Endothelial Dysfunction after Arterial Thrombosis Is Ameliorated by L-Arginine in Combination with Thrombolysis

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PURPOSE: To assess endothelial function after arterial thrombosis creation and after administration of a novel thrombolytic regimen in a new porcine model.

MATERIALS AND METHODS: Untreated arteries that had undergone thrombosis for 90 minutes were compared to arteries treated with tissue plasminogen activator (tPA, 4 mg) and a combination of tPA and L-arginine (L-arg; 600 mmol/L). External iliac artery luminal diameter was measured with use of duplex ultrasonography. Endothelial-dependent relaxation (EDR) and endothelial-independent relaxation (EIR) were measured with use of acetylcholine chloride (ACh) and sodium nitroprusside (NTP), respectively. Endothelial integrity was confirmed by scanning electron microscopy (SEM). Nitric oxide (NO) levels were determined with use of a chemiluminescent assay of its nitrite/nitrate metabolites (NO_x).

RESULTS: After thrombosis, EDR was decreased (69% ± 9.5; ACh = 15 μg/min; $n = 6$). EDR remained unchanged after thrombolysis with tPA despite complete dissolution of thrombus (67% ± 5.7; ACh = 15 μg/min; $n = 5$). Thrombolysis with use of tPA coupled with L-arg resulted in an increase in EDR (95% ± 4.9; ACh = 15 μg/min; $n = 5$; $P = .007$). EIR was preserved in all groups, with uniform response to NTP. SEM analysis revealed intact endothelium in all groups. Local NO_x levels were diminished after 90 minutes of thrombosis (49.3 μmol/L vs 40.8 μmol/L; $P = .0002$), but increased to 55.7 μmol/L after thrombolysis with tPA and L-arg (P = NS).

CONCLUSIONS: Thrombus induces arterial dysfunction acutely without altering endothelial integrity. This dysfunction is ameliorated through regional administration of L-arg in combination with standard thrombolytic therapy, which increases local NO levels. This model allows the in-vivo study of thrombosis and alternative thrombolytic regimens. Regional enhancement of NO levels may prove to be an attractive adjunct in thrombolytic therapy.

Index terms: Endothelial function • Thrombolysis • Thrombosis, experimental

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Abbreviations: ACh = acetylcholine chloride, EDR = endothelial-dependent relaxation, EIA = external iliac artery, EIR = endothelial-independent relaxation, L-arg = L-arginine, NO = nitric oxide, NO_x = nitric oxide metabolite, NTP = sodium nitroprusside, SEM = scanning electron microscopy, tPA = tissue plasminogen activator

ACUTE arterial occlusion secondary to thrombosis remains a dire clinical entity and is an active area of laboratory and clinical investigation at this time. Advances in treatment in the past few decades have included surgical thrombectomy and thrombolysis, popularized by multiple recent clinical trials (1,2). Catheter-directed thrombolytic therapy is a frequent and well-recognized treatment for thrombotic occlusions in coronary, cerebral, and limb ischemia. A major potential benefit of thrombolytic therapy is that thrombosis can be managed with less-invasive interventions. In addition, it can often decrease the extent of surgical revascularization.

Despite numerous advances in thrombolytic therapy, clinical outcomes are frequently less than opti-

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mal. Rethrombosis of an arterial segment is an important clinical problem in some patients in whom initial thrombolysis success has been achieved. Thrombosis complicates 5%–25% of thrombolytic cases (3,4). Reocclusion after arterial recanalization remains a clinical problem with all available thrombolytic regimens (5). In addition, clinically significant postintervention vasospasm is occasionally noted, which limits perfusion to dependent vascular beds and may itself place the vessel at increased risk for reocclusion (6).

Much attention has been devoted to the study of thrombolytic regimens, but less attention has been given to the physiologic responses of the arterial wall to acute arterial thrombosis. It is well established that the vascular endothelium plays a significant regulatory role in many physiologic processes, including the regulation of vasomotor tone. The endothelium produces vasomodulator substances; most important among them is endothelium-derived relaxing factor, now known as nitric oxide (NO) (7–9). NO is formed from the metabolism of L-arginine (L-arg) by the constitutive NO synthase of endothelial cells. NO is released by the endothelium and diffuses to underlying smooth muscle to induce vasorelaxation. There is increasing evidence that thrombus disrupts normal NO-dependent vascular function by diminishing the supply of NO to the underlying smooth muscle (10).

We have observed in recent studies that acute thrombosis causes endothelial dysfunction, as evidenced by decreased endothelial-dependent relaxation (EDR) (10,11). Results from these studies strongly implicate altered endothelial NO physiology in this dysfunction. This phenomenon may have significant relevance in the treatment of arterial thrombosis. The endothelial dysfunction induced by luminal thrombus may play a major role in suboptimal outcomes of current thrombolytic regimens. Previous studies have shown that L-arg supplementation of standard thrombolytic therapy ameliorates thrombus-induced endothelial dysfunction (10). In this study, we planned to investigate a novel thrombolytic regimen in a newly developed in-vivo porcine model of acute arterial thrombosis. Our goal was to study endothelial function after thrombolysis with targeted NO augmentation.

MATERIALS AND METHODS

Animal Model

Adult female Yorkshire swine (*Sus scrofa*; K-Bar Livestock, Sabinal, TX) weighing 59 kg ± 4.5 were used for all experiments. Animals were handled and cared for under institutional guidelines in compliance with the Guide for the Care and Use of Laboratory Animals and the American Association for the Accreditation of Laboratory Animal Care. Animals were entered into the study after a 1-week period of acclimation in the housing facilities of the Wilford Hall Medical Center Clinical Investigations Department. All animals were inspected before the study by a veterinarian and monitored daily by a technician. The experimental protocols were approved by the Wilford Hall Medical Center Clinical Investigations Division Institutional Animal Care and Use Committee panel and were performed in accordance with the recommendations of Good Laboratory Practices. All animals were killed with an overdose of sodium pentobarbital (100 mg/kg) given intravenously in an auricular vein after completion of each experiment.

Thirty minutes before instrumentation, the animals were sedated with ketamine (15–20 mg/kg) and atropine (0.04–0.4 mg/kg) intramuscularly. The intramuscular preanesthetic was given in the caudal gluteal region with a 20-gauge × 1.25-inch needle by a veterinary technologist. An 18-gauge × 1.25-inch intravenous catheter was placed in an ear vein and secured. Crystalloid intravenous fluids were administered at a rate of 2–4 mL/kg/h until initiation of the experimental protocol. Anesthesia was induced with mask ventilation with use of isoflurane (3.5%–5.0%) in 40% oxygen and 60% air. The animals were orally intubated with a 6.0–7.5-mm endotracheal tube. Anesthesia was maintained with inhaled isoflurane to provide slightly greater than the minimal alveolar concentration. A bladder catheter was placed for urinary drainage.

A large animal model was chosen with a cardiovascular system closely approximating that of humans in terms of caliber and physiology. Specifically, a porcine model (*Sus scrofa*, Yorskire strain) was used (**Fig 1**). The external iliac artery (EIA) was chosen as the study vessel for its moderate caliber and ease of access. The right side was selected as the study vessel for the sake of consistency. After adequate general anesthesia was obtained, a midline abdominal incision was made. The bowel was retracted superiorly and the bilateral deep circumflex iliac arteries were exposed and isolated. These vessels were carefully cannulated with 18-gauge × 1.25-inch catheters so the tip of the catheter was in the lumen of the EIA. This allowed proximal delivery of vasoactive agents to the arterial segment being studied. The common femoral arteries were exposed and isolated by bilateral 3-cm groin incisions and were cannulated with 18-gauge × 2-inch angiocaths. The femoral catheters allowed blood specimen removal just distal to the arterial study segment.

Duplex ultrasonography (US) was used to evaluate vasomotor function because of its noninvasive nature and precise imaging and measuring capabilities. Motion-mode ("M-mode") was used to allow assessment of changes in vessel diameter over three cardiac cycles (12–15). The average end-diastolic luminal diameter was determined, and this served as the diameter value for the given time point. US measurements were completed with the assistance of a US technician blinded to the experimental groups.

Thrombosis was induced in this model by placing small occlusive vascular clamps (1.25-inch DeBakey Classic cross action bulldog clamps; Codman and Shurtleff, Randolph, MA) at designated points on the right EIA (**Fig 1**). These clamps encompassed a 3-cm portion of the right EIA just distal to the origin of the deep circumflex iliac artery. During model development, 90 minutes of thrombosis consistently led to intraluminal thrombus formation, as revealed by excision and direct viewing of thrombus within test vessels. Duplex US documented no evidence of flow in the EIA in all study animals. All US measurements were taken in the central portion of the study segment delineated by the vascular clamps.

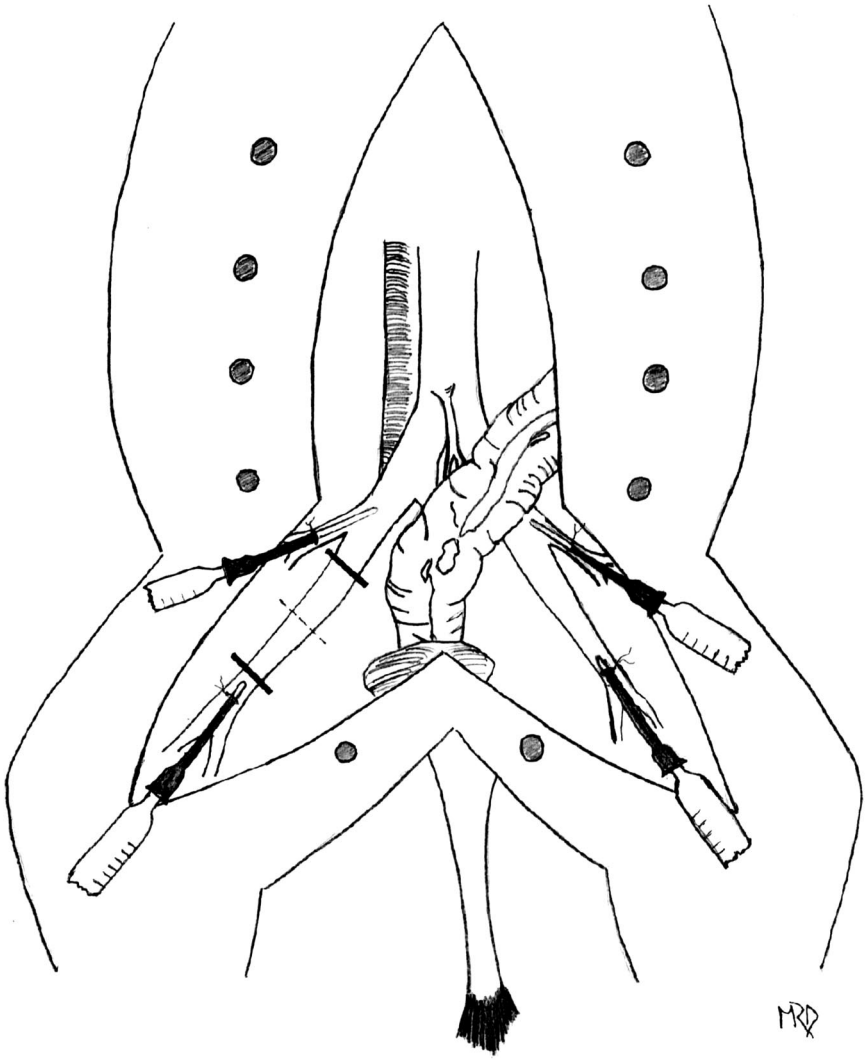

Figure 1. Illustration of model: representation of the ventral surface of a study animal with exposure of the aortoiliac system. Cannulas are placed through the bilateral deep circumflex iliac arteries and the bilateral common femoral arteries. The arterial segment studied is a 3-cm section of the right EIA bounded by the dark lines overlying the vessel. Duplex US measurements of vasomotor changes are made over the point designated by the dotted line. Proximal cannulas allow administration of agents upstream of the study segment whereas distal cannulas allow sampling of the immediate runoff from the segment.

Experimental Protocol

The experimental design included three study groups. The first group consisted of animals that underwent creation of thrombosis of the right EIA without further intervention ($n = 6$). In the second group, external iliac arterial thrombosis was induced, followed by standard thrombolytic therapy consisting of administration of tissue plasminogen activator (tPA, 4 mg; Genentech, South San Francisco, CA) through the deep circumflex iliac artery cannula over a period of 30 minutes ($n = 5$). The third group underwent creation of right EIA thrombosis with subsequent treatment with tPA (4 mg) coupled with simultaneous infusion of L-arginine (L-arg, 600 mmol/L; Pharmacia, Columbus, OH) over the course of 30 minutes ($n = 5$). In all animals, the left EIA served as an internal control and the right external iliac system functioned as the test vessel.

The tPA preparation included 4 mg of tPA in 20 mL of sterile water. This commercially obtained preparation of tPA contains 0.8 mmol of L-arg (0.14 g).

L-arg was obtained from a 10% stock solution in sterile water. The preparation of tPA plus L-arg was made by placing 4 mg of tPA in 20 mL of L-arg stock solution. Therefore, the final amount of L-arg in the experimental thrombolytic solution was 12.8 mmol (2.23 g), representing a 15-fold increase compared to the commercially available tPA preparation. Preexperimental studies with use of lower concentrations of L-arg (300 mmol/L) were found not to have a statistically significant effect.

After surgical exposure and cannulation, baseline measurements of bilateral EIA luminal diameters were taken via motion-mode duplex US (model 3000AP; Biosound, Indianapolis, IN) followed by a 90-minute period of thrombosis.

Study Endpoints

The catheter in the right deep circumflex iliac artery was used for administration of all vasoactive agents via a microinfusion pump (model AS40A; Baxter, Deerfield, IL). Acetylcholine chloride (ACh; Sigma, St. Louis, MO) at a concentration of 100 $\mu g/cm^3$ was delivered to measure EDR. A 60-mL syringe was used to deliver ACh to the iliac artery in graded rate increments of 5, 10, and 15 $\mu g/min$. Each concentration was delivered for a total of 10 minutes, followed by luminal diameter assessment via duplex US (**Fig 2**). A separate 20-mL syringe containing 20 cm^3 of 2.5-mg/mL sodium nitroprusside (NTP) (Gensia Pharmaceuticals, Irvine, CA) was used to determine endothelial-independent relaxation (EIR). NTP was administered at a rate of 5 mg/min for a total of 5 minutes. Luminal diameter was then reassessed via motion-mode US.

Local NO metabolite levels (NO_x) were measured at pre- and postthrombosis time points as well as after the respective periods of thrombolysis. Because no biologic boundary exists for diffusion at the level of the vessel wall, NO diffuses equally well in a luminal or abluminal manner. Analysis of the luminal outflow then reflects

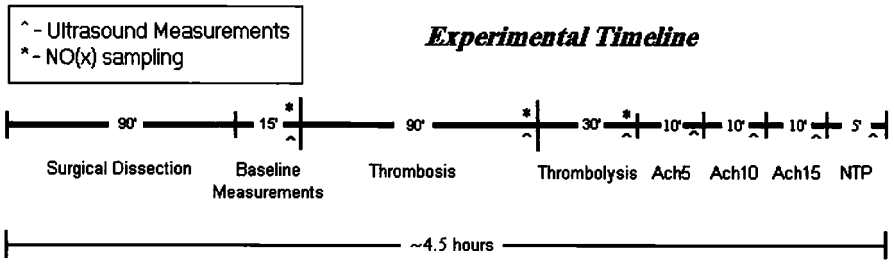

Figure 2. Experimental timeline: the interval denoted "thrombolysis" designates mock thrombolysis in the "thrombosis-alone" group, tPA (4 mg) infusion in the thrombolysis group, and tPA (4 mg) and L-arg (600 mmol/L) infusion in the L-arg/thrombolysis group. "ACh 5," "ACh 10," and "ACh 15" designate acetylcholine doses of 5, 10, and 15 μg/min. "NTP" designates nitroprusside infusion at 5 mg/min.

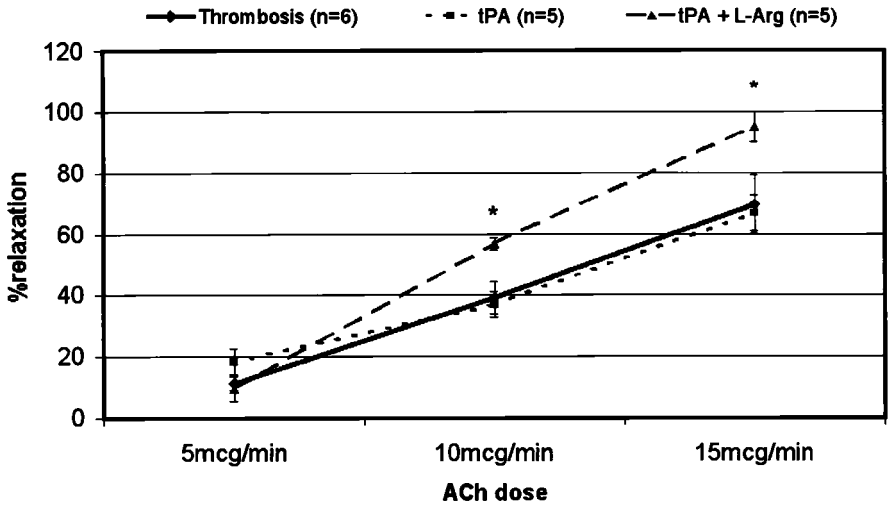

Figure 3. EDR was measured after administration of increasing doses of ACh. tPA supplemented with 600 mmol/L of L-arg resulted in significantly greater EDR than tPA alone or no pharmacologic thrombolysis (thrombosis alone). There were 5–6 animals per group. *$P < .01$, analysis of variance.

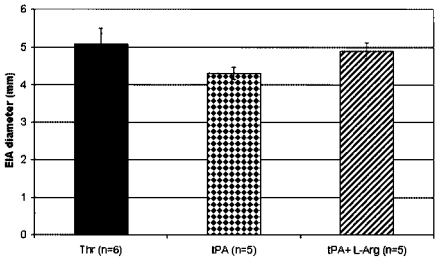

Figure 4. EIR was measured in response to NTP. Vessels in each respective group showed no significant difference in maximum relaxation in response to NTP (5 mg/min × 5 min). $P > .05$, analysis of variance.

the activities of the upstream endothelium in a similar manner to the venous drainage of the vessel wall. In a previous ex-vivo model, distal arterial sampling and venous sampling were conducted before vessel excision, with similar findings (10). Therefore, in this in-vivo model, arterial blood samples were obtained through the femoral artery cannula at baseline and immediately after 90 minutes of thrombosis to allow immediate distal sampling from the study segment. The plasma component was separated in these samples and evaluated by a chemoluminescent assay for NO_x (Department of Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA). This was accomplished with a chemiluminescence detector (NO_x analyzer, M Model 2108; Dosibi Environmental, Glendale, CA). The instrument underwent calibration with known NO_x values and blank samples on a routine basis.

Histologic Analysis

Intact endothelial presence and morphology were evaluated in all groups through the use of scanning electron microscopy (SEM; University of Texas Health Science Center, San Antonio, TX). External iliac artery segments were incised to allow en-face imaging. The rectangular segments of tissue were immediately placed in 2% glutaraldehyde and fixed overnight. The specimens were then transferred to 10% neutral buffered formalin. In preparation for SEM imaging, the samples were treated with a graded series of hexamethyldisilazane, air-dried, and gold sputter-coated.

Statistical Analysis

EDR is expressed as the percentage of relaxation from the amount of thrombus-induced vasoconstriction back to the baseline luminal diameter. EIR is expressed in terms of maximum luminal diameters after exposure to NTP. NO_x levels are expressed as absolute concentrations (in μM). All values are reported as means ± SE. Single comparisons were made with two-tailed Student paired and unpaired *t* tests. Multiple comparisons were made with analyses of variance. A *P* value lower than .05 was considered significant. Statistical analysis was performed with use of Excel (Microsoft, Redmond, WA) and Instat (GraphPad, San Diego, CA) software.

RESULTS

After 90 minutes of thrombosis, EDR was markedly decreased and reached a maximum of 69.9% ± 9.5 (ACh = 15 μg/min; $n = 6$) (**Fig 3**). Similarly, EDR in the tPA-treated group increased from 18.3% ± 4.1 to 37.0% ± 4.3 and last to 67.0% ± 5.7 in response to ACh doses of 5, 10, and 15 μg/min ($n = 5$). This represented no improvement in EDR after standard thrombolytic therapy despite clearing of all luminal thrombus. However, in the group that underwent treatment with tPA coupled with L-arg administration, EDR improved from 9.4% ± 4.0 to 56.8% ± 2.1 and finally to 95.2% ± 4.9 ($n = 5$) and was significantly higher than with standard thromboly-

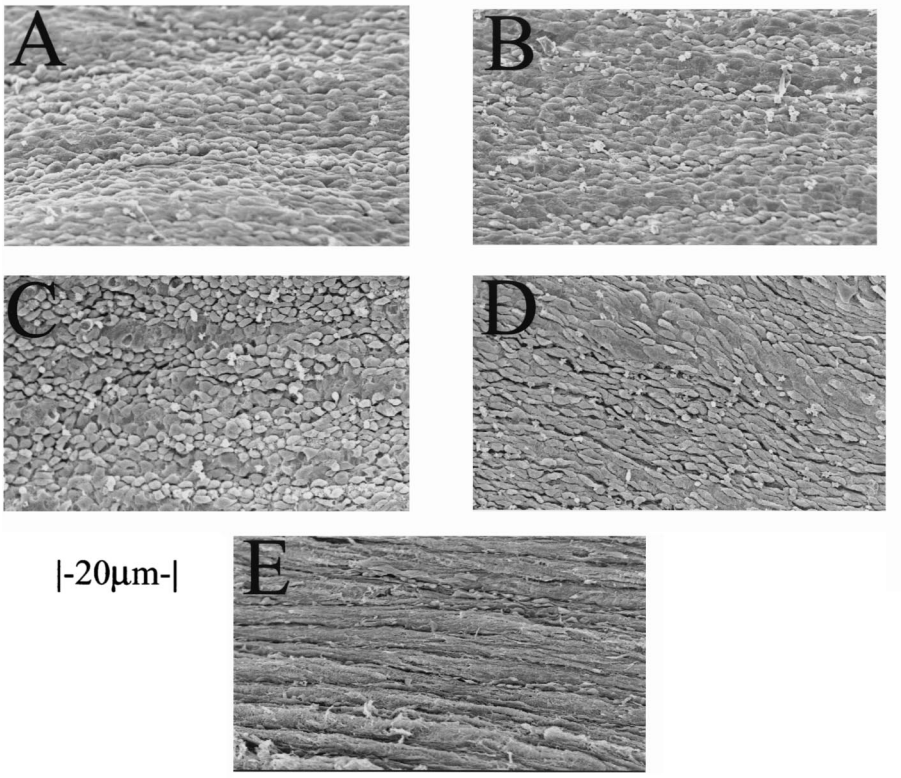

Figure 5. Sample SEM images of study porcine EIA endothelium. Normal endothelium **(A)**; endothelium after thrombosis alone **(B)**; after thrombosis and tPA infusion **(C)**; and after thrombosis and tPA and L-arg infusion **(D)**. Denuded endothelium **(E)** shown for comparison only.

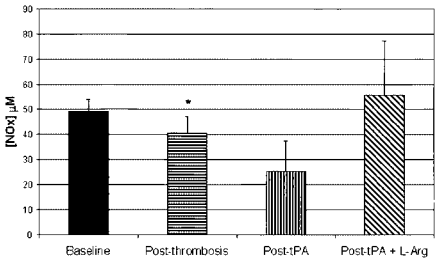

Figure 6. NO_x levels at baseline and post-thrombosis time points in all animals (n = 21) compared with postthrombolysis levels in the group treated with tPA alone (n = 5) and that treated with tPA and L-arg (n = 5). A significant decrease in NO_x from baseline is seen after thrombosis (asterisk; P = .002). An increase in NO_x is suggested in the group that received tPA and L-arg (P = NS).

sis alone. EIR was preserved in all groups, with uniform response to NTP (**Fig 4**). Maximum vessel dilation was not significantly different in response to NTP between the three groups.

SEM analysis revealed an intact monolayer of endothelium in all groups (**Fig 5**). SEM images from each group (n = 4) were independently evaluated by six blinded observers. When compared to normal pig EIA endothelium, there was no difference in endothelial morphology and no areas of denudation or endothelial cell loss.

Local levels of NO_x were measured with a chemiluminescent assay (**Fig 6**). The NO_x levels mirrored changes in EDR. NO_x levels at baseline were 49.3 µmol/L ± 4.7 but decreased significantly, by 17%, to 40.8 µmol/L ± 6.3 after thrombosis (n = 21; P = .0002). NO_x levels were 25.3 µmol/L ± 12.0 after tPA treatment but, in contrast, were 55.7 µmol/L ± 21.6 when thrombolytic therapy was coupled with L-arg (n = 5; P = NS), suggesting that an increase in substrate could lead to a local increase in NO levels.

DISCUSSION

Thrombolysis is a widely used option in the treatment of acute arterial thrombosis. Although thrombolytic regimens have been the focus of clinical refinement, limitations remain. Early rethrombosis and vasospasm are noted postintervention sequelae, especially in arterial beds with low flow.

Multiple clinical and preclinical studies have investigated the incidence and mechanism of rethrombosis after successful thrombolysis (1,2). The Thrombolysis and Peripheral Arterial Study investigators (1) have shown an acute amputation-free survival rate of 83.5% after thrombolysis for lower extremity occlusion, with a 6-month rate of 71.8%. The Surgery versus Thrombolysis for Ischemia of the Lower Extremity investigators (2) showed ongoing or recurrent ischemia to occur at a rate as high as 53% at 30 days after intervention. The possible etiologies for these frequent suboptimal outcomes are multiple. One probable contributory mechanism involves dysfunction of the arterial wall.

Yao et al (16), in an intracoronary animal model, showed a 25% reduction in reocclusion of arteries when initial thrombolysis was coupled with L-arg administration (100 µg/kg/min). In addition, time to reocclusion was extended fourfold when compared to controls (30 min ± 8 vs 123 min ± 26). This was correlated to an increase in NO_x levels 1 hour after thrombolysis. They concluded that increasing NO production may inhibit platelet aggregation and delay intracoronary thrombus formation and reocclusion after thrombolysis.

In another study, Mizuno et al (17) used a porcine model of myocardial ischemia to study blood cardioplegia solutions supplemented with L-arg. They found that the endothelial stunning seen in his model could be ameliorated through the addition of L-arg (2 mmol/L). Endothelial-dependent vasodilator responses recovered 75% ± 5 in the L-arg–treated group, but less than 20% in controls. Rates of NO_x production were twofold higher in the L-arg blood cardioplegia–treated group than in controls (0.6 vs 1.2 mmol/L/min/g of heart tissue).

Newby et al (18) investigated the influence of coronary artery atheroma on the release of tPA from the heart in

a recent study. They found a correlation between the degree of atheromatous plaque burden and endothelial dysfunction as measured by decreased native fibrinolytic activity. This, coupled with previous findings of the relationship between the L-arg/NO pathway and the acute release of tPA from the coronary system, expands the potential role L-arg may play in optimizing thrombolysis. Growing evidence also suggests NO and related molecules may exert a clinically important antiplatelet effect (19,20). These effects in concert help secure stable arterial recanalization after thrombolytic therapy and thereby help avoid acute complications. Therefore, our findings in a model of normal arteries may have more clinical relevance in the setting of atherosclerotic arteries.

Other key studies have looked at endothelial dysfunction after thrombosis (10,11). Previous studies have clearly displayed diminished EDR in response to luminal thrombus and have implicated alterations in the availability of endothelial-derived NO as the etiology for this dysfunction. In response to these findings, we hypothesized that endothelial dysfunction after arterial thrombosis can be ameliorated via local NO augmentation.

The aim of this study was to create an in-vivo model in a large animal with a cardiovascular system similar to that of humans in terms of caliber and physiology and to investigate the hypothesis of whether L-arg administration, when coupled with standard pharmacologic thrombolysis, could ameliorate thrombus-induced endothelial dysfunction. We used a porcine iliac occlusion model and assessed alterations in vasomotor tone via duplex US. Local administration of experimental agents was made possible via cannulation of branches while sparing direct puncture of the study vessel. In addition, sampling and analysis of the immediate downstream blood flow from the study segment made determination of local endothelial NO production feasible.

This investigation provides in-vivo relevance to the concept of amelioration of thrombus-induced endothelial dysfunction with L-arg. Standard thrombolytic therapy with use of tPA was shown to provide no discernible benefit to the known endothelial dysfunction produced by luminal thrombus. However, when standard therapy was coupled with L-arg administration, significant enhancement in EDR was seen. This study suggests that increased EDR after L-arg administration is correlated with improved local NO production.

Although local L-arg administration has shown promise in providing relief from thrombus-induced endothelial dysfunction in this and previous studies, questions remain. First, other mediators of vasomotor activity may have a role in vasospasm after acute thrombosis. Factors such as endothelin-1 may have an unforeseen impact in this study. Second, a single large L-arg dose was used in this study. This dose was determined based on review of the scant literature on this topic (16,17). To optimize the effect of L-arg, dose-response studies will be required. Last, this study was conducted in normal arteries with hyperacute thrombus. The question remains as to how these results will translate into diseased atherosclerotic vessels. The use of hyperlipidemic animal models may help answer the last question.

Much work remains in creating the ideal treatment for acute arterial thrombosis. New trends in antithrombotic therapy should reside in attention to mechanisms, specifically those of thrombogenesis and vascular dysfunction. We believe this large animal model will allow further progress in the development of novel treatments for arterial thrombosis. Regional NO enhancement may prove to be an attractive clinical adjunct in thrombolytic therapy.

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