Local Delivery of an Antithrombin Inhibits Platelet-Dependent Thrombosis

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Background Platelet-dependent thrombosis can be effectively inhibited by intravenous administration of direct thrombin antagonists. However, an increased propensity for abnormal bleeding has been associated with systemic administration of these agents. The goal of this study was to determine whether local delivery of a potent thrombin inhibitor, D-Phe-L-Pro-L-Arg chloromethyl ketone (PPACK), could inhibit platelet-dependent thrombosis without altering systemic hemostatic function.

Methods and Results Thrombus formation was measured by quantitative imaging of 111In-labeled platelet deposition on segments of thrombogenic vascular graft interposed in arteriovenous shunts in a porcine model. Intravenous administration of PPACK inhibited platelet deposition at a dose of 12.5 μg/kg per minute, which was associated with significant prolongations of both template bleeding times and activated partial thromboplastin times. By contrast, local infusion of PPACK at a dose of 0.02 μg/kg per minute (ie, a 600-fold smaller dose) into the fluid boundary layer at the interface between flowing blood and the thrombogenic segment produced equivalent inhibition of platelet deposition without prolonging either the bleeding time or the activated partial thromboplastin time. In addition, static exposure of a mural thrombus to solutions of PPACK at concentrations ≥2.5 mg/mL for 15 minutes produced sustained inhibition of platelet-dependent thrombosis with no change in hemostatic measurements.

Conclusions These results indicate that local delivery of the direct antithrombin PPACK, by either boundary layer infusion or static application techniques, effectively inhibits platelet-dependent thrombosis at doses that are several orders of magnitude less than the systemic dose required for an equivalent antithrombotic effect. In contrast to the systemic administration of PPACK, local delivery produced maximal inhibition of thrombosis without alterations in hemostasis. (Circulation. 1994;90:1951-1955.)

Key Words • platelets • thrombosis • coronary disease

Thrombosis plays a central role in the pathogenesis of acute coronary syndromes such as unstable angina and myocardial infarction.1,3 In addition, thrombosis frequently complicates mechanical interventional procedures used in the management of symptomatic vascular disease. Thrombi formed under arterial flow conditions consist primarily of platelets.4 The recruitment of platelets into growing thrombi critically depends on platelet activation by the procoagulant enzyme thrombin.5-7 Systemic administration of direct thrombin inhibitors can effectively block platelet-dependent thrombosis,8-11 although a corresponding impairment of hemostasis represents the principal complicating adverse effect limiting the use of these agents.7,9,10

While systemic administration of thrombin inhibitors is the only accepted method for drug delivery at the present time, catheter-based approaches for local drug delivery directly at sites of arterial injury are under development. Although anatomic demonstration of local drug delivery has been accomplished for some of these catheter systems,11 a careful evaluation of efficacy and safety for local versus systemic therapies has not been provided. The present study was designed to compare in a porcine thrombosis model the antithrombotic versus antihemostatic effects of a direct antithrombin peptide administered by local and intravenous infusions.

Methods

Surgical Procedures

Normal domestic swine were used in these studies. The animals weighed 28 to 35 kg and were observed to be disease free for at least 1 week before use. All procedures were approved by the Emory University Institutional Animal Care and Use Committee and were in accordance with Federal Guidelines.12 All animals had an arteriovenous shunt surgically implanted between the carotid artery and external jugular vein. Ketamine hydrochloride (10 mg/kg IM) was given as a preanesthetic agent, and the operation was performed under general 1% isoflurane anesthesia. The permanent shunt system consisted of two 10-cm lengths of silicone rubber tubing (Silastic, Dow Corning, Inc) with 3.0-mm inside diameter (ID). Blood flow was established by connecting the two Silastic shunt segments with a curved 2-cm length of Teflon tubing (2.8-mm ID). As described in detail previously, the permanent Teflon-Silastic shunts do not detectably shorten platelet survival or produce measurable platelet activation.13 After implantation of the chronic shunts, all studies were performed in conscious animals that were lightly sedated with ketamine and diazepam.

Thrombogenic Segments

As described previously,14,15 uncrimped, knitted Dacron grafts (2 cm in length, 4.0-mm ID) were obtained from C.R. Bard Inc. Impervious grafts rigidly constrained to a linear geometry and having an ID of precise dimensions (4.0 mm) were constructed with a flow channel that was smooth in its transition from the graft to proximal and distal Silastic tubing.
segments (4.0-mm ID) without imperfections in the flow surface due to the coupling procedure. Construction of the thrombogenic graft segment is illustrated in Fig 1. The Silastic tubing segments proximal and distal to the Dacron graft were subsequently connected to the 3.0-mm-ID Silastic tubing comprising the chronic arteriovenous shunt with 2-cm-long tapered Teflon connectors (Small Parts, Inc.).

Mean blood flow rates through vascular graft segments incorporated into the arteriovenous shunt system were measured continuously using an ultrasonic flowmeter (Transonics, Inc) with a cuff-type transducer probe that fitted snugly around the Silastic tubing comprising the chronic shunt. In all studies, initial blood flow rates were 160 to 240 mL/min. Dacron grafts were initially placed and imaged after the injection of autologous $^{111}$In-labeled platelets. The Dacron graft segments were imaged continuously for up to 2 hours and then removed.

**Platelet Labeling and Imaging**

Platelet count and hematocrit determinations were performed on whole blood collected in Na$_2$EDTA (2 mg/mL) using a J.T. Baker model 810 whole blood analyzer. Autologous porcine blood platelets were labeled with 1 mCi of $^{111}$In-oxine, as previously described. Labeling efficiencies averaged >85%. Images of the grafts, including proximal and distal Silastic segments, were acquired with a General Electric Starcam scintillation camera. Immediately before imaging the vascular grafts, images were also acquired of a segment of 4.0-mm-ID Silastic tubing filled with autologous blood and having the same lumenal volume as the graft segment (blood standard).

The radioactivities of the blood standard and 2-cm Dacron graft segments were measured in the same 3×6-cm region of interest (10×20 pixels) as defined by image analysis software routines. Images were acquired at 5-minute intervals. Deposited $^{111}$In-platelet radioactivity was calculated by subtracting the blood standard radioactivity from the dynamic study images. The total number of platelets (labeled plus unlabelled cells) deposited at each 5-minute interval over the study period was calculated by dividing the deposited platelet radioactivity (cpm) by the circulating blood radioactivity (cpm/mL) and multiplying by the circulating platelet count (platelets per milliliter).

**Intravenous Infusion of PPACK**

After a catheter procedure to isolate an external jugular vein, an 8.3F, 40-cm-long pigtail catheter (Cook, Inc) was positioned in the right atrium under fluoroscopic guidance. The catheter was flushed with heparinized saline immediately after insertion and capped. Just before the experiment, the catheter was flushed with unheparinized saline. All solutions were administered at a rate of 1.0 mL/min using a syringe pump that was calibrated with a stopwatch and a graduated cylinder. PPACK (D-Phe-L-Pro-L-Arg chloromethyl ketone) was obtained from Chemica Alta Ltd as the purified (94%) lyophilized powder (lot 256892). Immediately before administration, PPACK was dissolved in 0.9% NaCl.

**Local Delivery of PPACK**

**Static Local Delivery**

Extension tubing containing the Dacron graft was inserted into the arteriovenous shunt and exposed to blood flow for 15 minutes. Prior studies have shown that this period of time is sufficient for the formation of a nonocclusive mural thrombus that is well anchored to the Dacron substrate. The tubing was then disconnected from the animal and gently flushed with 0.9% NaCl. A solution containing PPACK, 2.5 mg/mL or 5.0 mg/mL, was gently flushed into the shunt so that it completely covered the Dacron graft. The solution was left in contact with the mural graft thrombus for 15 minutes. The PPACK solution in the shunt was then cleared and discarded by flushing the shunt with normal saline. The shunt was then reconnected to the animal and blood flow restored. Images were continuously acquired at 5-minute intervals over the 2-hour study period.

**Local Infusion of PPACK**

Local infusion of PPACK was performed using a device that was designed to deliver the infusate in a manner that achieved highest concentrations within the circumferential blood "boundary layer" adjacent to the wall of the tube (Fig 1). The device was constructed from microporous expanded polytetrafluoroethylene (ePTFE, 4.0-mm ID) clinical vascular graft material (Gore-Tex, W.L. Gore and Associates, Inc). A cuff reservoir composed of a 5-mm-long ring of silicone rubber tubing (Silastic, Dow Corning) was placed over the ePTFE graft and secured in place using Silastic adhesive. PPACK was infused into the reservoir space between the Silastic ring and ePTFE graft exterior through a length of 1-mm-ID Silastic tubing inserted through the wall of the 5-mm-ID Silastic cuff. The infused drug thus filled the reservoir, passed through the wall of the porous ePTFE graft, and entered the slow-moving blood layers at the interface between the blood and the graft wall (Fig 1). This approach was developed to regionally anticoagulate only the annular ring of blood adjacent to the wall of the device and distal Dacron graft, thereby achieving markedly greater efficiencies than could be obtained for agents delivered uniformly over the entire cross section of the blood flow column (eg, as would be obtained with systemic drug administration). The 4.0-mm-ID infusion device was connected proximally and distally to 4.0-mm-ID Silastic tubing using the Silastic adhesive to prevent blood leakage and to ensure smooth transitions without hemodynamic perturbations between the different device tubing components. The infusion device was placed 2 cm proximal to the thrombogenic Dacron graft segment, which was prepared as described previously. All PPACK solutions were infused at a total volume rate of 0.1 mL/min for 60 minutes, with PPACK concentrations in the infusate adjusted to provide infused doses of 0.005, 0.01, and 0.02 mg/kg per minute.

**Bleeding Time Measurements**

Bleeding time measurements were performed in duplicate on the shaved dorsal surface of the ear with a commercially available template bleeding time kit (Organon Teknika).

**Statistics**

All values are given as mean±SEM. Statistical analyses were performed using statistical analysis software and an IBM386 personal computer (SigmaStat). Statistical comparisons were
made using ANOVA with a repeated measures design and by Dunnett's test for comparisons of more than two groups. When two groups were compared, the Student's t test (two tailed) for unpaired sample groups was used.

**Results**

Values for platelet deposition onto thrombogenic Dacron grafts after exposure to flowing blood are given in Fig 2. In animals that received intravenous infusion of saline vehicle only, platelet deposition increased in a monotonous manner over 1 hour and plateaued at a value of 0.97±0.20 \times 10^9\text{ platelets}. Intravenous infusion of PPACK at doses of 1, 5, and 12.5 \mu\text{g/kg per minute} inhibited platelet deposition in a dose-related manner (Fig 2). With infusion of 12.5 \mu\text{g/kg per minute} PPACK, platelet deposition was reduced by 91\% versus the control results (0.09±0.04 \times 10^9\text{ platelets}; P<.001). When twice this dose was infused into two animals, there was no detectable platelet deposition over the study interval (data not shown).

Animals infused locally with saline for 1 hour showed values of platelet accumulation that were equivalent to those seen when saline was infused intravenously (0.92±0.16 \times 10^9\text{ platelets}; P>.5), indicating that thrombus formation was not impaired by the minimal local dilution of reactive blood elements produced by the boundary layer infusion approach. Platelet deposition was inhibited in a dose-response fashion when PPACK was locally infused at doses of 0.005, 0.01, and 0.02 \mu\text{g/kg per minute (Fig 2). At the intermediate dose, platelet deposition was reduced by 89\% versus the control results (0.10±0.08 \times 10^9\text{ platelets}; P<.001); at the highest dose, platelet thrombus formation was effectively abolished. For both local and intravenous PPACK therapy, the shapes of the dose-response curves were quite similar (Fig 2). However, in order to produce equivalent antithrombotic effects, the doses that were required using the local approach were reduced by a factor of approximately 600 versus intravenous therapy. Interestingly, after either the intravenous or local infusions were terminated, platelet deposition increased within 15 minutes, indicating that the capacity of the thrombogenic grafts to form thrombus was not abolished (data not shown).

Measurements of bleeding times and activated partial thromboplastin times in the treated animals are given in Fig 3. Compared with results in control animals, intravenous infusion of up to 5 \mu\text{g/kg per minute} PPACK did not significantly prolong the bleeding time (5.6±0.7 versus 4.0±0.3 minutes; P>.1) or clotting time (35.9±2.6 versus 42±3.6 seconds; P>.1). At the highest dose infused (25 \mu\text{g/kg per minute}), bleeding times averaged 18±3 minutes, and clotting times were >300 seconds. Local infusions of PPACK in the range 0.005 to 0.02 \mu\text{g/kg per minute} were without effect on either bleeding times or clotting times (Fig 3).

Static local treatment of preformed thrombus with PPACK produced a significant inhibition of subsequent platelet accumulation using concentrations of either 2.5 or 5.0 \text{mg/mL} (Fig 4). Although the duration of exposure of the graft-associated thrombus to PPACK was only 15 minutes, platelet deposition was inhibited throughout the 2-hour study interval (P<.05), indicating that a more sustained benefit was produced by this form of intensive local therapy. As expected, static exposure of thrombi to PPACK solutions (which were then discarded) produced no changes in bleeding time or clotting time parameters.

**Discussion**

It has been shown previously that the systemic administration of PPACK in a primate model of arterial

\[ \text{Platelet Deposition} = 0.9 \times 10^9 \]

\[ \text{Local Infusion} = 0.025 \mu\text{g/kg} \]

\[ \text{PPACK} = 0.5 \text{mg/mL} \]

\[ \text{Blood Flow} = 1.2 \text{mL/min} \]
thrombosis abolishes platelet-dependent thrombus formation at doses that markedly prolong bleeding times and activated partial thromboplastin times.7 The present results corroborate those findings and provide alternative strategies for delivering PPACK locally that effectively inhibit arterial thrombosis at doses several orders of magnitude lower than those required for systemic therapy, without compromising hemostasis.

Thrombin is the principal physiological agonist for platelet activation in vivo. Through a novel mechanism, thrombin cleaves the thrombin receptor on platelets, producing a neoinflammatory receptor sequence that acts as a tethered ligand to initiate receptor signaling.16 PPACK produces specific irreversible inhibition of thrombin enzymatic activity by initially forming a reversible active-site complex, followed by rapid, irreversible acylation of catalytic site histidine residues.7,17 In vitro, PPACK potently inhibits thrombin-induced platelet activation, secretion, and aggregation.17 In vivo, thrombin bound to fibrin may remain enzymatically active18,19 and inaccessible to antithrombin III and other serine protease inhibitors.20 Although thrombin bound to formed thrombus is relatively protected from inhibition by conventional heparin anticoagulation, it is susceptible to inactivation by low-molecular-weight, antithrombin-independent inhibitors because the sites of their interaction are not masked by thrombin binding to fibrin. Thus, the prolonged interruption of ongoing platelet accumulation onto preformed thrombus achieved by the static local delivery of PPACK was probably due to effective saturation of the thrombus by PPACK with inactivation of sequestered thrombin. Although PPACK is relatively specific for thrombin, it is also possible that the high local concentrations of PPACK that were used may have blocked other activated coagulation factors as well, such as factor Xa. In general, the results of the static delivery studies clearly demonstrate that thrombus reactivity depends critically on the availability of active coagulation proteases. Thrombus formation was also blocked by the infusion of PPACK, either systemically or locally, thereby reinforcing the conclusion that thrombogenicity per se may generally reflect the capacity of nonendothelialized surfaces to initiate reactions leading to the generation of thrombin.

In contrast to systemic infusion, local delivery of PPACK produced an inhibition of thrombus formation at doses that were several orders of magnitude (ie, 600-fold) below those required to achieve comparable antithrombotic effects with intravenous infusion. A major advantage of local drug administration is the potential to greatly reduce systemic side effects, since the absolute amount of compound administered is relatively small. The present study clearly documents this advantage because the local infusion rate of PPACK required to markedly inhibit thrombosis (0.01 μg/kg per minute; Fig 2) was at least 1000-fold lower than the infusion rate required to produce a significant impairment of hemostasis (10 to 15 μg/kg per minute; Fig 3).

The thrombosis model used in these studies involved blood exposure to thrombogenic Dacron vascular grafts that served to initiate clotting and anchor the growing thrombus.15 While this mechanism of thrombus initiation differs from that produced by angioplasty and other types of vessel injury, thrombus formation after the initiation phase proceeds through mechanisms that are quite similar to those triggered by natural tissue substrates; ie, thrombus growth occurs predominantly through platelet-platelet and not platelet-surface interactions.4-7 It is presumably for this reason that the Dacron graft model has predicted antithrombogenic efficacy for therapeutic agents studied in a variety of thrombosis models.8,9,21-23 On a mechanistic basis, PPACK might be expected to be globally effective for inhibiting platelet-dependent arterial thrombosis, a conclusion that is supported by previous studies with this compound. For example, systemic infusion of PPACK (45 μg/kg per minute) has been shown to block thrombus formation at sites of carotid artery endarterectomy22 and endovascular stent placement2 in primates, although hemostasis was profoundly impaired during PPACK infusion. Incorporation of PPACK into thin polymer films that were deployed with metallic stents also reduced stent thrombus formation in primates.23 After endarterectomy, even brief (1 hour) intensive systemic therapy with PPACK, which is cleared in vivo with a half-life of 2 to 3 minutes,2 produced a marked diminution of subsequent thrombus formation that persisted for at least 48 hours.22 Thus, while longer-term studies remain to be performed with locally infused PPACK, this approach is expected to provide the same lasting benefit as observed in the systemic infusion models but without affecting hemostasis.

Efficient local drug delivery was achieved by infusing PPACK through the porous wall of a conventional clinical vascular graft material. This novel method documented the remarkable efficiency of boundary layer drug administration and, to our knowledge, is the first approach described for active local delivery of substances along the wall of tubular blood conduits. The efficiency of this method derives from the fact that agents are infused only into the slowest-moving blood layers along the vessel wall. For example, in the case of steady tube flow, it is readily shown that the annular ring adjacent to the tube wall, which contains 10% of the total tube cross-sectional area, transports only about 1% of the total volume flow.24 Drug infusion into this hypothetical annular region is thus 100 times more efficient than approaches delivering the same amount of drug uniformly over the entire vessel cross section. In practice, the method may be several orders of magnitude more efficient than intravenous therapy (eg, 600-fold in the present study), depending on actual pharmacokinetic and hemodynamic variables. This infusion method also has the advantage that agents can be delivered continuously for extended periods, as opposed to static delivery or other approaches involving brief vessel occlusion, but is limited in application to vessels that can be surgically grafted. Clinically, the method could conceivably be applied in patients with peripheral arterial grafts or arteriovenous dialysis access grafts at risk for early occlusion. Experimentally, this method should more readily permit efficient studies in larger animals of both thrombosis and vascular lesion formation, with agents previously available only in relatively small amounts or that could produce harmful or confounding side effects. For example, in a preliminary study, local infusion by this method of the potent thrombin inhibitor hirudin was found to reduce early smooth muscle cell proliferation by approximately 80% at sites of carotid artery angioplasty in baboons.25 Thus, we believe that consideration should be given to exploiting the concept of boundary layer drug administration.
clinically, perhaps by adapting the method for catheter-based or other delivery systems having broader utility.

Summary

These data clearly demonstrate feasibility for the concept of local drug delivery in the treatment of arterial thrombosis, a complication that is frequently encountered after angioplasty and other interventional procedures. Acutely, thrombosis was effectively treated by local administration of the direct irreversible antithrombin PPACK in doses that were orders of magnitude below the systemically effective doses without altering hemostatic function. While the long-term benefits of this approach remains to be established and the relative effectiveness of other antithrombotic pharmacological agents remain to be determined, the continued development of catheter-based systems that can deliver drugs locally may hold considerable promise for safe and effective therapy.

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