Protein C Activation Following Coronary Artery Occlusion in the In Situ Porcine Heart

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Background. Activated protein C, the body's natural anticoagulant, may play a role in protecting the heart during and following an occlusion of the left anterior descending coronary artery (LAD) in the porcine heart.

Methods and Results. Infusion of 1 unit thrombin over 30 seconds into the LAD of juvenile pigs produced a prolongation of the Xa clotting time (153±14%) in blood drawn from the anterior interventricular vein (AIV). The action of the anticoagulant was blocked by a polyclonal immunoglobulin G antibody directed against porcine activated protein C. A brief (30 seconds) occlusion of the LAD induced a similar prolongation of the Xa clotting time (138±11%), which was also blocked by the polyclonal antibody. To determine whether activated protein C helps sustain the heart during and following a 2-minute occlusion, three groups of pigs were studied: 11 controls, six receiving activated porcine protein C, and nine receiving a monoclonal antibody (HPC4) that blocks protein C activation. Relative to the controls, HPC4-treated animals recovered function, as measured by the maximum time derivative of left ventricular pressure and segmental shortening, more slowly and were not able to sustain this recovery. Animals receiving activated protein C recovered more quickly and sustained this recovery.

Conclusions. These data indicate that an ischemic insult induces rapid activation of protein C in the coronary microcirculation and that blocking this activation impairs recovery. (Circulation 1991;84:293–299)

Until fairly recently, there was serious debate concerning the role of spontaneous thrombus formation in the initiation of an acute myocardial infarction. However, with the development of angiography, DeWood et al were able to demonstrate the presence of occlusive thrombi in 87% of patients experiencing an infarction. Subsequent studies have confirmed this and have established the importance of thrombus formation in a major coronary artery. However, thrombosis may not be limited to the large vessels. In fact, recent studies have provided evidence that microvascular thrombi may form in the ischemic area and impair recovery by reducing the blood supply to these tissues. Falk reported that 38% of the patients studied following sudden cardiac death showed histological evidence of microinfarcts. Indeed, the extent of microvascular platelet deposition has been reported to correlate with ST segment elevation, regional blood flow decrease, and clinical outcome. Given the potential role of microvascular thrombosis in determining recovery from ischemic injury, we examined potential mechanisms that may normally limit microvascular thrombosis in ischemic tissue.

There are several natural anticoagulant mechanisms. Two, the antithrombin-heparin system and the protein C anticoagulant pathway, are known to be clinically important. The protein C pathway is composed of the vitamin K-dependent zymogen protein C, thrombomodulin, and protein S, a critical regulatory protein that interacts with activated protein C to form the active anticoagulant. A more thorough description of this protein and its physiological role are provided in References 15 and 19. Deficiencies in either pathway lead to thrombosis. Deficiencies in the protein C pathway can lead to microvascular thrombosis, neonatal purpura fulminans, or warfarin-induced skin necrosis. These clinical observations suggest that protein C plays a major role in maintaining blood fluidity in the microcirculation. This is especially likely since the protein C activation complex probably functions primarily in the microcirculation. Based on this, we decided to investigate whether protein C is converted rapidly to the active anticoagulant form in ischemic areas and

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whether this activation process plays a role in reducing ischemic injury.

**Methods**

Juvenile pigs (Duroc, Poland) of either sex and weighing approximately 20 kg were initially sedated with 100 mg/kg ketamine and 0.5 mg/kg acepromazine delivered with a blowgun dart. By using this technique, the animal remained calm during the initial induction. Anesthesia was induced by the intravenous injection of 15 mg/kg thiamylal followed by 80 mg/kg α-chloralose. A proper plane of anesthesia (stage III, plane 2) was maintained throughout the experiment by the infusion of 8 mg/kg/hr α-chloralose, and the level was checked regularly by jaw tension and heart rate.

Following tracheostomy, respiration was maintained with a Harvard respirator (South Natick, Mass.). During surgical preparation, the pig was given 100% O₂ to maintain PaO₂ at 80 mm Hg or greater. A Millar pressure transducer (Millar Instruments, Houston, Tex.) was introduced into the left ventricle via the right carotid artery. The right femoral artery and vein were dissected free and cannulated. A left thoracotomy was performed through the fourth intercostal space. The pericardium was opened roughly parallel to the phrenic nerve, and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected free immediately distal to the first major diagonal branch and cannulated, and a pneumatic occluder was placed around it. The anterior interventricular vein (AIV), which runs parallel to the LAD, was cannulated with a Silastic catheter and held in place by two stay sutures. Finally, a pair of sonomicrometer crystals were placed in the subepicardium in the region at risk in a circumferential orientation.

At the completion of the surgical procedure, the left lung was inflated by providing a positive end-expiratory pressure of 5 cm H₂O and the respiratory gas was returned to room air. After 15 minutes, blood gases were measured (Corning 165/2, Corning, N.Y.) and supplemental oxygen was provided if necessary (PaO₂ less than 80 mm Hg). Blood gases were measured every 30 minutes thereafter to ensure that the animal stayed within physiological limits.

At the conclusion of the experiment, the anesthetized pig was killed by a lethal injection of 3 M KCl. All experiments were conducted in strict accordance with the guidelines of the American Physiological Society.

**Assays**

Blood samples from the AIV and femoral artery of each animal were collected in 3.8% sodium citrate (1:9), and platelet-poor plasma was prepared using a Beckman Model 12 microfuge (Fullerton, Calif.).

The activated partial thromboplastin time was determined using Simplastin (General Diagnostic) and a Clotek monitoring device (Hyland-Travenol Laboratories). The modified thrombin clotting time (TCT) of Penner was performed to ensure that fibrinogen levels were normal and that circulating heparin and heparinlike molecules were not present. Thrombin used in this assay was prepared by the activation of purified prothrombin as described by Owen et al. For the first two sets of experiments, the activated partial thromboplastin time and TCT were measured at the beginning and end of each experiment. There was no significant change in either.

The Xa one-stage clotting time was determined on citrated plasma samples according to the method of Walker et al. Bovine factor Xa was prepared according to the method of Skogen et al. Studies by Comp et al have shown that prolongation of the Xa clotting time is directly proportional to the activated protein C concentration in normal plasma. Although sensitive, this assay can give erroneous results if factor Xa or fibrin is present at high levels. To ensure that any prolongation was due to activated protein C, the Xa clotting time was also measured in samples incubated with a goat anti-porcine protein C immunoglobulin G antibody.

**Protein C Isolation**

Porcine blood (120 l) was collected into 0.1 volume of 0.1 M sodium oxalate and 10 mM benzamidine. Plasma was prepared by centrifugation through a continuous-flow centrifuge (Westfallia). The plasma concentration was adjusted to 10 mM in benzamidine HCl and the vitamin K-dependent proteins in 70 l plasma were adsorbed onto 1.6 kg BaSO₄, for 1 hour at room temperature with gentle stirring. The barium was removed by centrifugation; washed extensively with 20 l of 77 mM NaCl, 1 mM sodium citrate, 10 mM Tris, 5 mM benzamidine, and 0.02% azide; and eluted four times with a total of 8 l of 0.1 M sodium citrate (pH 7.5) in 10 mM benzamidine HCl. The eluate was precipitated by the addition of solid NH₄SO₄ at 4°C to a final concentration of 70% saturation. The precipitate was dissolved in a minimal volume of 20 mM Tris HCl and 10 mM benzamidine HCl and was desalted on a Sephadex G 75 column equilibrated in 0.1 M NaCl, 5 mM 2-[N-morpholino]ethanesulfonic acid (MES) HCl, and 10 mM benzamidine HCl (pH 6.0). The sample concentrations were adjusted to 2 mM CaCl₂ and 50 μM in PPACK before chromatography over a column of immobilized monoclonal antibody to protein C (HPC4) linked to Affigel 10 at a final concentration of 5 mg/ml.

Two 2.5×40 cm columns were employed. The columns were washed with 400 ml of 0.15 mM NaCl and 5 mM MES HCl; the 2 mM CaCl₂ was replaced with 2 mM ethylenediaminetetraacetic acid (EDTA). Protein C was activated by incubating a 2 mg/ml concentration with thrombin (5% by weight) for 3 hours at 37°C. The thrombin was then separated from the activated protein C by chromatography on a Mono Q HR5/5 column (Pharmacia Diagnostics Inc., Fairfield, N.J.) on an FPLC system. The column was developed with a 20-mL linear gradient from 0.1 to 0.6 M NaCl in 20 mM Tris HCl (pH 7.5). The eluate
was lyophilized and stored at 4°C. Either before or after activation the protein C emerged from the column as a single symmetrical peak, and dodecyl sulfate gel analysis revealed a two-chain (about 38 and 22 kDa) protein that migrated as a single higher-molecular-weight species (about 60 kDa) without disulfide bond reduction.

**Experimental Protocols**

This study comprised three sets of experiments. The first set \((n=5)\) determined whether the myocardial microvasculature of the pig would respond to direct thrombin infusion. Bovine thrombin (1,000 units/ml) was diluted in Tangen \(N\)-[2-hydroxyethyl]piperazine-\(N\)'-[2-ethanesulfonic acid] (HEPES) buffer to the appropriate activity as determined by the TCT using a standard human platelet-poor plasma. Immediately prior to the infusion of thrombin into the LAD, blood samples were drawn from the AIV and femoral artery. Bovine thrombin (1 unit in 0.5 ml) was then infused into the LAD between \(t=0\) and 30 seconds. As a control, 0.5 ml of the vehicle (Tangen HEPES) was infused into the five pigs 45 minutes after the infusion of thrombin. No change in the Xa cloting time was observed. To determine whether any anticoagulant response was due to activated protein C, blood samples were drawn from the AIV following the infusion of thrombin and incubated with goat anti-porcine protein C antibody. Although the Xa cloting time was adjusted at the beginning of surgery to approximately 35 seconds, there was variation in the Xa cloting time at \(t=0\) (28–42 seconds). Therefore, the postinfusion cloting times are expressed as a percentage of the value at \(t=0\).

The second set of experiments \((n=5)\) determined whether a brief occlusion of the LAD induced protein C activation. This question was approached in two steps. First, thrombin was infused into the LAD as before and the Xa cloting time was determined in plasma from blood drawn from the AIV essentially as described above. Having determined the baseline response of the heart to thrombin, the response to a 30-second occlusion was assessed. Blood samples were drawn from the AIV and femoral artery as described before. In addition, a second blood sample was drawn from the AIV following occlusion and incubated with the goat antibody against protein C as before.

The third set of experiments addressed the question of whether protein C activation is important in protecting the myocardium during and following an occlusion of the LAD. To adequately test this, it was necessary to extend the duration of the occlusion such that the heart would be seriously challenged. Thirty seconds (used in the second set of experiments) was too brief, and preliminary studies showed that no \((n=5)\) animals survived a 5-minute occlusion. For these reasons, occlusions of 2 minutes' duration were employed in this third set of experiments.

Three groups of pigs were studied in this set of experiments: controls that received vehicle only; those receiving HPC4, a murine monoclonal antibody that prevents protein C activation\(^{15,16}\); and those receiving porcine-activated protein C antibody\(^{15,16}\); and those receiving porcine-activated protein C just prior to the occlusion. All three groups were subjected to the same protocol. Specifically, after surgical preparation the animal was allowed to stabilize for 30 minutes. The LAD was occluded for 2 minutes and released. The animal was then allowed to recover for 58 minutes. Preliminary experiments showed that pigs

![Figure 1. Changes in Xa cloting time \((X_{a} CT)\) in response in injection over 30 seconds of 1 unit thrombin into left anterior descending coronary artery of pigs. Values are mean±SEM \((n=5)\) expressed as percentage of clotting time at \(t=0\) for blood drawn from anterior interventricular vein \((34±4 seconds)\). ○, Samples from anterior interventricular vein; □, samples from anterior interventricular vein incubated with goat immunoglobulin G antibody against activated protein C; △, samples from femoral artery.](image)
recovered full function following repeated 2-minute occlusions and 58 minutes of reperfusion. Vehicle, 1.5 mg/kg HPC4, or 1.5 mg/kg activated porcine protein C was then injected into the femoral vein and the LAD was again occluded for 2 minutes. The animal was observed for 58 minutes after release of the second occlusion. Use of this double-occlusion technique allowed each animal to serve as its own control.

During this set of experiments, the electrocardiogram, systemic arterial blood pressure, left ventricular pressure and its time derivative, and segmental length were continuously recorded on a Gould chart recorder (Glen Burnie, Md.). Percentage segmental shortening was subsequently calculated as (DL−SL)/DL, where DL=diastolic length and SL=systolic length.

In the third set of experiments, 35 pigs were studied. Of these, nine fibrillated during or immediately following the first occlusion. Of the 26 that survived the first occlusion, 11 were controls, six received activated protein C, and nine received HPC4. Of the nine that received HPC4, three fibrillated during or immediately following the second occlusion.

Analysis

All values were expressed as a percentage of the value at t=0 (i.e., just prior to the first occlusion), and comparisons were made using analysis of variance for repeated measures. A significance level of α<0.05 was adopted. Results are presented as mean±SEM.

Results

The increase in Xa one-stage clotting time following the infusion of 1.0 unit thrombin into the LAD (n=5) is shown in Figure 1. There was an immediate increase in the Xa clotting time in blood collected from the AIV; the increase persisted for 10 minutes. To test whether the vehicle used to dilute the thrombin could elicit a similar increase, the pigs were also infused with the vehicle alone and the Xa clotting time was determined as before. There was no effect of the vehicle on Xa clotting time (data not shown). Additional blood samples were drawn from the AIV and incubated with the goat polyclonal antibody to protein C. As shown in Figure 1, incubation with the antibody blocked the prolongation in Xa clotting time. The absence of any prolongation in the Xa clotting time in the presence of the goat antibody indicates that the anticoagulant was activated protein
C. Finally, blood samples were drawn from the femoral artery; as Figure 1 shows, Xa clotting time in these samples did not change over the period of interest.

The second set of experiments was designed to determine whether a 30-second occlusion of the LAD was accompanied by the activation of protein C. The response of the Xa clotting time (n=5) is shown in Figure 2. Note that the time scale is slightly different from that in Figure 1. There was a clear prolongation immediately following release of the occlusion; the prolongation persisted for at least 3 minutes. As before, additional samples of blood were drawn from the AIV and incubated with goat protein C antibody and the Xa clotting times were determined. The antibody blocked the anticoagulant activity that was formed during the occlusion. Again, there was no significant change in the Xa clotting time of blood from the femoral artery. In three animals, Xa clotting times were also determined at 45 and 60 minutes (data not shown). These did not change significantly from those recorded at 30 minutes.

Prior to beginning the third set of experiments, it was necessary to determine the systemic half-life of injected porcine activated protein C. Three animals received an intravenous injection of 1.5 mg/kg activated protein C after which arterial blood samples were taken at regular intervals to provide a measure of the systemic half-life of the injected activated protein C. The results from one pig, shown in Figure 3, are representative of those from the other two. As is evident, the Xa clotting time was still prolonged at 15 minutes after injection.

Responses of the maximum time derivative of left ventricular pressure (Pmx) to the second occlusion and reperfusion are shown in Figure 4. The recovery of Pmx for the HPC4-treated animals was slower than that for the controls and was not sustained. In contrast, the activated protein C-treated animals recovered almost immediately. Note that the HPC4-treated animals did not recover until approximately 5 minutes after occlusion. At 60 minutes, both the controls and the activated protein C-treated animals had recovered fully. The responses of segmental shortening in all three groups are quite similar to the response observed with Pmx. As with Pmx, segmental shortening initially recovered in the HPC4-treated animals, but this recovery was not sustained (data not shown).

The cumulative data at 60 minutes are presented in Table 1. For the HPC4-treated animals, all measures of ventricular function were significantly depressed. Values in the controls returned to preocclusion levels, although segmental shortening was depressed but not significantly. The activated protein C–treated animals clearly recovered ventricular function and did significantly better than the HPC4-treated animals.

**Discussion**

The results from the first set of experiments demonstrate that the infusion of thrombin at low levels

| Table 1. Ventricular Performance at 60 Minutes Following 2 Minutes of Occlusion in Pigs |
|-------------------------------|-----------|----------------|----------------|
| Parameter                     | Protein C | Controls (vehicle) | Activated protein C |
| Left ventricular pressure     | 87±6      | 104±6           | 107±7           |
| Time derivative               |           |                  |                 |
| Maximum                       | 84±5      | 96±7            | 102±5           |
| Minimum                       | 81±8      | 93±5            | 98±7            |
| Segmental shortening          | 43±15     | 82±12           | 114±11          |

Values are mean±SEM percentage of value just prior to first occlusion.
induces an anticoagulant response as measured by the Xa one-stage clotting time. This response is effectively neutralized if the blood sample is incubated with an antibody directed against activated protein C. Based on this, the prolongation in Xa clotting time is due to the activation of protein C. A similar response has been reported by Comp et al.26

In addition, this response was also blocked in pigs pretreated with HPC4, thus demonstrating that the antibody can prevent the activation of protein C in pigs.

The prolongation of the Xa clotting time following a 30-second occlusion of the LAD is not the response one would expect from the activation of platelets known to occur with an occlusion.27-29 This prolongation could be due to the release of plasminogen activators30 or to decreases in the concentrations of factors of the coagulation cascade. However, the ability of the goat polyclonal antibody to neutralize the anticoagulant demonstrates that this response is due to the activation of protein C. The absence of any appreciable prolongation in the systemic blood samples indicates that the activation occurs in the region drained by the AIV. Studies by Vinten-Johansen et al31 have shown that most of the blood entering the microvasculature from the LAD is drained by this vein. As with direct thrombin infusion, the activation of protein C occurs quickly following the brief insult and persists for 10 minutes. Thus, it would appear that activation of protein C is an early response of the heart to an ischemic challenge.

Based on this observation, it was important to ascertain whether the activation of protein C affected the response of the heart to more severe transient occlusive episodes and/or reperfusion. As Figure 4 and Table 1 show, the HPC4-treated animals recovered more slowly than the controls, though the difference was not significant, and were unable to sustain this recovery. The slower return in these animals could be due to the formation of microthrombi that would slow the reestablishment of nutritive flow throughout the region at risk. Our preliminary studies showed that the pig heart is very sensitive to the duration of an occlusion. Specifically, of five pigs subjected to a 5-minute occlusion, none survived. Therefore, any delay in the reestablishment of adequate local blood flow could result in an increased susceptibility to a fatal arrhythmia.

This could be the reason why three pigs receiving HPC4 fibrillated during reperfusion but none of the controls or those receiving activated protein C did. Contingency analysis indicated that the HPC4-treated animals were significantly (χ² = 6.41, df = 1) more likely to fibrillate than those in which protein C activation was not blocked. Specifically, blocking the activation of endogenous protein C could allow the formation of microthrombi in the region at risk during occlusion. Studies by Ruf et al21 have shown significant platelet trapping in an evolving infarct in baboons. With release, these platelets would impede reperfusion in the region at risk, thereby extending the effective time of the occlusion. As indicated above, our preliminary studies showed that pigs are very sensitive to the duration of an occlusion.

The inability of the HPC4-treated animals to sustain recovery of left ventricular function clearly shows that these hearts suffered greater injury. As is evident in Table 1, all four measures of performance were depressed at 60 minutes in the HPC4-treated animals. Relative to the activated protein C–treated animals, Pṃ and segmental shortening were significantly depressed at the last three time points (i.e., 40, 50, and 60 minutes). Mechanical function (e.g., left ventricular pressure, Pṃ, and minimum time derivative) initially recovered and then decreased by approximately 20% after 60 minutes of reperfusion. This response is similar to that observed in the stunned myocardium. Local function (i.e., segmental shortening) was even more depressed. These data suggest that blocking protein C activation during an occlusive insult seriously compromises the heart’s ability to recover.

The nearly complete recovery of the controls and full recovery of the activated protein C–treated animals supports the conclusion that protein C plays an important role in the restoration of function following a transient ischemic episode. As evident in Figure 2, even brief occlusions induce the activation of endogenous protein C. Thus, with the 2-minute occlusions studied here, endogenous protein C activation would appear sufficient to protect healthy animals since only a small additional protective response was obtained by supplemental activated protein C. However, this situation may be very different in disease states, when thrombomodulin may be downregulated and protein C activation may be impaired.32,33 Inflammatory mediators such as tumor necrosis factor, which are capable of downregulating thrombomodulin, have been demonstrated in patients with a myocardial infarct.34 This downregulation would decrease the capacity for protein C activation. This could lead to an increase in thrombin formation, which would induce platelet activation as well as increase the adhesiveness of the endothelium for neutrophils.35 Both of these could severely retard reestablishment of adequate local blood flow following resolution of the infarct.

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References


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