase I). Second, when this increase in protein synthesis is prevented, the development of late PC against myocardial stunning is completely blocked (phase II). This latter finding cannot be explained by a delayed toxic action of cycloheximide, because in group VI pretreatment with this agent on day 0 had no effect on the severity of myocardial stunning 24 h later (on day 1). The failure of cycloheximide-treated rabbits (group V) to exhibit PC on day 2 was not caused by an inherent resistance of these animals to the PC effect, because the protection became fully manifest on day 3. Taken together, these results demonstrate that the synthesis of new proteins is necessary for late PC against myocardial stunning to develop. Prior studies (32) have shown that protein synthesis is required for

the delayed cardioprotective effects observed in vitro after pretreatment of rats with norepinephrine; however, the delayed cardioprotection elicited by ischemia has not been previously examined. To our knowledge, this is the first demonstration that the late phase of ischemic PC in vivo is dependent on augmented synthesis of proteins.

A major concern in the design of this study was to exclude the possibility that the administration of cycloheximide produces toxic actions that could exacerbate myocardial stunning 24 h later (on day 2) in a nonspecific manner, i.e., irrespective of whether or not the myocardium is preconditioned. The dose of cycloheximide selected for the present experiments (2 mg/kg) had only mild side effects. As detailed in the RESULTS, on the day of administration cycloheximide did not produce any changes in arterial blood pressure or systolic wall thickening in normal myocardium and did not produce any appreciable behavioral changes in the animals. The only side effects noted were that in rabbits treated with cycloheximide on day 1 (group V), heart rate increased for the first 5 h of reperfusion (Table 1) and wall thickening decreased at 5 h after reperfusion on the same day (Fig. 4) (possibly as a result of the increased heart rate); nevertheless, the total deficit of wall thickening on day 1 was not augmented compared with that in control animals (Fig. 6), indicating that cycloheximide did not exert a detrimental influence on myocardial stunning on the same day. The increase in heart rate and the transient decrease in wall thickening noted in group V on day 1 are hardly surprising, because it is difficult to conceive of a situation in which protein synthesis is suppressed for several hours in an intact organism without any side effects. Conceptually, however, neither the tachycardia nor the transient deterioration of wall thickening seen in group V on day 1 can be invoked to explain the lack of a PC effect on day 2. Even more importantly, in group VI, which was treated with cycloheximide 24 h before day 1, the recovery of wall thickening on day 1 was essentially the same as that observed in control

animals (Fig. 5), demonstrating that the administration of cycloheximide did not aggravate stunning 24 h later in nonpreconditioned myocardium. Cycloheximide worsened the recovery of wall thickening 24 h later only in preconditioned myocardium (*group V*). Thus we were able to achieve the intended goal of suppressing protein synthesis on *day 1* without causing a delayed nonspecific exacerbation of myocardial stunning.

Whereas we made an effort to minimize the toxic effects of cycloheximide, we also sought to document that the dose employed was sufficient to block the stimulatory effect of ischemic PC on protein synthesis. The results of *phase I* demonstrate that the administration of 2 mg/kg of cycloheximide resulted in a marked decrease in the rate of protein synthesis in the heart, liver, and skeletal muscle. The decrease was particularly pronounced in the preconditioned myocardium (-79%), where the augmentation of protein synthesis associated with ischemic PC was totally ablated (Fig. 2). Because [³H]leucine was given at the end of the sequence of coronary occlusion-reperfusion cycles and the tissue samples were obtained 2 h later, the effect of cycloheximide documented in *phase I* occurred very early after the PC ischemia (within the first 2 h after the 6th reperfusion). Taken together, these results indicate that the heart responds to a PC stimulus with a rapid increase in protein synthesis and that this early response is indispensable for late PC against stunning to occur. Because reversible myocardial ischemia activates a battery of genes (15, 30), it seems likely that not all of the new proteins formed in response to the PC stimulus are involved in the cardioprotective effect. In fact, it is conceivable that the protective protein(s) may account for only a small fraction of all the new gene products that appear in preconditioned hearts.

The exact mechanism(s) whereby a brief ischemic stress stimulates protein synthesis remains to be elucidated. It seems plausible to hypothesize that such a mechanism(s) involves signaling pathways mediated by cellular kinases [such as protein kinase C (PKC) and/or mitogen-activated protein kinases (MAPKs)], which are activated by ischemic PC (3, 5, 10, 12, 15, 17, 30, 31, 33, 37, 38, 40, 56, 59). Activation of PKC and MAPKs could lead to activation of a number of transcription factors [such as nuclear factor (NF)- κ B (58)], resulting in increased gene transcription and protein synthesis (18, 22). Whatever the exact signal transduction cascade responsible for increased protein synthesis may be, it is likely to be triggered by nitric oxide (NO) and reactive oxygen species (ROS), because the generation of these species during the initial ischemic stimulus is essential for the development of the delayed protective effects of late PC (7, 37, 41, 47, 52). NO is known to modulate multiple cellular functions, signal transduction pathways, and transcription factors (25); furthermore, a large number of genes have been identified that are regulated by the intracellular redox state (14, 36, 42). Our working hypothesis is that enhanced formation of NO and NO-derived ROS during the initial PC ischemia causes gene upregulation, resulting

in the appearance of cardioprotective proteins, which then render the heart resistant to subsequent ischemia.

The identity of these proteins continues to be the focus of intense investigation. Although Mn SOD has been implicated in late PC in dogs (21), we found in conscious pigs that 24 h after the PC ischemia there was no detectable increase in any of the major antioxidant enzymes, including Mn-SOD, Cu,Zn-SOD, catalase, glutathione peroxidase, and glutathione reductase (51). Other proteins whose induction could be necessary for late PC include HSPs (30), transcription factors, ATP-sensitive K⁺ channels, and NO synthase (8, 48) as well as cellular kinase(s) involved in key regulatory functions. The finding that ischemic PC stimulates protein synthesis not only in the ischemic-reperfused region but also in the nonischemic (control) region (Fig. 2) was unexpected. Further research will be necessary to elucidate the mechanism for this phenomenon. In previous studies, the protective effect of the early phase of ischemic PC has been reported to extend to the nonischemic region (PC at a distance) (39), and this has been postulated to be caused by stimulation of stretch-activated ion channels (35). Perhaps the same mechanism(s) that leads to PC at a distance also leads to enhanced synthesis of proteins.

A number of previous studies have explored the relationship between the formation of new proteins and the development of the late phase of ischemic PC. Thornton et al. (53) could not demonstrate loss of early PC against myocardial infarction after administration of cycloheximide or actinomycin D and concluded that early PC against infarction is unrelated to synthesis of protective proteins. The late phase of PC was not addressed in that study. Hoshida and co-workers (21) reported in open-chest dogs that ischemic PC resulted in a delayed increase in the myocardial protein content of Mn-SOD 24 h after the PC stimulus. In contrast, Heads and colleagues (20) reported in open-chest rabbits that ischemic PC resulted in a delayed (24 h later) increase in the myocardial activity of Mn- and Cu,Zn-SOD with no change in protein content. Several investigations have documented increased expression of HSPs after ischemic PC (reviewed in Ref. 4). For example, Knowlton et al. (26) demonstrated in rabbits an increase in HSP70 mRNA at 1 h after brief ischemia, which persisted for 4 h; analysis of HSP70 protein levels demonstrated an increase as early as 2 h after the PC ischemia, which was more pronounced at 24 h. Marber and colleagues (29) reported that ischemic PC resulted in increased HSP70 expression 24 h later in rabbits, concomitant with protection against infarction. In contrast, Heads et al. (20) found no significant increase in HSP70 after ischemic PC in rabbits. In these previous experiments (21, 26, 29, 30), however, a cause-and-effect relationship between the appearance of new proteins and the development of protection after ischemic PC remained unproven. The present experiment expands these prior reports by demonstrating that protein synthesis is necessary for late PC to occur *in vivo*. Recent work by Meng et al. (32) has shown that

inhibitors of protein synthesis inhibit the pharmacological induction of a late PC effect with norepinephrine. Thus it appears that the requirement for augmented protein synthesis is shared by both ischemic PC and pharmacological PC.

Although in *group V* the administration of cycloheximide on *day 1* blocked the development of late PC on *day 2*, it did not prevent the protection from becoming manifest on *day 3* (Figs. 4 and 6). This pattern is similar to that observed in previous studies in this model in which the development of late PC was blocked by treating rabbits on *day 1* with antioxidants (52) or inhibitors of NO synthase (7), PKC (40), protein tyrosine kinases (16), and NF- κ B (58); in all of these studies, the protection was abrogated on *day 2* but reappeared on *day 3* (7, 40, 16, 52, 58). Thus, when the first stimulus (*day 1*) is neutralized, the second stimulus (*day 2*) is fully capable of triggering a late PC effect that becomes manifest 24 h later (on *day 3*). Whether the mechanism of the late PC effect elicited by the second stimulus is the same as that elicited by the first and whether repeat stimuli applied on subsequent days can also trigger a late PC response are unknown. Further studies will be required to elucidate these issues.

In conclusion, the present study demonstrates for the first time a cause-and-effect relationship between increased synthesis of proteins and development of protection during the late phase of ischemic PC in vivo. Kinase activation and posttranslational modification (e.g., phosphorylation) of key effector proteins during the second ischemic insult may well be important for the occurrence of late PC. However, our results indicate that this alone is not enough; *de novo* formation of proteins is required for the development of this cardioprotective phenomenon.

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