Inhibition of Thrombin With PPACK-Nanoparticles Restores Disrupted Endothelial Barriers and Attenuates Thrombotic Risk in Experimental Atherosclerosis

Rohun U. Palekar, Andrew P. Jallouk, Jacob W. Myerson, Hua Pan, Samuel A. Wickline

Objective—A role for thrombin in the pathogenesis of atherosclerosis has been suggested through clinical and experimental studies revealing a critical link between the coagulation system and inflammation. Although approved drugs for inhibition of thrombin and thrombin-related signaling have demonstrated efficacy, their clinical application to this end may be limited because of significant potential for bleeding side effects. Thus, we sought to implement a plaque-localizing nanoparticle-based approach to interdict thrombin-induced inflammation and hypercoagulability in atherosclerosis.

Approach and Results—We deployed a novel magnetic resonance spectroscopic method to quantify the severity of endothelial damage for correlation with traditional metrics of vessel procoagulant activity after dye-laser injury in fat-fed apolipoprotein E-null mice. We demonstrate that a 1-month course of treatment with antithrombin nanoparticles carrying the potent thrombin inhibitor PPACK (d-phenylalanyl-d-prolyl-d-arginyl chloromethylketone) nanoparticle (1) reduces the expression and secretion of proinflammatory and procoagulant molecules, (2) diminishes plaque procoagulant activity without the need for systemic anticoagulation, (3) rapidly restores disrupted vascular endothelial barriers, and (4) retards plaque progression in lesion-prone areas.

Conclusions—These observations illustrate the role of thrombin as a pleiotropic atherogenic molecule under conditions of hypercholesterolemia and suggest the utility of its inhibition with locally acting antithrombin nanoparticle therapeutics as a rapid-acting anti-inflammatory strategy in atherosclerosis to reduce thrombotic risk. (Arterioscler Thromb Vasc Biol. 2016;36:446-455. DOI: 10.1161/ATVBAHA.115.306697.)

Key Words: atherosclerosis ● endothelium ● inflammation ● nanoparticles ● thrombin inhibitors

Atherosclerosis, the leading cause of death in the Western world, is essentially a disease of inflammation from its inception through the evolution of vulnerable atheromas that eventually break down to induce focally occlusive thrombosis with consequential tissue death in subtended vascular beds. A direct link exists between inflammation and coagulation in atherosclerosis by way of the serine protease thrombin that plays a central role in both clot formation and inflammatory molecular signaling events that may instigate and potentiate plaque development. Thrombin’s vascular activity is mediated primarily by a family of G-protein coupled receptors known as protease-activated receptors (PARs). Activation of PAR-1 by thrombin initiates a signaling cascade that promotes proinflammatory, vasomotor, and cellular proliferative effects in various cell types, including endothelial cells, smooth muscle cells, and macrophages, among others. Thrombin signaling promotes the synthesis and release of procoagulant factors, such as tissue factor, which establishes the conditions for repeated cycles of endothelial disruption, coagulation, inflammation, and plaque expansion.

Given the abundance of thrombin in atherosclerotic plaques and its recognized contribution to plaque inflammation and hypercoagulability, we sought to investigate the hypothesis that focal inhibition of plaque thrombin to abrogate its inflammatory signaling actions would both attenuate plaque procoagulant activity and facilitate restoration of naturally anticoagulant endothelial vascular barriers. Furthermore, we sought to elucidate direct relationships between thrombin inhibition, regulation of inflammatory signaling, recovery of endothelial barriers, and reduction in thrombotic risk with the use of clinically translatable functional methods for quantifying vascular barrier integrity. Although clinically approved antithrombotic pharmaceuticals have been evaluated in experimental primary and clinical...
secondary prevention trials, no information exists as to their ability to directly attenuate focal plaque thrombotic propensity or to improve vascular barrier integrity, which could serve to deter acute vascular syndromes.

To those ends, we have reported recently that atherosclerotic endothelial damage can be quantified nondestructively with the in vivo use of semipermeant perfluorocarbon (PFC)-core nanoparticles that passively diffuse beyond disrupted endothelial barriers in plaques, allowing both fluorine (19F) magnetic resonance imaging and quantification of PFC nanoparticle deposition with 19F magnetic resonance spectroscopy. Using this method, we have delineated the temporal progression of endothelial barrier disruption in apolipoprotein E (ApoE)-null mice as a consequence of a prolonged high-fat diet and demonstrated that barrier damage was related directly to the propensity for thrombotic occlusion in the dye-laser vessel injury model. Moreover, dietary management by restoration of a normal chow diet simultaneously recovered vascular barrier integrity and rapidly reduced plaque hypercoagulability within 1 to 2 months. Although these nanoparticles previously have been conjugated to selected antithrombin agents to serve as potent and safe antagonists of thrombosis in acute clotting events, their therapeutic potential for chronic control of inflammatory signaling through thrombin inhibition is untested in atherosclerosis.

Accordingly, we hypothesize that the reported capability of PFC nanoparticle to localize to atherosclerotic plaques manifesting disrupted barriers and be retained for prolonged periods would set the stage for the formulation and focal deposition of a reservoir of antithrombin nanoparticles in plaques (Figure 1A) to exert prolonged surveillance against and rapid inactivation of any locally generated thrombin. The present work was designed to examine the efficacy and safety of nanoparticle-based strategies for focal inhibition of serine proteases, namely thrombin (Figure 1A) in atherosclerosis. The test agent used for this study is a PFC nanoparticle carrying the potent thrombin inhibitor l-phenylalanyl-l-prolyl-l-arginyl chloromethylketone (PPACK), which has been previously demonstrated to be efficacious in limiting the growth of acute thrombi in mouse models of carotid artery thrombosis, with limited bleeding side effects. Furthermore, PPACK itself is a highly potent inhibitor of thrombin with several orders of magnitude increased affinity for thrombin over other serine proteases. By delivering doses of antithrombin nanoparticles over the course of several weeks, we aim to define the role of focal thrombin inhibition in mediating inflammatory signaling events in vitro and in vivo how that might play a mechanistic role in diminishing vascular endothelial barrier damage and thrombotic risk. The observed efficacy of the approach indicates a significant direct contribution of thrombin signaling to the evolution of atherosclerosis and the emergence of thrombotic risk and implicates it as a key contributor to endothelial damage in this model.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Antithrombin Nanoparticles Reduce Vascular Procoagulant Activity In Vivo

To characterize the effects of PPACK nanoparticle on reducing procoagulant activity, groups of ApoE-null mice were fed a Western diet for 3 months followed by continuation of the diet with treatment (saline, control nanoparticle, and PPACK nanoparticle) for 1 additional month. At the conclusion of the treatment/feeding period, mice were subjected to a previously validated model for measuring thrombotic risk using photochemical injury of the carotid artery that yields a quantitative metric of coagulant activity (vessel occlusion time) with good dynamic range and monotonic responsiveness to therapeutic agents that affect clotting. Before performing the vessel injury, mice were maintained on their diets for 2 to 3 additional days without further treatment to allow wash-out of any residual antithrombin nanoparticles. After 1 month of PPACK nanoparticle treatment, carotid occlusion times increased significantly over control groups (Figure 2A), with occlusion times reaching 48.7±6.7 minutes (N=7) compared with saline treatment (26.1±4.63 minutes, N=9; P=0.005) and control nanoparticle treatment (25.5±4.17 minutes, N=9; P=0.004). We note that the occlusion times in the 1-month PPACK nanoparticle–treated group approximated those of previously reported fat-fed ApoE null mice after 2 months off diet, indicating that the PPACK nanoparticle therapy may more rapidly attenuate vessel procoagulant activity than does dietary management in this model, despite maintenance of the high-fat diet in the PPACK nanoparticle–treated group.

Antithrombin Nanoparticles Restore Vascular Barrier Integrity In Vivo

We tested the ability of PPACK nanoparticle treatment to restore functional endothelial barriers, which concomitantly might be expected to reduce plaque procoagulant activity as previously reported. Mice were injected with a dose of crown ether nanoparticle for magnetic resonance spectroscopy that were allowed to circulate for 2 hours before killing, at which time plaque saturation occurs. After this circulation time, the entire length of the aorta (from the aortic root to the bifurcation) was removed for ex vivo 19F magnetic resonance spectroscopy measurements at 11.7T. Figure 2B illustrates the beneficial effects of PPACK nanoparticle treatment for 1 month on plaque endothelial permeability according to the decreased deposition of crown ether nanoparticle (0.084±0.009 μL/g aorta, N=7) compared with saline (0.122±0.011 μL/g aorta, N=8; P=0.023) and control nanoparticle (0.132±0.013 μL/g aorta, N=10; P=0.014). Using paired samples, we observed an inverse correlation (R=−0.56; Figure I in the online-only Data Supplement) between plaque permeability and vessel procoagulant activity (P=0.004),

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consistent with our previous observation that thrombotic risk tracks with plaque permeability to PFC nanoparticle.

A subset of mice was allocated for measurements of plaque extent in the aortic arch by conventional Sudan IV staining and computer-assisted planimetry. Overall, PPACK nanoparticle treatment resulted in a 20.69% decrease in aortic arch plaque extent (Figure 2C): 40.24±3.21% plaque area for saline-treated mice (N=5) and 31.91±1.69% plaque area for PPACK nanoparticle-treated mice (N=7; P=0.03).

Antithrombin Nanoparticles Attenuate Inflammatory Signaling Molecules

PAR-1 Responses
To delineate molecular signaling events responsible for the beneficial effects of PPACK nanoparticle, cell culture studies were used to quantify the responses of activated endothelial and monocytic cell lines to thrombin inhibition. First, as thrombin’s effect on cell types is mediated primarily by cleavage of the PAR-1 receptor on cell surfaces, flow cytometry was used to determine the percentage of intact PAR-1 receptors that was left after treatment with thrombin in the various treatment groups. PPACK nanoparticle treatment completely prevented thrombin cleavage of PAR-1 receptors on human aortic endothelial cells (HAECs) compared with thrombin or thrombin/control nanoparticle groups, which manifested significantly decreased PAR-1 expression (N=3 for both groups; P=0.019 and P=0.000005, respectively) as compared with baseline (Figure 3A).

Tissue Factor Responses
Because PPACK nanoparticles successfully prevented PAR-1 activation, we tested the downstream signaling effects of
PAR-1 activation related to inflammation and coagulation. Expression of tissue factor on the surface of HAECs and THP-1 monocytes in response to thrombin stimulation was assayed using a functional assay of measuring Factor Xa generation as a result of the presence of tissue factor/factor VIIa (TF/VIIa) complexes. PPACK nanoparticle prevented thrombin-induced TF expression on the surface of both HAECs (Figure 3B) and THP-1 monocytes (Figure 3C), with no significant increase over baseline TF levels at both concentrations of thrombin used (1 and 4 μM). Whole excised aortic arch segments exhibited a marked reduction in TF-positive plaque area after PPACK nanoparticle treatment: 46.75±7.74% (N=3) in PPACK-treated mice (Figure 3D) versus 72.69±5.06% (N=3; P=0.027) for saline treatment and 72.32±4.34% (N=3; P=0.023) for control nanoparticle treatment as quantified in immunofluorescent staining using ImageJ (Figure 3E–3G).

**NF-κB Responses**

Because thrombin is known to stimulate nuclear factor kappa B (NF-κB) transcriptional regulation of a panoply of inflammatory genes through PAR-1 signaling, we delineated the effect of PPACK nanoparticle on the inhibition of NF-κB activation in HAEC (Figure 4A–4E) and THP-1 cells (Figure 4F–4J). Thrombin cleavage of PAR-1 results in the activation of Gq, and dissociation of the Gβγ complex, which subsequently results in the parallel activation of protein kinase C (PKC) δ and PI3-kinase/Akt pathways. These parallel pathways then converge to stimulate IKK (I kappa B kinase), which results in the binding of the p65 homodimer to IκB and subsequent phosphorylation and degradation of IκB. Activation of the PKCδ pathway results in activation of p38 which, in turn, phosphorylates p65 to induce the nuclear translocation and transcriptional activity of the p65.

Cell cultures were stained for phosphorylated p65 after 6 hours of thrombin stimulation and treatment. Treatment with PPACK nanoparticle resulted in little to no observable positive staining for intracellular phospho-p65 and preservation of IκB protein (Figure II in the online-only Data Supplement) compared with thrombin and thrombin/control nanoparticle treatment groups. The preservation of IκB indicates that this cytoplasmic regulatory component of p65/p50 retains control of the preexisting cytoplasmic stores of p65, thereby preventing subsequent p65 phosphorylation and translocation to the nucleus.

Next we quantified phosphorylated NF-κB p65 (pp65) in the endothelium (Figure 4K) and intraplaque regions (Figure 4L) by staining sections of the excised aortic arch for pp65 (Figure 4M–4O), where increased phosphorylation of p65 indicates increased NF-κB activity. After PPACK nanoparticle treatment, aortic plaques exhibited significantly decreased endothelial pp65 (11.49±3.66%, N=3) compared with saline (33.11±4.05%, N=3; P=0.017) and control nanoparticle treatments (33.25±4.33%, N=3; P=0.019). Decreased pp65 also was observed in the same regions as plaque macrophages after PPACK nanoparticle treatment (21.78±3.15%, N=3) compared with saline (43.31±6.55%, N=3) and control nanoparticle treatments (47.51±4.59%, N=3). To rule out loss of endothelial pp65 staining because of missing endothelium, selected neighboring slide sections where pp65 was noted to be reduced were stained for vWF (von Willebrand factor), indicating that endothelium was present, thus confirming the specificity for NF-κB downregulation by PPACK nanoparticle in endothelium in vivo (Figure 4P–4R).

**Systemic Coagulation and Inflammation Markers**

Thrombin–antithrombin complexes are correlative systemic harbingers of procoagulant activity. PPACK nanoparticle treatment significantly reduced serum thrombin–antithrombin complexes (5.43±0.64 ng/mL, N=5; P=0.0001 versus saline, P=0.032 versus control nanoparticle; Figure 5A) versus saline (13.32±1.01 ng/mL, N=6) and control nanoparticle (9.96±1.64 ng/mL, N=5; P=NS versus saline).

Because NF-κB is a known driver of endothelial adhesion molecule 1 (sVCAM-1) levels as biomarkers of activated endothelium in atherosclerosis, ELISA analysis of sVCAM-1 (Figure 5B) revealed a modest, but significant decrease in sVCAM-1 with PPACK nanoparticle treatment (1504.88±65.25 ng/mL, N=4) compared with saline treatment (1666.37±12.78 ng/mL, N=5; P=0.029).

**Macrophage Responses**

Excised aortic arch sections were stained for macrophages (Figure 6A–6C), and plaque macrophage content was quantified using ImageJ. We observed no significant difference

**Figure 2.** A, μ-phenylethyl–μ-prolyl–μ-arginyl chloromethylketone (PPACK) nanoparticle (NP) treatment (rightmost bar) significantly increases time to occlusion of the carotid artery by 46% over saline (P=0.005) and control NP (P=0.004) treatments. B, Plaque permeability is reduced with PPACK-NP treatment by 33% compared with saline (P=0.023) and control NP treatments (P=0.014). C, Sudan IV staining of the aortic arch of saline and PPACK-NP–treated mice demonstrates a 20.69% decrease in gross plaque deposition with PPACK-NP treatment vs saline treatment (P=0.03) as quantified with ImageJ.
between treatment groups in terms of overall plaque macrophage content (Figure 6D).

Systemic Responses to PPACK Nanoparticle
Activated partial thromboplastin time measurements conducted on serum collected at the time of killing indicated no persistent nonspecific effects of PPACK nanoparticle after the terminal treatment dose 2 to 3 days before killing (Figure 6E). Furthermore, no significant difference was observed in serum cholesterol after the PPACK nanoparticle treatment regimen (Figure 6F).

Pharmacokinetics
Quantitative 19F spectroscopy was used to estimate the clearance half-life of PPACK nanoparticle in vivo. The half-life of the nanoparticles was determined by measuring the exponential decay of 19F signal intensity emanating regionally from the tail blood pool (Figure III in the online-only Data Supplement) and fitting the data to a biexponential, 2-compartment model. PPACK nanoparticle exhibited a mean clearance half-life (Figure IV in the online-only Data Supplement) of 105.87±23.38 minutes (N=3) compared with clearance half-lives for plain PFC nanoparticle (181.3±40.7 minutes, N=3) and PEGylated PFC nanoparticle (240.16±23.42 minutes, N=3), indicative of only modest effects on pharmacokinetics with selected particle surface modifications. The anticipated clearance mechanism for PFC nanoparticle was the reticuloendothelial system as demonstrated by 19F magnetic resonance imaging of mice postmortem, which depicted

Figure 3. A, Flow cytometry for intact protease-activated receptor (PAR)-1 after thrombin stimulation in the presence of each treatment group demonstrates inhibition of thrombin-mediated cleavage of PAR-1 with L-phenylalanyl-L-prolyl-L-arginyl chloromethylketone (PPACK) nanoparticle (NP) treatment. *P<0.05. B, PPACK-NP inhibit expression of surface tissue factor on human aortic endothelial cells (HAECs). C, THP-1 cells in response to stimulation with different concentrations of thrombin (light gray bars, 1U/mL; dark gray bars, 4U/mL). **P<0.005, ***P<0.0005. D, Diminished tissue factor expression in PPACK-NP–treated mice (P=0.027 and 0.023 vs saline and control NP, respectively) as quantified by ImageJ in tissue sections stained for tissue factor (green) in ApoE-null mice treated with saline (E), control NP (F), and PPACK-NP (G).
accumulation of nanoparticles in the liver and spleen after 2 hours of nanoparticle circulation before killing (Figure V in the online-only Data Supplement).

**Discussion**

The principal new observation in this work is that focal inhibition of plaque thrombin, and possibly other trypsin-like proteases, in fat-fed ApoE-null mice results in rapid recovery of damaged endothelial barriers and attenuated vascular procoagulant activity in spite of a continued Western diet. These beneficial outcomes were achieved with the use of antithrombin nanoparticles that passively permeated plaque intimal regions after intravenous injection and were focally retained to exert sustained pleiotropic anti-inflammatory effects.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Immunocytochemistry for phosphorylated p65 demonstrates inhibition of NF-κB (nuclear factor kappa B) activation in human aortic endothelial cells (HAECs; A–E) and THP-1 cells (F–J) with d-phenylalanyl-d-prolyl-d-arginyl chloromethylketone (PPACK) nanoparticle (NP) treatment. ImageJ quantification of endothelial (K) and intraplaque phospho-p65 staining (L) on ApoE-null mouse plaques (M–O) demonstrate significantly less endothelial and macrophage phospho-p65 (green). Elastin autofluorescence is depicted in yellow. P–R, Von Willebrand factor staining was conducted on neighboring sections to rule out loss of endothelial phospho-p65 staining because of missing endothelium in this case.
effects. Additionally, the progression of atherosclerotic plaque in lesion-prone areas of the ascending aorta was forestalled during the 1 month treatment period. Potential mechanisms for promoting quiescence in activated endothelium related to downregulation of inflammatory NF-kB signaling activity through inhibition of the thrombin–PAR1 signaling are illustrated in Figure 7.

The direct relationship between thrombotic risk and endothelial barrier disruption (Figure I in the online-only Data Supplement and Figure 1D in Palekar et al11) according to the metrics used in these models confirms a primary role for intact endothelium in maintaining vascular homeostasis in atherosclerosis. Recent reports of the relationship between endothelial damage/sloughing and acute coronary syndromes in patients recalls original descriptions of hypercoagulable vascular erosions by the Virmani group and focuses attention on ways to measure and preserve endothelial integrity as a strategic path to the detection and reduction of thrombotic risk. Prior work in our laboratory indicates that the effects of cholesterol feeding elicit both procoagulant effects and barrier disruption only after some time on a sustained high-fat diet: >3 months in ApoE-null mice11 and >6 months in NZW rabbits. In ApoE-null mice, barrier disruption worsens progressively over time on a high-fat diet, but can resolve rapidly within 2 months after switching to normal chow. Although leaky vasculature and reduced vasodilatory capacity associated with endothelial dysfunction may occur within weeks of inception of an atherogenic diet in the ApoE-null model, the barrier disruption and procoagulant activity identified by our nanoparticle permeability metrics emerge later and may serve as more direct harbingers of thrombotic risk.

![Figure 5](image1)

**Figure 5.** A, ELISA evaluation of thrombin–antithrombin (TAT) complexes reveals a significant decrease in detected TAT complexes after 1 month of \(-\text{phenylalanyl}-\text{prolyl}-\text{arginyl chloromethylketone (PPACK) nanoparticle (NP) treatment. B, ELISA analysis for detection of serum soluble vascular cell adhesion molecule 1 (sVCAM-1) demonstrated a decrease in detectable sVCAM-1 after 1 month of PPACK-NP treatment.}

![Figure 6](image2)

**Figure 6.** Immunofluorescent staining for macrophages in apolipoprotein (ApoE)-null mouse plaques treated with saline (A), control nanoparticle (NP; B), and \(-\text{phenylalanyl}-\text{prolyl}-\text{arginyl chloromethylketone (PPACK)-NP (C) revealed no significant difference in detected plaque macrophages (D) as quantified with ImageJ. E, No significant difference in activated partial thromboplastin time (APTT) between treatment groups, indicating no persisting systemic effect of PPACK-NP on coagulation 2 to 3 days after the penultimate treatment dose. F, No significant difference was observed on serum cholesterol between treatment groups, indicating that therapeutic effects observed occurred without cholesterol lowering as a consequence of nanoparticle treatment.}
With respect to the underlying mechanisms responsible for endothelial damage, inflammatory signaling and immunomodulatory events orchestrated by various plaque cell types interacting with activated endothelium have been described in detail. Here we have focused on thrombin as a key instigator of plaque growth and instability contributing to endothelial activation, vessel inflammation, and hypercoagulability, as summarized in Figure 7. Surprisingly, after an aggressive 1-month treatment period with PPACK nanoparticle after 3 months of initial cholesterol feeding, marked benefits were observed even in the face of persistently elevated serum cholesterol. The role of thrombin not only as a principal prothrombotic agent, but also as an atherogenic molecule is not unexpected given that it drives many of the inflammatory molecules that participate in plaque growth, such as NF-κB, among many others. Thrombin’s role as a proinflammatory molecule through the activation of the NF-κB pathway results in numerous downstream effects that accelerate plaque development, cell infiltration, expression of inflammatory molecules, and promotion of hypercoagulability through stimulation and secretion of procoagulant enzymes.

Of particular interest is the role of endothelial-specific NF-κB activation in atherogenesis as demonstrated by Gareus et al with the use of genetically engineered conditional knockouts of endothelial NF-κB that markedly suppressed plaque formation in fat-fed ApoE−/− mice. Sun et al achieved similar results by delivering MiR-181b to fat-fed ApoE null mice for 12 weeks to inhibit nuclear translocation of NF-κB via importin, even in the face of high cholesterol levels, which is consistent with our present observations. The seminal observations of early upregulation of NF-κB in lesion-prone aortic arch regions by Cybulsky’s group raises the interesting speculation of a role for thrombin even at these incipient time points, particularly in the context of previously documented clusters of intense, albeit small, clusters of endothelial apoptosis and replication in these aortic arch regions even in normal subjects. The ability to achieve focal suppression of NF-κB with nanoparticle delivery systems that might abrogate endothelial PAR-1 activation could help to maintain a more quiescent endothelial phenotype (eg, reduced sVCAM; Figure 5B), preserve barrier integrity, and simultaneously reduce paracrine crosstalk with other inflammatory plaque components.

A pleiotropic response to the suppression of thrombin signaling in diverse cell types that participate in atherogenesis is evidenced by modulation of NF-κB in THP-1 as well as HAEC cells (Figure 4A–4J; Figure II in the online-only Data Supplement), and our previous reports of reduced platelet content in clots that are produced by vessel injury. Regarding platelet activation, synergistic benefits also could accrue by local inhibition of thrombin–PAR-1 signaling through the NF-κB axis. However, as a potential caveat, it is interesting to note that selective inhibition of NF-κB in macrophage populations has been associated with increased atherosclerosis as contrasted with more specific inhibition of endothelial NF-κB. Although we show that NF-κB may be downregulated in representative human monocyte cell lines in vitro by interrupting thrombin/PAR-1 activation (Figure 3A) and in vivo through quantification of pp65 staining in PPACK nanoparticle–treated mice, the exact relationships between macrophages and endothelial signaling and responses to this intervention remain to be defined.

Recent experimental reports have explored the role of thrombin and related coagulation enzymes in promoting atherosclerosis with the use of orally administered antithrombotic agents or genetically modified mice for primary prevention of atherosclerosis. All such studies report significant decreases in overall plaque extent and reduced expression of inflammatory mediators. Secondary prevention clinical trials in patients with acute coronary syndromes using oral antithrombotic agents have shown modest effects on subsequent.
clinical events related to atherosclerosis progression, but at the risk of significantly increased bleeding. Interestingly, nanoparticle-based thrombin inhibition exhibits similar therapeutic effects to that of the experimental studies mentioned above, but with significantly fewer treatments (12 doses over 4 weeks), no requirement for cholesterol reduction (Figure 6F), and a more promising safety profile because coagulation parameters and bleeding times have been shown to normalize within 30 to 60 minutes after intravenous injection.12

We observed no significant change in plaque macrophage content between treatment groups (Figure 6A–6D) in contrast to the 50% decrease in plaque macrophages reported by Hara et al after 5 months of Xa inhibition.37 However, despite our shorter 1-month time window of therapeutic intervention that may not have allowed for reduced plaque macrophage content, our observations of rapid downregulation of NF-κB in macrophage-rich regions and downstream inflammatory markers (TF, thrombin–antithrombin complexes, sVCAM) (Figure 4E–4H) is consistent with the similar observations of Kadoglou et al in dabigatran-treated ApoE-null mice.35 These results also accord with previously published data,38 demonstrating the effect of thrombin and PPack thrombin in modulating the expression of TF in human saphenous vein endothelial cells, which is thought to be caused by activation of NF-κB. Future work will aim to elucidate the effects of PPack nanoparticle on plaque healing, with respect to local vascular smooth muscle cell populations and fibrosis in response to therapy over longer follow-up intervals.

The optimal dosing interval for this therapy and the duration of the local effect on barrier integrity and procoagulant activity remain to be defined. Fortunately, the pharmacokinetics (nanoparticle clearance half-life: ≈2 hours; Figure III in the online-only Data Supplement) is dominated primarily by the nanoparticle itself, with only modest alterations induced by conjugation of the active pharmacological ingredient (Figure IV in the online-only Data Supplement) or other surface constituents. This minor difference in pharmacokinetics may allow for convenient swapping of alternative anticoagulants, such as bivalirudin, as we have shown previously,13 while allowing for adequate circulation time to allow for sufficient plaque uptake of nanoparticles, demonstrated in mouse, rabbit, and human samples through prior work in our laboratory.10,11 These pharmacokinetic parameters are advantageous for potential clinical applications, as we have shown in mice that nanoparticle clearance through the reticuloendothelial system (Figure V in the online-only Data Supplement) results in reduction of residual circulating (ie, nontrapped) bioactive conjugated PPack or bivalirudin mieties within 30 to 60 minutes to a level below that required to alter systemic clotting and bleeding parameters.12,13 The potential disadvantage of intravenous nanotherapy also is notable, but there are several clinical scenarios that might benefit from early and aggressive treatment for a period of time before effective cholesterol control could be established. It is also important to note that the particular thrombin inhibitor PPack may not be entirely specific to thrombin as a serine protease inhibitor, with PPack exerting inhibitory effects on other tryptic-like proteases, such as Factor Xa. Despite its effects on other proteases, however, PPack remains a strong inhibitor of thrombin, where the inhibitory effect of PPack on Xa has been previously shown to be 3 orders of magnitude less than on thrombin.39 Nevertheless, local inhibition of other coagulation proteases may in fact exert a synergistic but still locally constrained effect in preventing the activation of thrombin.

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Disclosures

S.A. Wickline reports financial disclosures for Acupla, LLC (founder and equity). The other authors report no conflicts.

References

Thrombin-mediated inflammation and atherosclerosis progression that depends in part on its deleterious consequences for vascular barrier function. The direct and progressive relationship between endothelial barrier disruption and vessel thrombotic risk provides a clear rationale for designing therapeutic approaches that seek to preserve or restore endothelial integrity as a strategy to ameliorate atherosclerosis. To this end, the application of thrombin-inhibiting nanoparticles for focal control of procoagulant plaque activity without the need for systemic anticoagulation is demonstrated for the first time.
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Supplemental Material

Figure I

Figure I: Paired samples of aortic nanoparticle accumulation measurements and carotid occlusion times demonstrates a significant inverse correlation between the two metrics, confirming prior work\(^1\) indicating a relationship between increased endothelial permeability and increased vessel hypercoagulability.

Figure II

Figure II: Immunocytochemistry for IkB on HAECs demonstrates diminished degradation of IkB in response to thrombin stimulation with PPACK-NP treatment, consistent with inhibited activation of NF-κB.
Figure III: Temporal series of $^{19}$F spectra for one mouse at increasing times after a bolus dose of PFC-NP. Signal decay can be monitored through decrease in the $^{19}$F signal intensity and fit to a bi-exponential model to estimate clearance half-life. Depicted spectra reflect an arbitrary scale for signal strength. Frequencies are given as PPM (parts per million) assuming a 190 MHz spectrometer frequency.

Figure IV: Clearance half-life for selected nanoparticles. $^{19}$F MRS determined clearance half-lives for PPACK-functionalized PFC nanoparticles (105.87±23.38 minutes), PFC nanoparticles with a surface-
conjugated carboxy-PEG spacer (240.16±23.42 minutes), and non-functionalized PFC nanoparticles (181.3±40.7 minutes).

Figure V

Figure V: $^{19}$F spin echo coronal projection image (false color) overlaid on a proton spin echo coronal slice image (grayscale). The image, demonstrating accumulation of nontargeted PFC-NP in the spleen and liver, was acquired post-mortem in a mouse sacrificed two hours after injection.
Figure VI: (A-C) Staining of mouse aortic arch sections for the macrophage F4/80 antigen provides secondary confirmation of the presence of macrophage populations in plaques. (D-F) Isotype control staining confirms lack of nonspecific binding of the MOMA-2 antibody (see Fig. 6A-D).

Work Cited

Materials and Methods

Animal Model

To induce atherosclerosis in mice, 4-6 week old male ApoE-null mice (The Jackson Laboratory, Bar Harbor, ME), were fed a Western diet (TD-88137, Harlan Laboratories, Madison, WI) for 3 months continuously. Following 3 months of Western diet, mice were continued on diet for 1 more month with treatments of either 1 ml/kg saline, control NP, or PPACK-NP three times per week for a total of 12 doses (Fig. 1B). In animals used for plaque permeability measurements, a 1ml/kg dose of crown ether (CE) NP was administered 2 hours prior to sacrifice for single detection by MRI/MRS (see below). Blood was harvested from the left ventricle and serum was submitted to the Washington University Department of Comparative Medicine for cholesterol measurements.

Nanoparticle Formulation

Therapeutic Nanoparticles: PPACK-NP were formulated using previously described emulsification and conjugation techniques. Briefly, NP were first formulated as carboxyl-terminated perfluorocarbon nanoparticles composed of a 20% (vol/vol) perfluorooctylbromide (PFOB) core, 2% (wt/vol) surfactant, 1.7% (wt/vol) glycerin, and water. The surfactant used in this formulation consisted of 99% egg phosphatidylcholine, EPC (Avanti Polar Lipids, Alabaster, AL) and 1 mol% 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000], DSPE-PEG2000- COOH (Avanti Polar Lipids, Alabaster, AL). Following emulsification of the mixed surfactant, PFOB, glycerin, and water, the resulting precursor nanoparticle was activated for coupling of PPACK to the surface carboxyl groups with the use of 2 mg/ml 1-ethyl-3-(3-dimethylaminopropyl carbodiimide HCl, EDC (Pierce, Rockford, IL). Amine coupling of PPACK to the nanoparticle was then completed with the addition of 12.5 mg/ml PPACK (American Peptide Company, Sunnyvale, CA) to the activated precursor formulation following overnight mixing. The conjugated nanoparticle formulation was then dialyzed against MilliQ water with a MWCO of 3000-5000 for 4 hours with stirring. Size and zeta potential were measured on a ZetaPlus analyzer (Brookhaven Instruments Corporation, Holtsville, NY) and were determined to be 245.3 ± 3.5 nm and -6.13 ± 0.64 mV, respectively.

Imaging Nanoparticles: NPs were formulated as described above with the following composition: 20% (vol/vol) perfluoro-15-crown-5-ether (CE) core, 2% (wt/vol) surfactant, 1.7% (wt/vol) glycerin, and water. The lipid composition for the imaging nanoparticles consisted of 99% egg phosphatidylcholine, EPC, and 1 mol% 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL, USA).

19F Magnetic Resonance Spectroscopy For Quantification of Nanoparticle Deposition in Plaques

Fluorine magnetic resonance spectroscopy (19F-MRS) enables absolute quantification of plaque endothelial barrier disruption (Fig. 1C) by measuring the deposition of fluorine-core nanoparticles into aortic intimal plaques as described in previous work. There are several practical advantages of this method over traditional
staining approaches. The semipermeant 250 nm diameter PFC NP circulating in vivo provide a functional and selective metric for plaque endothelial barrier damage as they do not penetrate adjacent vascular segments devoid of plaque. Nor do they register the very early stages of endothelial dysfunction (e.g., weakening of tight junctions), but rather correspond to a later stage of endothelial disruption that correlates directly with procoagulant activity. The measurement is fully quantitative based on 19F spectroscopy, and objective because an entire unprocessed aortic segment is utilized without need for subjective selection of regions of interest or interpretation of selected microscopic sections. Finally, the permeation of NP is entirely passive and does not involve cell trafficking after NP uptake as it operates similarly ex vivo in excised and formalin fixed specimens.

Measurements were carried out on an 11.7T Varian MR scanner using a custom-built single solenoid RF coil. All measurements were conducted with the following parameters in each fluorine measurement: TR = 2.5s, 1024 signal averages, and a total scan time of ~42 minutes per sample. A reference standard of 0.1% trifluoroacetic acid (TFA) was included in each aorta sample to allow for absolute quantification of the perfluoro-crown ether signal from the nanoparticles compared to the known amount of TFA. For each sample, the detected amount of nanoparticles was normalized based on the weight of the aorta.

Photochemical Injury of the Carotid Artery to Assess Vascular Procoagulant Activity

Mice were anesthetized with ketamine (87 mg/kg) and xylazine (37 mg/kg) followed by isolation of the right common carotid artery through a midline cervical incision. A Doppler ultrasound probe (Transonic Systems, Inc., Ithaca, NY) was placed on the carotid artery to monitor blood flow rate for the duration of the experiment. Mice were administered a dose of 50mg/kg Rose Bengal (Sigma-Aldrich, St. Louis, MO) in saline to initiate thrombus growth following illumination of the injury site with a 1.5 mW 540 nm HeNe laser. The injury procedure concluded upon achieving a >85% decrease in measured carotid blood flow rate that was maintained for >5 minutes, indicative of stable occlusion of the carotid artery. Time to carotid occlusion was measured as a metric of coagulability where increased time to occlusion indicated increased potential for coagulation (Fig. 1D).

Cell Culture

Human aortic endothelial cells (HAECs) were obtained from Lifeline Cell Technology (Frederick, MD) and cultured in VascuLife EnGS Endothelial Cell Culture Medium (Lifeline Cell Technology), which was composed of Vasculife Basal Medium supplemented with 0.2% Endothelial Cell Growth Supplement, rhEGF (5 ng/ml), ascorbic acid (50 µg/ml), L-glutamine (10 mM), hydrocortisone hemisuccinate (1 µg/ml), heparin sulfate (0.75 U/ml) and 2% fetal bovine serum (FBS). THP-1 cells were obtained from ATCC and cultured in RPMI-1640 supplemented with L-glutamine (300 µg/ml) and 10% FBS. Cells were used at passages 2-4 for all experiments.

Inflammatory Signaling Events

PAR-1 Assay: A previously described method was utilized on HAECs to assay the inhibition of PAR-1 activation by thrombin. HAECs were incubated with 10 nM human thrombin (Haematologic Technologies, Essex Junction, VT) in assay medium (consisting of Vasculife Basal Medium) supplemented with either PBS, 10 nM PPACK,
0.8 nM plain PFOB NPs, or 0.8 nM PPACK NP for 2 hours with N=3 per group. The molar amounts of treatments were determined based on a 1:1 ratio of thrombin to inhibitor. Following incubation of HAECs with thrombin and each respective treatment group, HAECs were washed three times using PBS without Ca\(^{2+}\) and Mg\(^{2+}\) followed by cell harvesting with Nonenzymatic Cell Dissociation Solution (Sigma-Aldrich, St. Louis, MO). The harvested cells were washed and pelleted in FACS Incubation Buffer (0.5% bovine serum albumin in PBS without Ca\(^{2+}\) and Mg\(^{2+}\)), followed by staining with a phycoerythrin-labeled antibody against residues 35-46 of the PAR-1 receptor, corresponding to the cleavage site of PAR-1 (SPAN12, Beckman-Coulter, Brea, CA) for 30 minutes. Following staining, cells were washed in FACS Incubation Buffer three times each prior to and following fixation with 1% paraformaldehyde. Samples were analyzed on a BD FACScan Analytic Flow Cytometer, with ten thousand events collected per sample. Data was analyzed with FlowJo Collectors Edition.

**Surface Tissue Factor Activity Assay:** To measure the activity of surface exposed tissue factor on thrombin-activated cells, a functional assay of factor Xa generation was utilized. Cells were plated in 96 well plates at 30,000 cells per well and allowed to adhere overnight. Following overnight acclimation, cells were exposed to thrombin at 1 U/ml or 4 U/ml in serum-free medium containing equimolar amounts of either saline, free PPACK, control NP or PPACK-NP for 6 hours. Following stimulation with thrombin and each respective treatment, cells were rinsed three times with TF Activity Assay Buffer containing 50 mM HEPES, 150 mM NaCl, and 5 mM CaCl\(_2\) at pH 7.5. Cells were then exposed to Factor VIIa (4 nM, Haematologic Technologies) and Factor X (300 nM, Haematologic Technologies) in 150 µl of TF Activity Assay Buffer for 30 minutes. Following factor VIIa/X incubation, the reaction conversion was stopped with 5 µl of 100 mM EDTA. Generated Factor Xa was then assayed with the addition of 100 µl of 500 µM Chromogenix S-2222 (Diapharma, West Chester, Ohio), and allowed to incubate for 20 minutes. Cleavage of S-2222 was stopped with 10 µl of 30% acetic acid and the plate was then read at 405 nm on a BioRad Model 550 Microplate Reader.

**NF-κB Assay:** HAECs and THP-1 cells were seeded on to coverslips at 100,000 cells/coverlisp. For THP-1 seeded coverslips, coverslips were treated with 0.01% poly-L-lysine solution for 15 minutes, followed by 2 hours of drying prior to cell seeding. Following overnight incubation to allow for cell attachment, cells were stimulated with either thrombin alone or thrombin treated with PPACK, control NP, or PPACK-NP for 6 hours. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS. Cells were stained for phosphorylated p65 and IkB as markers of NF-κB activation using a phospho-p65 primary antibody (1:200 dilution, ab28856, Abcam, Cambridge, MA) or an IkB primary antibody (1:200 dilution, ab7217, Abcam) and and Dylight 488 secondary antibody (1:500 dilution, ab96899, Abcam). Coverslips were mounted onto slides with DAPI loaded mounting medium to counterstain for nuclei and cells were imaged using fluorescent microscopy.

**TAT and sVCAM Assays:** For measurements of thrombin-antithrombin (TAT) complexes and soluble VCAM-1 (sVCAM-1), blood was harvested from the left ventricle and serum was stored for ELISA analysis. TAT-complexes were measured on serum using an ELISA kit (ab137994, Abcam) as per the manufacturers instructions. For sVCAM-1 measurements, serum was assayed using an ELISA kit (MVC00, R&D Systems Inc., Minneapolis, MN) as per the manufacturers instructions.

**Histological Analyses**
For evaluation of gross plaque deposition in the aortic arch, aortas were removed a pinned en face for staining of plaques with Sudan IV. Tissues were fixed in 10% formalin for 24 hours, then stained with Sudan IV. Quantification of positive fat staining was carried out in the aortic arch for saline treated mice (N=5) and PPACK-NP treated mice (N=7) using ImageJ. Arch segments were selected using the top 1/3 portion of the aorta and uniformity of segments for analysis was verified through ImageJ measurements of total surface area for the region of interest. For histological analysis of plaque characteristics, tissues were stored in in optical cutting medium (OCT) and frozen sectioned at a thickness of 5 µm. Antibodies against Tissue Factor (1:100 dilution, sc30201, Santa Cruz Biotechnology, Dallas, Texas), phospho-p65 (1:200 dilution, ab28856, Abcam), von Willebrand Factor (1:100 dilution, ab11713, Abcam), and macrophages (MOMA-2, 1:100 dilution, ab33451, Abcam) where used to stain tissue sections. Quantification of positive staining for tissue factor, phospho-p65 and macrophages was accomplished using ImageJ with N=3 mice per treatment group. Additionally, secondary confirmation of macrophage staining was conducted with an antibody against the F4/80 antigen (1:100 dilution, ab6640, Abcam) along with isotype control staining (Rat IG2b, 1:100, ab18536, Abcam) to rule out nonspecific binding of the MOMA-2 antibody to mouse aortic tissue.

Activated Partial Thromboplastin Time

For measurement of activated partial thromboplastin time (APTT), blood was drawn from the ventricle at the time of sacrifice into a syringe containing 4% sodium citrate for APTT measurements at a final dilution of 1 part sodium citrate to 9 parts whole blood. Blood samples were centrifuged for 15 minutes at 1000g and the supernatant was saved for APTT measurements. APTT kits (HemosIL, Lexington, MA) were used as per the manufacturers instructions.

Pharmacokinetics of PPACK-NP using in vivo $^{19}$F MRI

To elucidate the pharmacokinetics of PPACK-NP, C57/BL6 mice under anestheia (Ketamine, 54mg/kg/hr and Xylazine (8.2mg/kg/hr)) were administered a 1mL/kg bolus dose of plain PFC-NP, PEGylated PFC-NP or PPACK-NP using a jugular vein catheter and $^{19}$F MR signal in the tail was measured on a 4.7T MR imaging system (Varian). The $^{19}$F spectra was obtained using a custom-built four-turn solenoid coil through $^{19}$F spectroscopy (256 signal averages, TR=1.05s). The decay of in vivo $^{19}$F signal intensity detected in the tail was fit to a bi-exponential curve, with the resulting exponents interpreted as half-lives in a two compartment model. For in situ imaging of nanoparticle biodistribution, mice were given a single bolus dose of PFC-NP and sacrificed two hours after administration. Proton (TR=0.5s, TE=0.02s, 256x256 matrix, field of view = 8 cm x 5 cm, 2.2 minute acquisition time) and $^{19}$F (TR=0.8s, TE=0.121s, 64 signal averages, 32x32 matrix, field of view = 8 cm x 5 cm, 2 cm image depth, acquisition time = 27.3 minutes) spin echo images were obtained post-mortem on a 11.7T MR imaging system (Varian).

Statistics

All statistical tests were performed on R, version 3.0.1. Student's t test was utilized for all data, with p<0.05 denoting statistical significance. The Pearson's product-moment correlation test was utilized to determine the relationship between NP accumulation and carotid occlusion time. Error bars represent standard error of the mean.
Study Approval

All animal experimental procedures were performed with approval from the Washington University Animal Studies Committee.

Work Cited


