Flavone-8-Acetic Acid (Flavonoid) Profoundly Reduces Platelet-Dependent Thrombosis and Vasoconstriction After Deep Arterial Injury In Vivo

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Background—Flavone-8-acetic acid (FAA; Flavonoid), an adjuvant antitumor drug, inhibits ristocetin-induced aggregation of human platelets. The effect of FAA on platelet-dependent thrombosis was studied in vivo in the porcine carotid artery after deep arterial injury by balloon angioplasty.

Methods and Results—In-labeled autologous platelet and 125I-labeled porcine fibrinogen deposition, and the incidence of macroscopic mural thrombosis onto deeply injured artery (tunica media) were compared in 20 pigs (40±1 kg [mean±SEM], body surface area=1.0±0.1 m²), randomized to FAA bolus (n=10) of 5.5g/m², followed by an infusion at 0.14g · m⁻² · min⁻¹ or placebo (n=10). Vasoconstriction was measured immediately beyond the dilated segment using quantitative angiography. Platelet deposition (×10⁷/cm² of carotid artery) was reduced over 12-fold in pigs treated with FAA (13±3 versus 164±51, P=0.001) compared with placebo. Fibrinogen deposition (×10¹² molecules/cm² of carotid artery) did not significantly differ in FAA-treated pigs versus placebo (40±8 versus 140±69, P=0.08). Large mural thrombi were present in 100% of placebo-treated pigs versus very small thrombi in 40% of FAA-treated pigs (P=0.005). Vasoconstriction was reduced from 46±6% in the placebo group to 15±3% in the FAA group (P<0.001). Plasma level of FAA before angioplasty was 515±23 μg/mL. The activated partial thromboplastin time was unchanged. The bleeding time was >2SD above the normal mean in 4 of 5 treated pigs (increased from 157±29 to 522±123 s).

Conclusions—FAA markedly reduced platelet deposition, mural thrombi, and injury-induced vasoconstriction after deep arterial injury, suggesting that a major inhibition of platelet glycoprotein Ibα may be beneficial therapy. (Circulation. 2000;101:324-328.)

Key Words: angioplasty ■ platelets ■ platelet aggregation inhibitors ■ thrombus ■ vasoconstriction

Von Willebrand (vWF) factor is necessary for normal platelet adhesion over an area of damaged vessel wall as well as for platelet-platelet interaction (aggregation) under high-shear flow conditions.¹⁻⁹ These interactions involve the platelet membrane glycoprotein (GP) complexes Ib-α and Iib-IIIa, and also fibrinogen, fibronectin, and vitronectin.¹⁻⁹ Flavone-8-acetic acid (FAA; Flavonoid)¹⁰⁻¹¹ is an adjuvant antitumor drug which inhibits implantation of solid tumors in the mouse but also inhibits ristocetin-induced, vWF-dependent platelet aggregation in humans.¹² This may cause a profound reduction in platelet-rich arterial thrombosis after deep arterial injury. In Phase II clinical studies in humans, no clinically significant toxicity was observed. Thus the effect of FAA, at the maximal dose tolerated by humans,¹⁰⁻¹² on platelet-dependent thrombosis was studied in vivo in the deeply injured porcine carotid artery produced by balloon angioplasty as a model of mainly GP Ib inhibition.

Methods

Twenty normal pigs of Babcock 4-way cross stock (a mixture of Landrace, Yorkshire, Hampshire, and Durock breeds), ~4 months old with a mean weight of 40±1 kg (~1 m² body surface area),¹³ were obtained from local farmers. They were randomly assigned to treatment with either placebo (0.9% saline) or FAA (National Cancer Institute), administered as a bolus of 5.5 g/m² followed immediately by an infusion at 0.14g · m⁻² · min⁻¹. Loading dose and maintenance infusion were calculated on the basis of preliminary pharmacokinetic experiments in pigs. Monoexponential declines in plasma concentrations of FAA were fitted to the equation C= Ae⁻ᵃᵗ, where A is the intercept at t=0, and α is the elimination rate constant. A weighting factor of 1/C, where C is the plasma concentration of FAA at time t, was employed.

Drug administration during the balloon dilatation procedure was not blinded, but all subsequent tissue and sample analysis was performed without knowledge of the treatment administered. This
study was approved by the Mayo Clinic Animal Care Committee and conformed to American Heart Association guidelines.

**Experimental Protocol**

The model of deep arterial injury in the porcine carotid artery has been described in detail previously.14–16 Autologous platelets were labeled with 300 μCu of 111In-tropolone and reinjected together with 250 μCu of 125I-labeled porcine fibrinogen on the day before the procedure.15–17 On the day of surgery, the pigs were sedated with 1g intramuscular ketamine (Ketaset, Bristol Laboratories), intubated and mechanically ventilated with room air (Harvard respirator, Harvard Apparatus). Anesthesia was maintained with a continuous infusion of etomidate (Abbott Laboratories, North Chicago) 40 mg/L, fentanyl (Elkins-Sinn, Inc) 10 mg/L, and ketamine 1000 mg/L, at about 5 mL/min. The ECG and intra-arterial pressure were continuously monitored throughout the procedure.

The left femoral vein and artery and the right femoral vein were dissected. A 9F sheath was placed in the left femoral artery, and a 3-cm polyethylene angioplasty catheter (Blue Max, Med-Tech) was advanced under fluoroscopic guidance. An 8-mm×3-cm polyethylene angioplasty catheter (Blue Max, Med-Tech) was advanced under fluoroscopic guidance to the left carotid artery for arterial dilatation. Angioplasty was performed 30 minutes after starting treatment, between the first and third cervical vertebrae, using a standardized procedure (5 inflations of 30 s duration at 7 atm, with 60 s between inflations).14–16 Carotid angiography was performed by injecting 6 mL of ionic contrast material (Renografin 76, Squibb) just before the site of carotid dilatation, using a catheter (8F) with a metal ring of known dimension (Cordis Corporation). Spot films were also taken during balloon dilatation. After the series of 5 inflations, the balloon catheter was withdrawn to the proximal carotid artery and angiography was repeated.

Fifteen minutes after dilatation of the right carotid artery, 120 mL of 0.5% Evans blue in 0.9% saline was injected into the descending aorta to demarcate the extent of arterial injury. Animals then received an overdose of pentobarbital and were euthanized. The proximal descending aorta was immediately cannulated and the carotid arteries perfused with buffered 0.9% saline until eluent from the external jugular veins was clear. The vessels were then perfused with buffered 2% glutaraldehyde for 15 minutes. All perfusions were at physiological pressure. After fixation in situ, the carotid arteries were harvested and cleaned of all adventitia. The dilated portions were divided into 2 equal segments and 2 similar-sized segments were taken proximal and distal to the dilated areas.14–16

**Tissue Analysis**

Platelet and fibrin(ogen) deposition on the arterial segments were quantified by the method of Dewanjee et al.17,19,20 Counting for 111In was performed on the day of surgery and for 125I 2 to 3 weeks later after the 111In had decayed.

The extent of deep arterial injury (defined as a tear through the internal elastic lamina into the arterial media) in the dilated portion of the vessel was assessed histologically as previously described.14–16 Each segment was cut open, pinned, and color photographed. Computer-assisted planimetric measurements of the area of deep injury and the total segment area were then obtained. The presence of macroscopic mural thrombus was assessed using a 2-fold magnifying glass.

**Vasoconstriction**

The severity of localized vasoconstriction was determined immediately distal to the dilatation site from angiograms of the common carotid arteries obtained before and after the dilatation procedure. Computer-assisted planimetry was used to measure the mean maximal narrowing in lumen diameter before and after the procedure, expressed as a percentage of the respective arterial dimension before dilatation.16

**Laboratory Tests**

All blood samples were collected with the 2-syringe technique (0.13 mol/L trisodium citrate as anticoagulant; anticoagulant:blood = 1:10). Samples for platelet count, fibrinogen, hematocrit, aPTT, and FAA concentration were drawn before drug administration, 30 minutes after starting the infusions, and immediately before euthanasia.

Platelet count, hematocrit, aPTT, and fibrinogen were determined using standard laboratory methods. Blood for FAA levels was mixed 9 parts to 1 with 0.13 mol/L trisodium citrate solution, centrifuged to obtain plasma, and stored at −70°C. Assays were performed as a single batch. The method of determination of FAA in plasma was that of L. Malspeis (written communication; 1987). Briefly, plasma (0.25 to 0.50 mL) was diluted to 1 mL with 0.5 mL of 0.5 mol/L sodium acetate (pH 3.0) and normal saline. After addition of diethyl ether (4 mL), tubes were shaken on a mechanical shaker for 15 minutes. Following low speed centrifugation, the ether phase was evaporated to dryness under a stream of nitrogen and the residue dissolved in mobile solvent before high-performance liquid chromatography (HPLC) analysis. Samples were analyzed by reversed-phase HPLC on an IBM C18 (10 μm) column with a mobile solvent of methanol/water (60/40) containing 20 mL glacial acetic acid per liter of the methanol/water mixture. Detection was by UV absorbance at 254 nm. Standard curves were prepared by adding known amounts of FAA to blank plasma and analyzing as described above. Concentrations of FAA were determined by fitting known sample peak areas to equations derived from standard curves.

**Statistical Methods**

Results are presented as mean±SEM. Two dilated segments per artery per animal were analyzed. Because of the variability of platelet and fibrinogen deposition and to use the animal as the unit of study (because all segments in the pig were exposed to the same treatment), analysis was performed on the natural logarithm of these values (per cm² of total area) averaged over all deeply injured segments. Treated and control groups were then compared using the Student’s t test for continuous variables. Pearson’s χ² test was used to test for a difference between groups in the incidence of mural thrombus.

**Results**

**Platelet and Fibrinogen Deposition**

Deep arterial injury occurred in 70% of segments in the dilated region, the remainder had subendothelial injury. Platelet deposition in deeply injured segments in animals treated with FAA was >12-fold lower than those treated with placebo (13±3 versus 164±51×10³/cm², P=0.001). Fibrinogen deposition was similar but slightly less in treated animals (40±8 versus 140±69×10³ molecules/cm², P=0.08; Figure 1).

**Mural Thrombus**

Large macroscopic mural thrombi were present in all pigs treated with placebo. FAA produced a reduction in the incidence and size of thrombus formation. Very small mural thrombi occurred in 40% of treated pigs (P=0.005). There were large thrombi in 85% of the deeply injured segments in the placebo group and very small thrombi in 30% of the treated group.
Vasoconstriction
Vasoconstriction immediately distal to the area of dilatation was significantly greater in the placebo group than in FAA-treated animals (46±6% versus 15±3%, P<0.001; Figures 1 through 3).

FAA Pharmacokinetics
Plasma elimination of FAA in 2 animals administered with an intravenous bolus dose of 1 g/m² was fit to a 1-compartment open model. Plasma half-life and plasma clearance values were 27.9 minutes and 279 mL min⁻¹·m⁻², respectively. The intravenous bolus and continuous infusion doses to maintain a plasma concentration of 500 μg/mL calculated from these values were 5.5 g/m² and 140 mg min⁻¹·m⁻², respectively.

Laboratory Tests
The plasma level of FAA before angioplasty was 515±23 μg/mL; at the end of the procedure, it was 575±36 μg/mL. The aPTT was only slightly increased in the treated animals (1.0 to 1.2 times baseline), but the bleeding time in the 5 animals in which it was measured increased from 157±29 to 522±123 s. In 4 of the animals the bleeding time was prolonged >210 s, (2 SD above the mean laboratory value) after the administration of FAA.

Discussion
This study demonstrates that platelet-dependent thrombus formation following deep arterial injury by balloon dilatation is profoundly reduced by FAA (Flavonoid) which appears to block vWF platelet glycoprotein Ibα-dependent platelet aggregation. This suggests that this mechanism of antithrombotic therapy may be clinically useful. We evaluated a dosage of FAA in the upper therapeutic range in humans as assessed by plasma concentrations.10–12 Platelet deposition and the incidence of mural thrombosis in pigs treated with FAA was significantly lower than those treated with placebo. Fibrinogen deposition was similar and not significantly decreased by FAA compared with placebo.

FAA is an adjuvant antitumor agent that inhibits implantation and causes necrosis of solid tumors in mice by an unknown mechanism.10,11,12 Necrosis of solid tumors by FAA triggers intravascular coagulation21–26 and thus, reduced tumor blood flow. Prolonged treatment causes reduced tumor blood flow, which may lead to hemorrhagic necrosis of these tumors.21–22 These changes were not seen in normal tissue and are thought to be secondary to necrosis in the solid tumors.

Rubin et al found that FAA administered to patients with cancer inhibited ristocetin-induced platelet aggregation (vWF-GP Ibα-dependent aggregation) and prolonged the bleeding time.12 Ex vivo and in vitro platelet aggregation studies with human platelet-rich plasma showed that in the presence of FAA, aggregation induced by adenosine diphosphate (ADP), collagen, arachidonic acid, and adrenaline was not inhibited.12 Plasma ristocetin cofactor activity was unchanged.12

vWF interacts with human platelets through 2 different mechanisms.1,2,5,6 Under high-shear flow conditions, the vessel-wall bound vWF binds to the platelet GPIb-α in the early phases of hemostasis (platelet adhesion), a process independent of ADP and induced by ristocetin. The other interaction, of soluble vWF with platelets involves glycoprotein IIb-IIIa complex exposed on activated platelets (platelet aggregation). This process requires ADP and Ca²⁺ and is not induced by ristocetin, in common with other adhesive proteins like fibrinogen, fibronectin, and probably with thrombospondin. Interactions of the glycoprotein IIb-IIIa complex, a member of a large family of related molecules known as integrins, with adhesive proteins involves the RDG (Arg-Gly-Asp) amino acid recognition sequence, is necessary for

Figure 1. Platelet deposition (left), fibrinogen deposition (middle), and percent vasoconstriction of deeply injured segments (right) in FAA and placebo pigs.

Figure 2. Platelet deposition and vasoconstriction in placebo-treated (n=10) and FAA-treated (n=10) pigs.
platelet-platelet adhesion (platelet aggregation).\textsuperscript{1–8,27} Interaction and binding of proteins to the glycoprotein Ib-\(\alpha\) does not involve the RDG recognition sequence.\textsuperscript{1,2} The antithrombotic mode of action of FAA (Flavonoid) remains unknown, but aggregation studies with ristocetin and prolongation of the bleeding time in humans and in our study suggest that FAA interferes with platelets in the formation of the initial platelet hemostatic plug. This is probably achieved by inhibition of binding of vWF to its binding site on the platelet GP Ib-\(\alpha\).

It was recently discovered that thrombin binds with high affinity to platelet GP Ib-\(\alpha\).\textsuperscript{28,29} Thrombin binding site on GP Ib-\(\alpha\) is distinct from, but in close proximity to, that involved in binding of the adhesive protein vWF.\textsuperscript{28,29} The proposed role of GP Ib-\(\alpha\) in thrombin binding includes acting as a high-affinity receptor for bringing thrombin near the platelet surface.\textsuperscript{30} It was suggested that initiating event in thrombin-induced platelet activation occurs via the GP Ib-\(\alpha\).\textsuperscript{31} FAA binding to vWF site on GP Ib-\(\alpha\), owing to proximity, could partially cover high-affinity binding site for thrombin on GP Ib-\(\alpha\). This could be another plausible explanation for our findings. FAA significantly decreased platelet deposition and macroscopic thrombosis (antiplatelet effect, solid-phase platelet GP Ib-\(\alpha\)), but did not have a significant effect on fibrinogen deposition or prolongation of aPTT (no anticoagulant effect; there was no inhibitory effect on the action of thrombin on the soluble-phase fibrinogen).

During administration of FAA, vasoconstriction just distal to the site of dilatation was significantly reduced. We previously showed that the degree of platelet deposition directly correlates with the degree of arterial vasoconstriction.\textsuperscript{16} Whether the current reduced vasoconstriction relates mainly to platelet deposition or to a direct action of FAA on the vessel wall, the endothelium, increased nitric oxide (which increases guanosine 3',5'-cGMP levels in vascular tissue [similar to other flavonoids]), or to some other mechanism, is unclear.\textsuperscript{16,32–34}

In conclusion, complex mechanisms are involved in the formation of arterial thrombi. At dosages used in clinical practice FAA (Flavonoid) is an effective agent for reducing platelet-dependent thrombosis in vivo over areas of deep arterial injury. There may be a role for interaction of vWF and GP Ib-\(\alpha\) in acute ischemic coronary syndromes.\textsuperscript{35–37} FAA also reduces the vasoconstriction associated with arterial balloon angioplasty probably related to the reduction in platelet deposition.\textsuperscript{16}

Potentially, FAA could be used for short periods (it also has short plasma half-life) during vascular interventions, in combination with other antithrombotics/anticoagulants, for primary prevention of platelet dependent thrombosis in the areas of deep arterial injury.

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