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Determinants of Delayed Preconditioning Against Myocardial Stunning in Chronically-Instrumented Pigs

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Abstract

To test the hypothesis that a critical stenosis prevents delayed preconditioning against stunning, studies were conducted in pigs chronically-instrumented with occluders and segment-shortening crystals. In the setting of a critical stenosis, a preconditioning stimulus of repetitive brief occlusions resulted in infarction. Thereafter, a single 10-minute occlusion was used as the preconditioning stimulus. Delayed preconditioning against stunning was documented on subsequent days by the deficit-of-function following brief repetitive occlusions. In contrast to experiments in the naïve heart, the deficit-of-function improved on the day after a single 10-minute occlusion (from 60 ± 14 to 24 ± 6 arbitrary units, $p=0.003$), and similar improvement occurred when reperfusion was performed through a critical stenosis (32 ± 6 units, $p=0.02$ vs. naïve and $p=0.34$ vs. no stenosis). Delayed preconditioning also reduced the frequency of ventricular fibrillation, and produced a 4-fold increase in both calcium-dependent and calcium-independent NOS activity. Thus, a critical stenosis did not prevent delayed preconditioning against stunning.

Keywords

Late or Delayed Preconditioning; Preconditioning against Stunning; Ischemia; Arrhythmias

INTRODUCTION

It is well recognized that episodes of reversible ischemia prior to prolonged coronary occlusion confer protection that results in a limitation in infarct size[19,20]. The window of preconditioning (PC) lasts for a few hours after the PC stimulus, with a second window of protection developing over the course of hours and continuing for a few days[26]. In addition, to protection against infarction, the late-window or delayed PC also hastens functional recovery from reversible ischemia that results in myocardial stunning[2]. This PC against stunning has been elegantly described in multiple investigations of pigs[24,26], dogs[5,22], rabbits[3,23] and mice[8] using serial studies of repetitive occlusion/reperfusion protocols.

Nevertheless, the available data suggest that the more clinically relevant situation of reperfusion in the presence of a residual stenosis may not elicit PC against stunning. For example, De Curzon et al. have shown that exercise in dogs with an acute, critical stenosis failed to PC against the stunning that occurred following a similar protocol repeated 24 hours later[5]. The absence of PC did not appear to be due to insufficient ischemia since even a more

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severe stenosis failed to elicit a PC effect[5]. Based on this observation it was hypothesized that delayed PC was dependent upon an increase in shear stress during reactive hyperemia [18], and thus PC against stunning would not occur distal to a critical stenosis. The studies to test this hypothesis led to additional experiments to explain the apparent lack of PC against stunning following a single prolonged occlusion in pigs[22]. The subsequent results show that a critical stenosis does not prevent PC against stunning. Furthermore, a single 10-minute occlusion does induce PC against stunning, but documentation of the protective effect requires stunning from a different stimulus.

METHODS

All experimental procedures conformed to institutional guidelines for the care and use of animals in research, and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo.

Chronic Instrumentation

Farm bred pigs (n=19, weight 40±1 kg) were chronically instrumented in order to permit physiological studies to be conducted in the closed-chest state[7]. After an overnight fast, pigs were premedicated with a mixture of Telazol [tiletamine (50 mg/ml) plus zolazepam (50 mg/ml)] and xylazine (100 mg/ml) (0.04 ml/kg IM), intubated, and then anesthetized with isoflurane (2-3%). A thoracotomy was performed in the 4th left intercostal space. Catheters were placed in the left internal mammary artery, left atrium, and pulmonary artery. A high-fidelity micromanometer (Kongsberg Instruments) was secured in the left ventricular apex. The proximal left anterior descending coronary artery (LAD) was instrumented with two hydraulic occluders, with a Doppler flow probe (Transonic Systems, Inc.) secured between them. A pair of piezoelectric crystals was placed in the subendocardium of the anterior wall for the quantification of regional function by segment shortening. Subendocardial crystal location was confirmed at the conclusion of the study. All instrumentation exited the chest through the 6th intercostal space, was tunneled subcutaneously to the posterior chest wall, and then secured in a pocketed jacket. The chest was closed in a standard fashion and the pneumothorax was evacuated. Analgesia was achieved with an intercostal nerve block and intravenous butorphanol, followed by 3-5 days of intramuscular doses buprenex and flunixin. Prophylactic antibiotics (cefazolin and gentamicin) were given prior to surgery and repeated once postoperatively. Aspirin (325 mg p.o.) was given daily. Catheters were flushed every 2-3 days with heparin (10,000 units/ml). Animals were caged individually and fed a standard diet.

Experimental Protocols

Experimental protocols (Figure 1) were performed at least one week following instrumentation surgery. Animals were initially sedated with Telazol/xylazine (0.04 ml/kg IM), and after prophylactic intubation they were maintained on propofol (2-5 mg/kg/min IV) supplemented with additional doses of Telazol/xylazine (0.02 ml/kg IM). Supplemental oxygen was administered, and blood gas analyses confirmed adequate ventilation without mechanical assistance. Self-adhesive defibrillator pads were prophylactically applied to the chest wall (right parasternal and left lateral chest). When ventricular fibrillation was documented by the sudden loss of phasic ventricular pressure, defibrillation was performed with a biphasic shock of 150-200J (LifePak 12, Medtronic Instruments).

The most widely studied porcine model of delayed PC against stunning consists of a series of 10 two-minute occlusion/two-minute reperfusion cycles performed on serial days (**Protocol 1 - Repetitive Brief Occlusions**, Figure 1)[1,24,26]. Using this approach, the ischemic stimulus on Day 1 induces PC. PC against stunning is documented on Day 2 when the myocardial stunning that occurs following the same stimulus demonstrates more rapid improvement in

function than on Day 1 (e.g. Figure 2). Although previous studies have followed the recovery of function for at least 5 hours after reperfusion[1,22,24], we were required by our IACUC to limit monitoring to 4 hours after reperfusion in order to allay concerns about prolonged daily anesthesia.

The primary aim of this investigation was to determine the necessity of complete reperfusion to mediate delayed PC against stunning. Thus, in Protocol 2 - Repetitive Brief Occlusions with Critical Stenosis (Figure 1) we attempted to maintain an acute critical stenosis while performing repetitive brief occlusions (10 cycles of two-minute occlusion/two-minute reperfusion) to induce PC. In the first experiment of this protocol, a critical stenosis was applied to the LAD by adjusting one hydraulic occluder such that reactive hyperemia following a 20-second occlusion of the second occluder resulted in only a 1-1.5 fold increase in LAD flow[5]. Subsequently, repetitive brief occlusions were performed with the second occluder. At the conclusion of 4 hours of reperfusion the critical stenosis was released. However, repeat studies on the next two days revealed resting dysfunction below the initial baseline, and post-mortem examination confirmed infarction in the distribution of the LAD (data not shown).

Since infarction would confound any assessment of PC against stunning this protocol was abandoned. In its place, a prolonged single occlusion as the PC inducing stimulus was considered. In dogs, a single 10-minute occlusion has been shown to induce and demonstrate PC against stunning[22], and to upregulate iNOS[11] as further evidence of a PC effect[25]. A single 10-minute occlusion in pigs was previously shown to result in a prolonged period of myocardial stunning without infarction[22]. However, when these investigators repeated this protocol on subsequent days they found no change in the recovery of function, and concluded that this protocol does not produce PC against stunning[22].

Nevertheless, for this investigation it was hypothesized that the severity of ischemia following a prolonged single occlusion should be sufficient to induce PC against stunning (although the stunning that it causes may not demonstrate PC against stunning). The presence or absence of PC against stunning could be tested on subsequent days by the recovery of function following repetitive brief occlusions (which had previously been shown to demonstrate PC against stunning in pigs[1,24,26]). Thus, in **Protocol 3 - Prolonged Single Occlusion** (Figures 1 and 3) a single 10-minute occlusion with complete reperfusion was performed on Day 1 to induce PC. On Days 2 and 3, repetitive brief occlusions (10 two-minute occlusion/two-minute reperfusion cycles) were performed, with function monitored for 4 hours after reperfusion to demonstrate PC against stunning. Protocol 4 - Prolonged Single Occlusion with Critical Stenosis (Figure 1 and Figure 4) was performed in the same way with the exception that a critical stenosis was maintained during the occlusion/reperfusion protocol used to induce PC on Day 1. Therefore on Day 1, one hydraulic occluder was used to produce a critical LAD stenosis in order to limit the increase in LAD flow following a 20-second complete occlusion with the second occluder to 1-1.5 times baseline perfusion[5]. After this was achieved, a 10-minute occlusion was performed. At the conclusion of 4 hours of reperfusion the critical stenosis was released. On Days 2 and 3, brief repetitive occlusions (10 two-minute occlusion/two-minute reperfusion cycles) were performed with complete reperfusion. For both **Protocol 3 - Prolonged Single Occlusion** and Protocol 4 - Prolonged Single Occlusion with Critical Stenosis, the presence of PC against stunning would be defined as: a) an improvement in the deficit-of-function on Day 2 as compared to control experiments in the naïve heart (**Protocol 1 - Repetitive Brief Occlusions** Day 1) and b) no further improvement in the deficit-of-function on Day 3 as compared to Day 2.

Quantitative Assessment of Regional Function and Stunning

Regional function was assessed with % segment shortening, defined as $[(\text{end-diastolic length}) - (\text{end-systolic length})]/(\text{end-diastolic length}) \cdot 100$. End-diastole was defined by the onset of

positive dP/dt, and end-systole was defined as 20 ms prior to minimum dP/dt. The recovery of function following the final reperfusion was quantified as the total deficit of segment shortening (the integrated severity of stunning)[24]. This was calculated (in arbitrary units) as the area between actual segment shortening and baseline function for the period from the final occlusion to function at 4 hours, normalized to baseline function. Function greater than that at baseline was truncated at the baseline level.

Quantification of Nitric Oxide Synthase Activity

Myocardial nitric oxide synthase (NOS) activity was quantified as previously published[16]. Myocardial samples were obtained from the central ischemic region immediately adjacent to the LAD 4 to 72 hours following the most recent PC stimulus. Myocardial samples were homogenized in ice-cold buffer and centrifuged at 20,000 g for 45 minutes. NOS activity in the supernatant was determined by the conversion of L-¹⁴C-arginine to L-¹⁴C-citrulline using an assay buffer, and expressed as nanomoles L-¹⁴C-citrulline per gram of protein per minute. Calcium-independent NOS (iNOS) was determined in the presence of 3mM EGTA, and calcium-dependent NOS (cNOS) was determined in the absence of EGTA.

Post-Mortem and Data Analyses

After all studies were completed, animals were euthanized under general anesthesia. The heart was serially sectioned and stained with triphenyltetrazolium chloride (TTC)[7]. With the exclusion of the initial study previously discussed, there was only minor irreversible injury in 6 of 18 animals. Areas of infarction were quantified using the trapezoid formula[4], and averaged 0.3±0.2% of left ventricular mass (range 0.4 - 2.5%).

Hemodynamic and functional data were digitized at 500 Hz, averaged over 30 seconds, and reported as the mean ± SEM. Hemodynamic parameters on serial days were compared with an analysis of variance (ANOVA, SigmaStat 3.0). Regional function following repetitive brief occlusions was assessed with a two-way repeated-measure ANOVA, in order to account for study day and time. Three separate analyses were performed for the periods of occlusion, reperfusion 1-9, and the reperfusion following the final occlusion. When significant differences were present, the Holm-Sidak method was used for post-hoc testing (SigmaStat 3.0). The deficits of function following brief repetitive occlusions on successive days were compared with paired t-tests. The deficit-of-function following repetitive brief occlusions was compared across all protocols using a one-way ANOVA with post-hoc Holm-Sidak testing (SigmaStat 3.0). Statistical significance was defined as $p \leq 0.05$.

RESULTS

All animals were in good health at the time that study protocols were performed. Five animals that underwent initial instrumentation were not used in subsequent studies due to piezoelectric crystal or occluder malfunction (n=3), myocardial infarction (n=1) or stroke (n=1). LAD flow was not quantified for two animals in **Protocol 3 - Prolonged Single Occlusion** due to flow probe malfunction. Baseline hemodynamic parameters for each of the three protocols are shown in Table 1. Baseline LAD segment shortening averaged 22.9±1.0 % at a heart rate of 120±3 bpm and a mean arterial pressure of 103±3 mm Hg.

Protocol 1 - Repetitive Brief Occlusions Induces Delayed Preconditioning Against Stunning

Control studies were performed to document improvement in the recovery of function (delayed PC against stunning) following brief repetitive occlusions on two successive days. As illustrated in Figure 2, each of the ten occlusions produced a similar level of dysfunction, and there were no differences in the degree of dysfunction during occlusion between the two days of study ($p=0.65$). However, during each brief reperfusion period (Reperfusion 1-9) regional

function relative to baseline was greater on Day 2 than Day 1 (ANOVA $p<0.001$). This was not due to a lower baseline as a result of persistent stunning since baseline function on Day 2 was similar to that on Day 1 (Table 1). After the 10th occlusion, the time course of recovery of function was more rapid on Day 2 (ANOVA $p<0.001$), and the deficit-of-function was significantly reduced on Day 2 (27 ± 14 arbitrary units) as compared to Day 1 (60 ± 14 arbitrary units, $p=0.03$). These results confirm that brief repetitive occlusions on successive days can both induce and demonstrate delayed PC against stunning.

Protocol 3 - Single Prolonged Occlusion Induces Delayed PC Against Stunning

In order to determine whether a prolonged single occlusion on Day 1 could produce delayed PC against stunning, repetitive brief occlusions were performed on successive days (Days 2 and 3). The functional response to the 10-minute occlusion on Day 1 is shown in **panel a of Figure 3**, and regional function associated with the repetitive brief occlusions on Days 2 and 3 are shown in **panel b of Figure 3**. Hemodynamic parameters prior to each daily study remained stable, with the exception of a higher heart rate on Day 1 (Table 1). During each occlusion (Figure 3) there was dyskinesia, with no differences between days of study. During the two-minute reperfusion periods between the repetitive occlusions on Days 2 and 3 (Figure 3), regional function was significantly reduced on the third day of the protocol (ANOVA $p<0.001$). This contrasts with the results of **Protocol 1 - Repetitive Brief Occlusions** in which the second consecutive day of repetitive brief occlusion was associated with improved function during reperfusion (Figure 2).

Recovery of function following brief repetitive occlusions in **Protocol 3 - Prolonged Single Occlusion** (Figure 3, **panel b**) also contrasted with the results of **Protocol 1 - Repetitive Brief Occlusions** (Figure 2). On Day 2 of **Protocol 3 - Prolonged Single Occlusion**, both the time-course of functional improvement (ANOVA $p<0.001$) and the integrated deficit-of-function ($p=0.003$, Figure 5) were improved as compared to the corresponding control experiment on Day 1 of **Protocol 1 - Brief Repetitive Occlusions**. Furthermore, the time course and degree of functional recovery was almost identical on Days 2 and 3 in **Protocol 3 - Prolonged Single Occlusion** (ANOVA $p=0.13$), and there was no difference in the integrated deficits of function (24 ± 6 vs. 27 ± 4 arbitrary units, $p=0.36$). These findings are consistent with the presence of delayed PC against stunning on Day 2 induced by the single 10-minute occlusion on Day 1 of **Protocol 3 - Prolonged Single Occlusion**.

Protocol 4 - A Critical Stenosis Does Not Abolish Delayed PC Against Stunning

Since **Protocol 3 - Prolonged Single Occlusion** demonstrated that a prolonged single occlusion could induce delayed PC against stunning, the primary hypothesis regarding the necessity of complete reperfusion could be tested by applying a critical stenosis on Day 1 of **Protocol 4 - Prolonged Single Occlusion with Critical Stenosis**. The critical stenosis blunted maximal mean flow during reperfusion to 85 ± 9 ml/min as compared to 249 ± 39 ml/min in **Protocol 3 - Prolonged Single Occlusion** ($p<0.001$), a 1.6 ± 0.1 fold increase over baseline flow as compared to a 4.0 ± 0.7 fold increase in **Protocol 3 - Prolonged Single Occlusion** ($p<0.001$). As expected, the critical stenosis resulted in no change in the degree of dyskinesia associated with the 10-minute occlusion (Figure 4, **panel a**). However, the recovery of function was significantly delayed in comparison to animals without a stenosis during reperfusion (**Protocol 3 - Prolonged Single Occlusion**, ANOVA $p<0.001$). Although the deficit-of-function was also greater when reperfusion was performed in the presence of a critical stenosis, the difference was not statistically significant (161 ± 16 vs. 108 ± 39 arbitrary units in **Protocol 3 - Prolonged Single Occlusion**, $p=0.19$).

Despite the delayed recovery of function following reperfusion through a critical stenosis (Figure 4, **panel a**), regional function had improved to baseline by the next day, and there were

no differences in hemodynamic parameters prior to each daily study (Table 1). When repetitive brief occlusions were performed on Days 2 and 3, there was no difference in the integrated deficits of function (32 ± 6 vs. 39 ± 4 arbitrary units, $p=0.28$; Figure 4, **panel b**). The small difference in the temporal recovery of function was statistically significant (ANOVA $p=0.008$); however, the rate of functional recovery was actually more rapid on Day 2 than Day 3 (Figure 4, **panel b**). Furthermore, on Day 2 of **Protocol 4 - Prolonged Single Occlusion with Critical Stenosis** there was improvement in both the time-course of functional improvement (ANOVA $p<0.001$) and the integrated deficit-of-function (Figure 5, $p=0.019$), as compared to the corresponding control experiment on Day 1 of **Protocol 1 - Repetitive Brief Occlusions**. Thus in **Protocol 4 - Prolonged Single Occlusion with Critical Stenosis**, PC against stunning was already present on Day 2 as a result of the 10-minute occlusion even when reperfusion was performed through a critical stenosis.

The hydraulic occluder did not remain perfectly stable for the duration of the occlusion and in two animals maximum LAD flow during reperfusion exceeded the initial threshold value of 1.5 times the baseline. When these two animals were excluded, flow during reperfusion increased to only 1.4 ± 0.0 fold over baseline. In these animals there was still evidence of delayed PC against stunning as the deficit-of-function on Day 3 (40 ± 4 arbitrary units) was identical to that on Day 2 (40 ± 4 arbitrary units, $p=0.98$). Therefore, the results were not confounded by less severe stenoses in a subgroup of animals.

Preconditioning Against Ventricular Fibrillation

There were 34 episodes of ventricular fibrillation which resulted in a loss of ventricular pressure for 26 ± 2 seconds (range 14 to 64 seconds). The majority of these events were associated with reperfusion ($n=29$, 28 with brief repetitive occlusions and 1 following a 10-minute occlusion), while the remaining 5 episodes occurred during the 10-minute occlusions (in 4 of 14 studies). Among the 34 repetitive brief occlusion studies (10 cycles of two-minute occlusion/two-minute reperfusion), ventricular fibrillation occurred 28 times (mean of 0.8 times per study, range 0 to 5; most frequently following the first or the last occlusion). The frequency of ventricular fibrillation was significantly reduced following a PC stimulus (from 4.0 ± 1.0 to 0.5 ± 0.2 per study, $p<0.001$). Although the likelihood of ventricular fibrillation was greater in animals on the first day of study (8 out of 17 studies or 47%) than following a PC stimulus (7 out of 31 studies or 23%), the difference was not statistically significant (Chi-square = 2.6, $p=0.11$). Transthoracic defibrillation was always successful, and only 9 episodes required more than one defibrillation attempt (mean of 1.3 ± 0.1 shocks, range 1 to 3).

Nitric Oxide Synthase Activity in Preconditioned Myocardium

Since previous studies have documented up-regulation of NOS protein in association with delayed PC, NOS activity was used to confirm the presence of PC myocardium. As shown in Figure 6, total NOS activity in PC myocardium was increased 4-fold over normal control tissue (1.41 ± 0.29 vs. 0.35 ± 0.09 activity units/mg protein, $p<0.01$). A quantitatively similar up-regulation occurred in calcium-dependent (cNOS, 0.90 ± 0.24 vs. 0.23 ± 0.08 activity units/mg protein, $p=0.02$) and calcium-independent NOS activity (iNOS, 0.52 ± 0.11 vs. 0.14 ± 0.06 activity units/mg protein, $p=0.01$).

DISCUSSION

The results of this study provide important new information regarding the late-window or delayed PC and specifically delayed PC against stunning. First, in contrast to a basic study that suggested that the PC against stunning may not be induced distal to a chronic stenosis[5], the present study demonstrated that a single 10-minute occlusion with limited hyperemia during reperfusion can induce PC against stunning. Second, a previous study in pigs using single 10-

minute occlusions showed no improvement in the rate of recovery of function on successive days, and concluded that it did not induce a “preconditioning-like effect”[22]. However, the results of the present investigation clearly show that a single 10-minute occlusion can induce PC against stunning when the recovery of function was quantified following brief repetitive occlusions (10 two-minute occlusion/two-minute cycles)[24]. Since a prolonged single occlusion resulted in greater stunning than brief repetitive occlusions, this suggests that there may be a threshold level of ischemia (or stunning) beyond which there is no longer protection afforded by a prior ischemic stimulus. Third, this study extends the scarce clinical data that suggest an antiarrhythmic effect of delayed PC[14,28]. In addition to its protective effects on stunning, the late-window of PC also conferred protection against reperfusion related ventricular fibrillation. Finally, we have shown a 4-fold up-regulation of both cNOS and iNOS activity in PC myocardium. This complements the previous studies which have shown an up-regulation of both eNOS[1] and iNOS[1,11] protein following ischemic protocols associated with delayed PC against stunning.

Delayed PC Against Stunning Occurs Distal to a Critical Stenosis

The necessity of complete reperfusion to induce delayed preconditioning is clinically important since the vast majority of ischemic events in patients with coronary artery disease occur distal to a fixed epicardial stenosis that limits reperfusion. The primary finding from the present study, that delayed PC against stunning can be induced in the setting of a critical stenosis, refutes the basic investigation in exercising dogs[5], but extends the limited clinical evidence of delayed PC effects in patients with coronary artery disease[9,14,15], and thus strengthens the potential clinical significance of this phenomenon. For example, Lambiase et al. have shown that exercise-induced ischemia (in the presence of significant single vessel coronary disease) 24 hours prior to a second series of stress tests could prolong the time to significant ST segment deviation[14]. Furthermore, the exercise-induced ischemia ameliorated the ST segment shifts induced by the initial coronary occlusion during coronary intervention on the second day[14]. Finally, these results are also consistent with the previous report that a critical stenosis does not abolish acute PC against infarction[10]; and with clinical studies that have correlated the presence of recent angina (within 48 hours) with smaller acute myocardial infarct size[12,13], a trend toward lower in-hospital mortality[12,13], and with reduced release of CK-MB and up-regulation of HSP-72 protein expression associated with coronary artery bypass surgery[27].

Is There a Threshold of Ischemia (or Stunning) Beyond Which Delayed PC Offers No Protection Against Stunning?

In order to test the hypothesis that PC against stunning was dependent upon complete reperfusion, it was necessary to reconcile the fact that two different ischemic protocols in pigs produced molecular markers of PC[1,11], but only the protocol consisting of repetitive brief occlusions was previously shown to demonstrate delayed PC against stunning[24,26]. In contrast, when Shen and Vatner performed a single 10-minute occlusion in pigs on successive days, they found no improvement in the time-course of functional recovery after reperfusion [22]. Nevertheless, the results of **Protocol 3 - Prolonged Single Occlusion** clearly show that a single 10-minute occlusion did induce PC against stunning when tested on subsequent days by the recovery of function following repetitive brief occlusions (Figures 3 and 4).

The explanation for why a single 10-minute occlusion in pigs can *induce* delayed PC against stunning, but the same stimulus on the subsequent day does not *demonstrate* PC against stunning is not readily apparent. One possibility could be related to the severity of ischemia and the resultant stunning. The single 10-minute occlusion resulted in more severe ischemia than repetitive brief occlusions as evidenced by both a lower nadir of dysfunction during reperfusion ($16 \pm 8\%$ vs. $53 \pm 10\%$ of control function, $p=0.049$; **panel a of Figure 3 and panel**

a of Figure 4 vs. Figure 2 **Day 1**) and a greater deficit-of-function following reperfusion (139 ± 19 arbitrary units vs. 60 ± 14 arbitrary units, $p=0.07$). This suggests the possibility of a threshold level of ischemia or stunning beyond which a PC stimulus cannot provide protection. This would be analogous to the previous demonstration that an ischemic stimulus that can provide PC against infarction following a subsequent 40-minute coronary occlusion, but offered no benefit when the occlusion was extended to 3 hours duration[17]. This finding was interpreted as showing that PC merely delayed rather than prevented myocyte necrosis[17][6]. The present investigation suggests that a similar situation may occur with delayed PC against stunning, in which a PC stimulus can improve the deficit-of-function following a relatively modest ischemic episode (repetitive brief occlusions), but a more severe episode (single prolonged occlusion) is afforded no protection. Formal testing of this hypothesis will obviously require further study.

Is PC Against Stunning an All-or-None Phenomenon?

Acute PC against infarction appears to be a graded phenomenon[21]; however, the available data suggest that delayed PC against stunning is “all-or-none”. The Bolli group has shown that repetitive brief occlusions in pigs consistently resulted in ~50% reduction in the integrated deficit-of-function. This same level of improvement occurred on days 2 and 3 of a protocol repeated on three successive days (identical to **Protocol 1 - Brief Repetitive Occlusions**, in the present investigation)[24] or when the protocols were separated by 3 days, but there was no change in the deficit-of-function when the protocol was repeated at intervals of 6 hours or 6 days[26]. When the protocol was repeated after 12 hours, the average results would appear to suggest an intermediate level of improvement (Figure 6 in reference[26]); however, the narrative clearly described that ~50% improvement in the deficit-of-function occurred in 3 of 6 animals, with minimal improvement in the remaining 3 pigs. An all-or-none phenomenon of PC against stunning was further supported by the experiments in the present investigation that showed a similar improvement in the deficit-of-function to that described by the Bolli group (~55% in **Protocol 1 - Brief Repetitive Occlusions**), with no differences among all groups with PC myocardium (Figure 5).

Preconditioning Against Arrhythmias

The early-window or acute PC has been shown to protect against ischemically-mediated arrhythmias; however, there has been little previous evidence to support a protective effect of late PC against arrhythmias[2,14,28]. In the present investigation we noted a marked reduction in the number of episodes of ventricular fibrillation during repetitive brief occlusions in animals with PC myocardium (from 4.0 ± 1.0 to 0.5 ± 0.2 , $p<0.001$), and a trend toward a reduction in the number of studies complicated by fibrillation (from 47% to 23%, $p=0.11$). However, it is important to note that in the present study, a majority of the initial ischemic stimuli consisted of a single prolonged occlusion, whereas the ischemia induced in PC myocardium was entirely the result of repetitive brief occlusions. Nevertheless, this data is consistent with and extends the limited clinical findings that suggest that delayed PC reduces arrhythmias. For example, ischemic PC prior to coronary bypass surgery has been shown to reduce the incidence of ventricular tachycardia even during the period of 24 to 48 hours after surgery[28], and exercise-induced ischemia reduced the rate of ectopic complexes during repeat exercise 24 hours later [14].

Methodological Limitations

In the present study delayed PC against stunning still developed when reperfusion occurred in the presence of a critical stenosis. However, the possibility that a more severe stenosis might have prevented delayed PC against stunning cannot be completely excluded. Nevertheless, even when we restricted our analysis of **Protocol 4 - Prolonged Single Occlusion with Critical Stenosis** to the five animals with hyperemic flow of <1.5 times baseline perfusion,

the deficit-of-function was no different on Days 2 and 3 (analogous to the results in Figure 4) and significantly less than that in the naïve heart (Figure 5), consistent with a PC effect induced from the ischemia on Day 1. In further support of this conclusion, a critical stenosis has been previously shown to have no significant effect on acute PC against infarction[10].

Aspirin was administered to the chronically-instrumented animals in order to reduce catheter occlusion, and high dose aspirin (25 mg/kg) has been shown to block delayed PC against stunning by the inhibition of cyclooxygenase-2 up-regulation[23]. However, the dose of aspirin used in this study (8.3 ± 0.3 mg/kg) was similar to the low (5 mg/kg) or medium (10 mg/kg) doses that were previously shown to have no effect on PC against stunning[23]. Furthermore, since the aspirin was consistently given to all animals (including those in **Protocol 1 - Brief Repetitive Occlusions**), this effect was unlikely to have confounded our results.

Finally, in this investigation an acute stenosis was used to limit reactive hyperemia and the effects of PC were assessed in the context of myocardial stunning. Although a critical stenosis can develop in the setting of an acute coronary syndrome, the more typical clinical scenario would be demand-induced ischemia in the presence of a chronic stenosis. Furthermore, clinical investigations of delayed PC have focused on changes in ST segment responses and infarct size rather than the rate of recovery from myocardial stunning. Thus, caution is warranted when considering the clinical implications of this investigation.

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Study Protocols

Figure 1. Study Protocols

This schematic of the four study protocols illustrates the relationships between periods of occlusion (shaded boxes) and periods of reperfusion (clear boxes). Brief repetitive occlusions consisted of 10 cycles of two-minute occlusion/two-minute reperfusion and were performed in each of the protocols. A prolonged single occlusion (of 10-minutes duration) was only performed on Day 1 of **Protocols 3 and 4**. In **Protocols 2 and 4** a critical stenosis was applied before the initial occlusion and was maintained through the 240 minutes of monitored reperfusion. **Protocol 2** was abandoned after the first study resulted in a large LAD myocardial infarction.

Protocol 1 (Repetitive Brief Occlusions)

Figure 2. Protocol 1 - Repetitive Brief Occlusions

Ten two-minute occlusion/two-minute reperfusion cycles were performed on successive days. During the ten occlusions there was no difference in the degree of dysfunction between days ($p=0.65$), but during reperfusion #1-9 function was significantly improved on the second day (dark gray circles) as compared to the first (white diamonds, $p<0.001$). Furthermore, the deficit-of-function following the final reperfusion was significantly reduced on Day 2 (27 ± 14 arbitrary units) as compared to Day 1 (60 ± 14 arbitrary units, $p=0.03$). This is consistent with delayed PC against stunning on Day 2 induced by repetitive brief occlusions on Day 1.

Figure 3. Protocol 3 - Single Prolonged Occlusion Followed by Repetitive Brief Occlusions
Protocol 3 tested the hypothesis that a prolonged single occlusion could induce PC against stunning. On Day 1 a single prolonged (10 minute) occlusion was performed (panel a). The presence of PC against stunning was determined using stunning induced by repetitive brief occlusions (panel b, 10 two-minute occlusion/two-minute reperfusion cycles) on Day 2 (black diamonds) and Day 3 (white circles). The time course of recovery of function following the final occlusion was no different on the two days, resulting in a similar integrated deficit-of-function (24 ± 6 vs. 27 ± 4 arbitrary units, $p=0.36$). Thus, PC against stunning was already present on Day 2, and had been induced by the single prolonged occlusion on Day 1.

Figure 4. Protocol 4 - Single Prolonged Occlusion with a Critical Stenosis Followed by Repetitive Brief Occlusions

In **Protocol 2** a critical stenosis during repetitive brief occlusion resulted in myocardial infarction. Thus in **Protocol 4**, on Day 1 (panel a) a critical stenosis was placed during a single prolonged occlusion to test the hypothesis that this would abolish PC against stunning. On Day 2 (black diamonds) and Day 3 (white circles) repetitive brief occlusions (panel b, 10 cycles of two-minute occlusion/two-minute reperfusion) were performed. The time course of recovery of function was slightly but significantly different between Days 2 and 3 ($p=0.008$); however, recovery of function was actually more rapid on Day 2. The deficit-of-function following the final occlusion was not different between Days 2 and 3 (32 ± 6 vs. 39 ± 4 arbitrary units, $p=0.28$).

Thus, PC against stunning was already present on Day 2 and had been induced by the prolonged single occlusion on Day 1 even in the presence of a critical stenosis.

Deficit of Function Following Repetitive Brief Occlusions

Figure 5. Deficit-of-Function Following Brief Repetitive Occlusions

The deficit-of-function following brief repetitive occlusions (10 two-minute occlusion/two-minute reperfusion cycles) was calculated as the reduction in regional function relative to baseline function for 4 hours after reperfusion. This deficit was significantly greater in the naïve heart (white bar, corresponding to **Protocol 1** Day 1) than when the same protocol was performed following a PC stimulus (ANOVA $p=0.03$, post-hoc $p<0.05$ for each group vs. Naive). Regardless of the PC inducing stimulus, the deficit-of-function improved by ~50% with no significant differences between groups. Thus, the deficit-of-function was similar the day after repetitive brief occlusions (dark gray bar, corresponding to **Protocol 1** Day 2), or the day after a prolonged single occlusion with or without a critical stenosis (black bars; corresponding to **Protocol 3** Day 2 and **Protocol 4** Day 2, respectively).

Nitric Oxide Synthase Activity

Figure 6. Myocardial Nitric Oxide Synthase Activity in Preconditioned Myocardium

There was a 4-fold increase in total NOS (tNOS) activity in PC myocardium (gray bars) as compared to normal myocardium (white bars, 1.41 ± 0.29 vs. 0.35 ± 0.09 activity units/mg protein in Normal, $p < 0.01$), with quantitatively similar increases in both calcium-dependent (cNOS) and calcium-independent (iNOS) NOS activity.

Table 1

Baseline Hemodynamic Parameters

	Heart Rate (bpm)	Mean Aortic Pressure (mm Hg)	Mean Left Atrial Pressure (mm Hg)	Left Ventricular dp/dt		LAD Mean Flow (ml/min)	End-Diastolic Dimension (mm)		Segment Shortening (%)
				Maximum (mm Hg/s)	Minimum (mm Hg/s)				
Protocol 1									
	n=3								
	Day 1	125±6	110±4	12±2	2964±8	-3268±98	56±4	14.8±2.2	23.8±0.5
	Day 2	107±15	91±10	10±6	2529±289	-2860±112	46±8	14.5±2.1	22.7±3.7
Protocol 2									
	n=7								
	(10-min Occlusion)								
	Day 1	114±6	96±6	15±2	2484±99	2933±109	58±6	17.2±0.5	23.5±1.9
	Day 2	92±4*	104±6	14±2	2087±167	2932±114	60±6	17.5±0.4	20.5±1.9
	Day 3	92±4*	107±5	17±1	2357±188	3084±139	61±4	17.4±0.4	23.6±1.7
Protocol 3									
	n=7								
	(CS & 10-min Occlusion)								
	Day 1	123±5	107±4	13±2	2166±136	3012±127	56±8	16.3±0.6	21.8±1.3
	Day 2	109±7	112±3	17±2	1978±116	2914±148	54±7	16.6±0.6	19.1±1.7
	Day 3	106±4	109±5	14±2	2386±93	3208±205	57±7	16.2±0.6	24.4±1.7

CS - Critical stenosis.

* p < 0.05 vs. Day 1.