Atherosclerotic plaque rupture leads to acute thrombus formation and may trigger serious clinical events such as myocardial infarction or stroke. Therefore, it would be valuable to identify atherothrombosis and vulnerable plaques before the onset of such clinical events. We sought to determine whether the noninvasive in vivo visualization of activated platelets was effective when using a target-specific MRI contrast agent to identify thrombi, hallmarks of vulnerable or high-risk atherosclerotic plaques.

Methods and Results—Inflammatory thrombi were induced in mice via topical application of arachidonic acid on the carotid. Thrombus formation was imaged with intravital fluorescence microscopy and molecular MRI. To accomplish the latter, a paramagnetic contrast agent (P975) that targets the glycoprotein $\alpha_{\text{IIb}}\beta_3$, expressed on activated platelets, was investigated. The specificity of P975 for activated platelets was studied in vitro. In vivo, high spatial-resolution MRI was performed at baseline and longitudinally over 2 hours after injecting P975 or a nonspecific agent. The contralateral carotid, a sham surgery group, and a competitive inhibition experiment served as controls. P975 showed a good affinity for activated platelets, with an $IC_{50}$ (concentration of dose that produces 50% inhibition) value of 2.6 $\mu$mol/L. In thrombosed animals, P975 produced an immediate and sustained increase in MRI signal, whereas none of the control groups revealed any significant increase in MRI signal 2 hours after injection. More important, the competitive inhibition experiment with an $\alpha_{\text{IIb}}\beta_3$ antagonist suppressed the MRI signal enhancement, which is indicative for the specificity of P975 for the activated platelets.


Key Words: arterial thrombosis ■ atherosclerosis ■ magnetic resonance imaging ■ platelets ■ thrombosis

Acut e thrombus formation is an outcome of atherosclerosis and may trigger the onset of serious clinical events, such as myocardial infarction or stroke. The rupture or erosion of atherosclerotic plaques exposes the prothrombotic core to the blood and is regarded as the pivotal event for thrombus formation. Activated platelets play a critical role in thrombogenesis, through both platelet aggregation and activation of the coagulation cascade. Consequently, platelets have been a target for aggressive treatments in patients experiencing acute coronary syndrome (ACS) and stroke. A specific signature of platelet activation is the externalization of integrins $\alpha_{\text{IIb}}\beta_3$ that play a pivotal role in platelet aggregation and thrombogenesis. Indeed, $\alpha_{\text{IIb}}\beta_1$ inhibitors are recommended in patients with unstable angina. Therefore, $\alpha_{\text{IIb}}\beta_3$ might be a good biomarker of nascent thrombosis. Therefore, using $\alpha_{\text{IIb}}\beta_3$ to image adherent or activated platelets represents a unique opportunity to identify atherothrombosis and vulnerable plaques before they induce dramatic clinical events.

Most of the imaging techniques for thrombus detection are invasive or are characterized by a relatively poor spatial resolution. However, major advances in medical imaging techniques in general and in noninvasive magnetic

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resonance techniques in particular have been achieved in recent years; they allow the visualization of both the vessel wall and atherosclerotic plaques.6–11 Indeed, researchers have shown that the visualization of atherothrombotic plaques and the identification of vulnerable lesions were achievable through non–contrast-enhanced MRI.12 Nevertheless, complete characterization of plaque components and particularly the accurate identification of thrombi in vivo remain a major challenge. Recently, encouraging studies13,14 have been published using magnetic resonance (MR) molecular imaging with gadolinium (Gd) contrast agents targeting fibrin to allow thrombi detection. These studies hold special promises for imaging large occlusive thrombi displaying a high fibrin buildup. Moreover, Von zur Muhlen et al15 reported the use of iron oxide nanoparticles attached to a single chain antibody targeting activated platelets to image thrombi induced in mice carotid arteries via MRI. The researchers showed that contrast agent targeted to activated platelets allowed the sensitive and rapid detection of smaller mural thrombi.15 Molecular imaging of platelets has been demonstrated in patients using nuclear imaging approaches, such as single positron emission computed tomography.16–18 In fact, Acutect, a technetium99m label, is Food and Drug Administration approved for this purpose.19 However, MRI will offer superior spatial resolutions and concomitant anatomical information compared with single positron emission computed tomography and nuclear imaging techniques in general.

Most of the studies about experimental thrombosis have used a ferric chloride model of thrombogenesis.20 This model does not involve the physiological mechanisms of thrombi formation; rather, it creates a severe endothelial injury that leads to thrombosis. Recently, an alternative model of thrombosis, in which arachidonic acid (AA) is topically applied to mice carotid arteries, was introduced.21 One of the major advantages of this model is that it more closely mimics inflammatory thrombogenesis through platelet activation and activation of the coagulation cascade.

In the current study, after emphasizing the critical role of platelets in AA-induced thrombi by using intravital microscopy, we used an activated platelet–targeted MRI contrast agent to noninvasively monitor the formation of platelet-rich thrombi in the previously mentioned model of thrombosis. This contrast agent (P975; Guerbet, Roissy Charles-de-Gaulle, France) is composed of a peptide targeting α₁bβ₃ (P977) conjugated to a Gd chelate (Gd plus 1,4,7,10-tetraazacyclododecane-N,N',N',N'-tetraacetic acid [DOTA]). The specificity of P975 to activated human platelets is shown herein both in vitro and in vivo.

**Methods**

**Contrast Agent**

P975 is a Gd-based contrast agent obtained by coupling a peptide (P977) via a small linker to Gd-DOTA at a 1:1 molar ratio. P977 is a cyclic peptide (cyclo(Cys-Arg-Gly-Asp-Cys)) of 878 Da, known to bind to the integrin α₅β₃ expressed on tumor cells.22 This conformation was also reported to provide a good affinity for α₁bβ₃; therefore, it was selected for the design of P975. The targeted contrast agent (P975) has a molecular weight of 1344 Da, which is approximately 2.5 times greater than Gd-DOTA.

**Measurement of P975 and P977 Binding to Thrombin Receptor–Activating Peptide–Stimulated Platelets**

The binding of P975 and P977 to the α₁bβ₃ integrin was evaluated by inhibition measurements of specific fluorescein isothiocyanate (FITC)–fibrinogen (Fg) binding to thrombin receptor–activating peptide (TRAP)–stimulated platelets using flow cytometry. Resting platelets, 15 µL, corresponding to 6.0×10⁶ to 7.5×10⁶ platelets per vial (calculated by counting), were activated by 100-µmol/L TRAP-6 (Bachem; Heidelberg, Germany) and coincubated with both increasing concentrations of test compounds P975 and P977 and 200 nmol/L of FITC-Fg²⁴ for 5 minutes at 37°C. All the reagents were dissolved in Tyrode solution, and the binding protocol was performed in a 100-µL reaction volume. The FITC-Fg binding was then measured by flow cytometry (FC500; Beckman Coulter; Paris, France) specifically gated on the population of platelets (without leukocytes) after excitation with a laser at 488 nm and emission at 525 nm. The specific binding of FITC-Fg to α₁bβ₃, expressed by stimulated platelets, was calculated by subtracting the mean of fluorescence intensity of background from the mean of fluorescence intensity of platelets showing specific FITC-Fg binding to α₁bβ₃. 

**Volume of the AA-Induced Thrombus**

Measurement of P975 and P977 binding to α₁bβ₃ using flow cytometry with 100-µmol/L TRAP-6 (Bachem; Heidelberg, Germany) and coincubated with both increasing concentrations of test compounds P975 and P977 and 200 nmol/L of FITC-Fg²⁴ for 5 minutes at 37°C. All the reagents were dissolved in Tyrode solution, and the binding protocol was performed in a 100-µL reaction volume. The FITC-Fg binding was then measured by flow cytometry (FC500; Beckman Coulter; Paris, France) specifically gated on the population of platelets (without leukocytes) after excitation with a laser at 488 nm and emission at 525 nm. The specific binding of FITC-Fg to α₁bβ₃, expressed by stimulated platelets, was calculated by subtracting the mean of fluorescence intensity of background from the mean of fluorescence intensity of platelets showing specific FITC-Fg binding to α₁bβ₃. 

**Kᵢ = IC₅₀/[1 + ([L]/K_d)]

where IC₅₀ represents the concentration yielding 50% inhibition of specific FITC-Fg binding to platelets; [L], the FITC-Fg concentration (200 nmol/L); and K_d, the dissociation constant (466 nmol/L in our conditions, data not shown). IC₅₀ values were calculated by plotting a graph of fluorescence intensity as a function of the concentration of the inhibitor, and by estimating the concentration that corresponded to a 50% reduction of the maximum of specific fluorescence. The Ki and IC₅₀ values were calculated using computer software (Graph Pad PRISM software v4.0.; San Diego, Calif).

**MRI Detection of the AA-Induced Thrombus**

Male C57BL/6 mice, aged 10 to 11 weeks (n=17; Jackson Laboratories, Bar Harbor, Maine), were used. Care and use of laboratory animals followed the national guidelines, and the study was approved by The Mount Sinai School of Medicine Animal Committee (Institutional Animal Care and Use Committee). Immediately after AA-associated thrombus induction (Supplemental Methods), a venous catheter connected with a 1-mL syringe containing a 100-µmol Gd/kg dose of the contrast agent P975 was placed into the tail vein. The animals (n=5) were
placed at the center of a whole-body coil (35-mm inner diameter) under continuous isoflurane anesthesia and positioned in a 9.4-T MRI system (Bruker BioSpec; Bruker; Rheinstetten, Germany). The animals were connected to a respiratory rate monitor, and the flow of anesthetic gas was constantly regulated to maintain a breathing rate of 60±20/min (mean±SD). The imaging protocol consisted of a pilot scan with 3 orthogonal slices, followed by a spin-echo T1-weighted sequence with an in-plane spatial resolution of 117 μm (slice thickness, 1 mm; field of view, 300 mm; matrix size, 256×256), a repetition time of 800 milliseconds, an echo time of 10.2 milliseconds, and 4 averages, resulting in an imaging time of 13.26 minutes. The area covered the common carotid artery, the carotid bifurcation, and the distal carotid artery. Contiguous cross-sectional images were obtained perpendicular to the long axis of the neck. The animals were scanned at baseline (20 minutes after thrombus induction) and longitudinally over a 2-hour period (after a bolus injection of 150 μL of the contrast agent) to follow the course of enhancement. In each animal, the contralateral carotid was used as a negative control.

A second group of mice (n=5) was used to compare P975 with the conventional Gd chelate (Gd-DOTA, 100-μmol Gd/kg, IV). In addition, a third sham surgery group (n=2), in which the diameter of the carotid was reduced upstream and the AA application was substituted by an application of ethanol alcohol, was studied.

To demonstrate the in vivo specificity of P975 to the integrin αIIbβ3 expressed on activated platelets, a competitive inhibition experiment on a fourth group of mice was performed (n=5). After thrombus induction, a baseline MRI scan was performed. Subsequently, epifibatide (IntegriVen; Schering-Plough; Essex Chemical, Lucerne, Switzerland), was injected 10 minutes before euthanasia. Animals were euthanized to avoid postmortem thrombus formation, heparin, 100 IU/kg, was injected 10 minutes before euthanasia. Animals were euthanized via an overdose of isoflurane. Transcardiac perfusions through the left ventricle were performed with saline and 4% paraformaldehyde. Carotid arteries were excised, collected in OCT freezing medium, and frozen for histology. The specimens were counterstained with a combined Masson elastin technique and hematoxylin-eosin.

Quantification of MRI Signal Enhancement
Image analysis was performed using software (OsiriX DICOM reader v3.0; OsiriX Foundation; Geneva, Switzerland). At the site of thrombogenesis, the inner vessel boundary was traced to determine the average signal intensity of the lumen in both the thrombosed and the contralateral carotid (SCarotid). Additional regions of interest were placed in the paravertebral muscles and in a motion-free region outside the animal to determine the signal intensity of the reference tissue (SImuscle) and the SD of the noise signal, respectively. Individual contrast to noise ratios (CNRs) were calculated from 3 contiguous MR images at each imaging point (CNR=[SCarotid−SImuscle]/SD of the noise signal). Their average resulted in a mean CNR value (mCNR). The contrast to the background signal (P975 or Gd-DOTA) was determined and plotted over time (ΔCNR=mCNR[imaging time point−mCNR[baseline]]). The evolution of ΔCNR values was compared between all groups. In addition, the ΔCNR values at 2 hours after injection were plotted, and statistical analyses were performed between each group to evaluate late enhancement. A 1-way ANOVA was performed using computer software (Graph Pad PRISM software v4.0.) to compare the groups. P<0.05 was considered to indicate a significant difference between the groups.

Histopathology
Histological evaluation of the thrombus and correlation of thrombus localization with MRI were systematically performed. Coregistration was performed using the carotid bifurcation as an anatomical landmark. Briefly, the out-of-plane resolution (slice thickness) was 1 mm, which allowed us to measure the position from the bifurcation and match the histology with the MRI. To avoid postmortem thrombus formation, heparin, 100 IU/kg, was injected 10 minutes before euthanasia. Animals were euthanized

Results

In Vitro Binding of P975 and P977
The rI value of P975 was measured on a relaxometer (Minispec; Bruker) and was shown to be 9 s−1·mmol/L−1 at 40°C and 60 MHz. The specificity of P975, P977, and tirofiban to αIIbβ3 integrin was evaluated by injection measurements of FITC-Fg binding to TRAP-stimulated platelets. We observed a concentration-dependent inhibition of FITC-Fg binding in the presence of P975, P977, or tirofiban (Figure 1). This inhibition was complete at higher concentrations; and the calculated IC50 values were 2.1±0.3 μmol/L, 1.6±0.2 μmol/L, and 20.4±0.3 mmol/L for P975, P977, and tirofiban, respectively. The Ki values for P975, P977, and tirofiban, calculated according to the Cheng-Prusoff equation, were 1.5 μmol/L, 1.1 μmol/L, and 14.3 mmol/L, respectively. Gd-DOTA, used as a reference, displayed no affinity for αIIbβ3 (data not shown).

Imaging of Thrombus Formation With Intravital Fluorescence Macroscopy
To study thrombus formation and emphasize the critical role of platelets in the AA model of thrombosis, intravital fluorescence microscopy was used after injection of fluorescently labeled platelets and AA thrombus induction. As
shown in Figure 2 and the video (Supplemental Data), activated platelets immediately started adhering to the vessel wall after AA application onto the carotid. The conversion of AA by cyclooxygenases and particularly cyclooxygenase 2 into prostaglandin H2 is known to yield thromboxane A2 and prostaglandin E2, which induce platelet activation and thrombosis. Accordingly, we observed small thrombi on the luminal side of the vessel wall, clustering and eventually forming a surface-occluding thrombus. As the pulsatile blood flow inside the carotid was maintained, the formed thrombi eventually detached from the arterial wall and embolized in the circulation. However, the process of thrombosis kept forming as long as the AA surrounding the carotid was still present in sufficient concentrations. The presence of intra-arterial thrombosis was subsequently confirmed by histology (Figure 2C). In addition to demonstrating the critical role of platelets in AA-induced thrombosis, these observations emphasized the necessity to more or less retain thrombi for subsequent in vivo MRI. Thus, a loose but permanent ligation was applied on the carotid before thrombus induction.

In Vivo MRI of Activated Platelets in the Thrombosis Model

AA, 6 µL, at 200 mg/mL (or ethanol alcohol sham surgery) was applied periadventitially for 1 minute to induce intra-arterial thrombosis in the mouse right carotid. Mice were placed in the MRI scanner, and baseline scans were performed to obtain images before contrast agent injection. Subsequently, P975 or Gd-DOTA was injected via a catheter placed in the tail vein. Consecutive MRI scans were acquired every 14 minutes up to 2 hours postinjection, and signal enhancement was calculated in both the thrombosed carotid and the contralateral carotid (negative control). In both groups, MRI allowed clear identification of both carotids at baseline by black blood imaging (Figure 3). The presence of a thrombus was already visible in the right carotid artery. Thirty minutes after contrast agent injection, an initial signal enhancement in the lumen of the thrombosed carotid artery and in the perivascular area was observed in both groups (Figure 3). This initial luminal enhancement was attributed to contrast
agent molecules trapped in the mesh of the thrombus. However, the initial signal increase obtained with P975 was visibly more pronounced compared with Gd-DOTA. More important, in the animals injected with P975, the signal enhancement persisted over time and was still present 120 minutes after injection (Figure 3). On the other hand, the animals injected with Gd-DOTA exhibited a rapid washout of the contrast agent from the lesion after 30 minutes. This was confirmed by MRI data quantification (Figure 5). Indeed, the CNR values obtained in the injured carotid with P975 injection were significantly increased at each imaging point compared with the values obtained with Gd-DOTA injection and the control and sham carotids (Figure 5). This was especially visible 2 hours after injection when the CNR values in the injured carotid of animals injected with P975 showed a 4-fold increase compared with the Gd-DOTA group, a 7-fold increase compared with the sham surgery group, and an 8-fold increase compared with the control group \((P<0.01)\). Therefore, P975 allowed accurate and efficient imaging of thrombosis.

**In Vivo Study of P975 Specificity to Activated Platelets**

To evaluate the specificity of P975 to activated platelet-rich thrombi in vivo, we performed a competitive inhibition experiment. Eptifibatide, a clinical antagonist for \(\alpha_{IIb}\beta_3\) receptors, was injected at a saturating dose before P975 injection. MRI scans were performed to obtain precontrast images, eptifibatide and P975 were injected, and the postcontrast MRI routine was applied. The injection of eptifibatide did not induce thrombolysis. An initial signal increase in the thrombosed carotid artery was observed 30 minutes after contrast agent injection. However, the signal increase did not persist throughout the 120-minute window of imaging time, and the washout kinetics of P975 in this cohort were similar to those of the nonspecific contrast agent Gd-DOTA (Figure 4). The absence of sustained contrast enhancement is likely the result of saturation of activated \(\alpha_{IIb}\beta_3\) integrins on activated platelets by eptifibatide. Data quantification at 120 minutes after injection in this cohort corroborated the observations because the increase in CNR values was comparable to those obtained in the control animals injected with Gd-DOTA (Figure 5).

**Discussion**

In the present study, we demonstrated the in vitro affinity of a novel contrast agent (P975) to the activated integrin \(\alpha_{IIb}\beta_3\)
specifically expressed on activated platelets. We then showed its in vivo specificity in an AA model of thrombosis induced in the mouse carotid artery.

Real-time observation of thrombi formation and embolization with intravital fluorescence microscopy demonstrated the critical role of activated platelets in the process of AA-induced thrombosis. The observation of embolization showed the necessity of keeping the thrombi collected to obtain sufficient temporal resolution to perform MRI for thrombus detection before and after contrast. To address this, the diameter of the vessel was slightly reduced upstream, with a loosely applied permanent ligation. This ensured that even in case of emboli, the thrombi formed would be retained at the site of imaging. To ensure that the ligation did not induce thrombi formation, a sham surgery group in which animals only underwent slight carotid ligature and received ethanol alcohol instead of AA showed an absence of intra-arterial thrombosis. This confirmed that the ligation did not affect thrombosis.

P975 is a contrast agent directed against the activated platelet fibrinogen receptor \( \alpha_{\text{IIb}}\beta_3 \). \( \alpha_{\text{IIb}}\beta_3 \) is an important therapeutic target in ACSs as the result of its central role in the pathway of platelet activation.\(^3\) The abundance of \( \alpha_{\text{IIb}}\beta_3 \) and its specific change of conformation on activated platelets make it an ideal target for imaging. The binding of adhesive proteins to the \( \alpha_{\text{IIb}}\beta_3 \) integrin has been shown to occur through the peptide sequence Arg-Gly-Asp (RGD), among others.\(^26\) The RGD sequence is found on fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin, and type I collagen.\(^27\)–\(^30\) The contrast effect provided by P975 in the lumen of the thrombosed carotid at 2 hours after injection. P975 only induced an initial enhancement of P975 in the lumen of the thrombosed carotid at 2 hours after P975 injection suppressed the sustained signal of P975 in the lumen of the thrombosed carotid at 2 hours after injection. P975 only induced an initial enhancement of the thrombus 30 minutes after injection before rapidly clearing out of the circulation. Contrary to what has been demonstrated ex vivo,\(^34\) the injection of epifibatide did not dissolve thrombi in vivo because the carotid still appeared occluded on the MRI performed postinjection. This different behavior is likely to be explained by the formation of fibrin during the in vivo clotting process. Indeed, fibrin plays a crucial role in stabilizing thrombi and might prevent thrombi from dissolving in the presence of anti-\( \alpha_{\text{IIb}}\beta_3 \) without the conjugate use of anticoagulant or thrombolytic therapy. In addition, the use of a ligation likely reduced the flow in the carotid and may have limited the potential for embolization in the first place. In this cohort, P975 displayed the behavior of an unbound circulating contrast agent that is almost identical to the non-specific contrast agent, Gd-DOTA. This phenomenon can be attributed to the profusion of anti-\( \alpha_{\text{IIb}}\beta_3 \) agent binding the activated \( \alpha_{\text{IIb}}\beta_3 \) receptors, therefore prohibiting its ligation by P975 molecules. This experiment was particularly critical because \( \alpha_{\text{IIb}}\beta_3 \) can be expressed by smooth muscle
cells after vascular injury and may have resulted in a competing binding source of P975. In addition, it would be of interest for future experiments to study the effect of physiological doses of aspirin or thienopyridine on P975-enhanced imaging because they both can achieve platelet inhibition.35

Other strategies to image thrombi via MRI were considered and published in the past. In particular, the high amount of fibrin formation during the clotting process resulted in a target of choice for the molecular imaging of thrombosis. Consequently, the MR compound EP-2104R showed promising clinical applications.13,14,36–38 Published data39 indicate that 50% of patients with acute ST-segment elevation myocardial infarction bore coronary thrombi for days or weeks before the event. Therefore, the detection of thrombi via MRI may be a valuable diagnostic approach for the early detection and prevention of cardiovascular events in the most at-risk patients. However, further investigations are still required before translating the presented technology to the clinic. The atherosclerotic plaque is characterized by chronic inflammation, which produces prostaglandin E2, among other mediators. The AA-induced model of thrombosis offers the advantage of exploring a model in which inflammation is predominant and is, thus, closer to atherothrombosis than other models. For example, the ferric chloride–induced model is characterized by large areas of endothelial necrosis that are usually not seen in human pathological studies. However, the AA model of thrombosis does not necessarily account for the complex process of thrombosis on spontaneous plaque rupture. Moreover, the expression of vitronectin may result in a source of nonspecific background signal because we expect P975, an RGD analogue, to bind to \( \alpha_{IIb}\beta_{3} \)-integrin. Nevertheless, smooth muscle cells in the intima of potentially vulnerable plaques compose only a small fraction compared with the formed thrombi. Overall, the exploration of P975 in a model of atherothrombosis is of high interest to determine the clinical relevance of the probe. In addition, the translation from high-field imager to clinical imager would result in a loss of spatial resolution and signal to noise ratio, although the relaxation of P975 is significantly higher at these lower field strengths. How these changes would affect the detection of P975 and the identification of microthrombi that may precede ACS/ST-segment elevation myocardial infarction should be investigated. Indeed, this study demonstrated the enhancement of nearly occlusive thrombi; given the much lower spatial resolution of clinical MRI systems, it is unclear whether P975-enhanced MRI would allow the clinical detection of platelet-rich microthrombi in small vessels, such as the coronary. Another limitation of this study is that many of these microthrombi are likely to be of subacute age. Previously, platelet-targeted imaging of subacute thrombi yielded lower signal intensities when compared with acute thrombi.40 Therefore, this might impair the detection of subacute thrombi via an activated platelet-targeted approach, such as P975-enhanced MRI. We will focus future studies on imaging variously aged thrombi in a variety of animal models to further investigate the overall potential of P975.

In conclusion, the detection of activated platelets via noninvasive molecular MRI could not only become an important tool for the clinic but could also provide information on the pathophysiology of thrombosis, embolism, and atherothrombosis. We were able to image AA-induced thrombosis using a contrast agent that specifically binds to the activated \( \alpha_{IIb}\beta_{3} \) expressed on activated platelets. P975 displayed excellent contrast properties and high-resolution and sensitive in vivo imaging of platelet-rich acute thrombi. Thus, P975 represents a novel noninvasive imaging tool for thrombi detection. However, further investigations are still required to demonstrate the overall clinical implications of this study.

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References


Magnetic Resonance Molecular Imaging of Thrombosis in an Arachidonic Acid Mouse Model Using an Activated Platelet Targeted Probe


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Platelet preparation

Freshly drawn whole blood from a normal healthy donor was collected in acid citrate dextrose in plastic vials and immediately fractionated by centrifugation at 25 °C to separate platelet-rich plasma (PRP) (Etablissement Français du Sang, France). The PRP fraction was stored at 22 °C under continuous and slow agitation until the beginning of the experiments. The isolated platelets were counted in 1% acetic acid solution using microscopy and contained no detectable erythrocytes as well as a very low amount of leukocytes. The platelets were then dispersed in Tyrode solution (pH = 7.5, NaCl 137 mM, KCl 2.7 mM, NaHCO3 1.2 mM NaH2PO4 0.36 mM, MgCl2 1.0 mM, CaCl2 2.0 mM, glucose 5.5 mM) containing 0.35% bovine serum albumin, fraction V.

Preparation of FITC-fibrinogen (Fg)

FITC-Fg was synthesized as previously described. Briefly, thawed human Fg (20 mg/mL, Sigma-Aldrich, France) was incubated with celite-fluorescein isothiocyanate (FITC) (1 mg/mL) at room temperature for 15 minutes with intermittent vortexing. The FITC-Fg was separated from unreacted free FITC by passing through a Sephadex G25 column in phosphate-buffered saline.

Model of arachidonic acid-induced thrombosis

As previously described, the right common carotid artery of isoflurane-anesthetized animals was exposed. 4 to 6 μl of AA (Sigma Aldrich, St-Louis) at 200 mg/ml (or ethanol for sham surgery) was applied for 1 minute to the carotid isolated from surrounding tissues by a piece of parafilm. The diameter of the carotid upstream was slightly reduced by a loose permanent ligation. Blood flow was never interrupted.