Short-term and Long-term Role of Platelet Activating Factor as a Mediator of In Vivo Platelet Aggregation

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**Background.** Platelet activating factor (PAF) is a phospholipid released upon stimulation by a variety of cells and has been implicated in several pathophysiological events such as asthma and inflammatory diseases. However, although the ability to aggregate platelets in vitro was the first biological activity ascribed to PAF, its role in contributing to the in vivo formation of arterial thrombi has not been thoroughly clarified.

**Methods and Results.** Intravascular platelet aggregation was initiated in two different animal models of arterial stenosis and endothelial injury. An external constrictor was positioned around rabbit carotid arteries and canine coronary arteries. After placement of the constrictor, a typical pattern of flow developed in the stenotic vessels. This pattern of flow, characterized by progressive reductions of carotid or coronary blood flow followed by spontaneous or induced restorations of flow (cyclic flow variations, CFVs), is related to recurrent platelet aggregation at the site of the stenosis followed by dislodgment of the thrombus. After observing CFVs for 30 minutes, BN52021 (up to 1.2 mg/kg), a potent and selective PAF antagonist, was given intravenously to rabbits (n=12) and dogs (n=10). BN52021 completely inhibited CFVs in 10 of 12 rabbits, whereas it was relatively ineffective in abolishing CFVs in dogs (only 2 of 10 animals inhibited). This different effect of BN52021 was not explained by too small a dose of the drug to achieve a complete blockade of PAF receptors in dogs, since ex vivo platelet aggregation was completely inhibited in both rabbits and dogs in response to exogenous PAF at concentrations up to 10^{-5} mol/L. In a second group of 10 dogs, the hypothesis that PAF may become an important mediator of CFVs in dogs only several hours after endothelial injury was tested. After 30 minutes of baseline CFVs, these animals received a bolus of BN52021 up to 1.2 mg/kg. After this treatment, CFVs were completely abolished in 2 of 10 animals. The remaining 8 dogs were followed for an additional 8-hour period, at the end of which a second bolus of BN52021 was given. At this time, BN52021 was effective, as CFVs were abolished in 6 of 8 animals. These effects of BN52021 at 8 hours were not the consequence of a cumulative dose of the compound, since ex vivo platelet aggregation in response to PAF returned to baseline values immediately before administering the second dose. To identify possible sources of PAF other than aggregating platelets at the site of arterial stenosis, dogs in a third group were killed after 30 minutes (n=7) and after 8 hours (n=8) of CFVs. Histological sections of the stenotic coronary artery showed a marked leukocyte infiltration in these arterial segments after 8 hours of CFVs, whereas sections from dogs killed after 30 minutes showed only moderate or no infiltration.

**Conclusions.** These data demonstrate that PAF plays an important role as a mediator of platelet aggregation in vivo in rabbits and dogs. In the canine model, PAF appears to become more important after leukocyte infiltration of the arterial wall, as it may contribute to initiating enough platelet activation to lead to cyclic flow variations at sites of arterial stenosis and endothelial injury. Data from the present study suggest that PAF antagonists may be used as antiplatelet agents. *(Circulation. 1993;88:1205-1214.)*

**Key Words** • platelet activating factor • platelets • thrombosis • leukocytes

An increasing number of studies indicate that interactions between the coronary vessel wall and circulating platelets may be responsible for the sudden conversion from chronic to acute coronary artery disease syndromes, including sudden cardiac death, acute myocardial infarction, and unstable angina. This work was presented in part at the 64th Scientific Sessions of the American Heart Association, Anaheim, Calif, 1991. Correspondence to Dr Golino, Division of Cardiology, 2nd School of Medicine, University of Naples, Via S Pansini 5, 80131 Naples, Italy.
as well as by the finding that antiplatelet therapy reduces the risk of myocardial infarction in patients with this syndrome.4,5

Further support for the role of platelet–vessel wall interactions in acute coronary artery disease syndromes has come from an experimental model of coronary stenosis.6 This model, as originally described,6 involves placement of an external constrictor around a segment of coronary artery where the endothelium had been damaged previously in order to mimic the clinical situation of unstable angina.1 After implantation of the constrictor, the stenosed vessels usually develop cyclic variations of coronary blood flow followed by spontaneous or induced restorations of flow to basal levels. This pattern of flow, called cyclic flow variations (CFVs), is related to recurrent platelet aggregation at the site of the stenosis followed by dislodgment of the thrombus.6,7 Several chemical mediators have been shown to be involved in initiating and/or sustaining CFVs, including thromboxane A2,8 serotonin,9 thrombin,10 and adenosine diphosphate (ADP).11 by either recruiting platelets from the circulation and/or causing coronary vasoconstriction.1

Another potential mediator of CFVs is platelet activating factor (PAF), a phospholipid released upon activation by a number of cells, including endothelial cells, leukocytes, and platelets. This substance has been shown to participate in several inflammatory reactions and is one of the most potent activators of platelets as yet described in vitro.12 However, although the ability to activate platelets in vitro was the first biological activity ascribed to PAF,13 its role in mediating platelet activation and thrombus formation in vivo has not been fully elucidated. Accordingly, the present study was designed to assess whether PAF is a mediator of CFVs acutely in two different animal models, namely, rabbit carotid and canine coronary arteries with experimental stenosis and endothelial injury.

Methods

Short-term Cyclic Flow Variations

To test the hypothesis that PAF is a mediator of CFVs, two different animal models were used in this arm of the study.

Rabbit studies. This model represents a modification14 of the well-described coronary canine model.1 Briefly, 12 New Zealand White rabbits of either sex were anesthetized with a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) given intramuscularly. Anesthesia was maintained during the course of the experiment by an intravenous infusion of ketamine sufficient to abolish the corneal reflex. Through a median incision of the neck, the left or right common carotid artery was exposed and carefully isolated from the surrounding tissue. Polyethylene catheters were placed into a jugular vein and a femoral artery for drug administration and continuous blood pressure monitoring, respectively. Thereafter, a segment of the exposed carotid artery was deendothelialized by gently squeezing the artery between a pair of rubber-covered forceps, and an external plastic constrictor was placed around it. Carotid blood flow velocity was measured continuously by a Doppler flow probe placed proximally to the constrictor (Fig 1).

After instrumentation, all animals developed a typical pattern of blood flow (CFVs) characterized by gradual declines of flow to almost zero values, followed by either spontaneous or induced (by gently shaking the constrictor) restorations of flow (Fig 1). Once CFVs were obtained, they were observed for 30 minutes. During this period, CFV frequency (cycles per hour) and severity (carotid blood flow at its nadir, as a percent of baseline) as well as heart rate and arterial blood pressure were continuously recorded. After having observed CFVs for 30 minutes, an intravenous bolus of BN52021 (a generous gift of Dr Pierre Braquet, Institut Henri Beaufour, Les Plessis Robinson, France), a potent and selective PAF receptor antagonist,15,16 was administered at an initial dose of 0.6 mg/kg, and, to make sure that a maximal degree of PAF receptor blockade was achieved, it was eventually repeated up to 1.2 mg/kg only in those animals in which CFVs were not abolished. The animals in which CFVs were abolished by BN52021 were followed for an additional 30 minutes.

Canine studies. The importance of PAF in mediating CFVs was also tested in the canine model of coronary artery stenosis and endothelial injury. Ten mongrel dogs (weighing 20 to 36 kg) of either sex were anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with room air by a Harvard respirator (South Natick, Mass). Arterial and venous catheters were inserted into a common carotid artery and a jugular vein for measurements of systemic arterial pressure and administration of intravenous fluids or drugs, respectively. The heart was exposed through a thoracotomy at the fifth left intercostal space and suspended in a pericardial cradle. A segment of the left anterior descending coronary artery (LAD) was gently dissected free from the surrounding tissue, and a pulsed-wave Doppler flow probe was placed around the LAD proximal to the area to be constricted. A plastic cylindrical constrictor was placed around the LAD below the Doppler flow probe. The constrictor had a residual lumen diameter that abolished or markedly attenuated reactive hyperemia after 10 seconds of total coronary artery occlusion. Once CFVs were established, they were observed for 30 minutes. During this period, CFV frequency, phasic and mean coronary blood flow velocities, and systemic hemodynamics were continuously recorded. Also in this case, after 30 minutes of baseline CFVs, an intravenous bolus of BN52021 was administered at an initial dose of 0.6 mg/kg, up to 1.2 mg/kg only in those animals in which the compound was ineffective in abolishing CFVs. The animals in which CFVs were abolished by BN52021 were followed for an additional 30 minutes.

Ex vivo platelet aggregation. To assess whether BN52021 resulted in complete blockade of PAF receptors on platelets, aggregation in response to PAF was evaluated ex vivo under baseline conditions and after administration of the last bolus of BN52021. Additionally, to determine that BN52021 is a selective PAF receptor antagonist, platelet aggregation was also tested in response to ADP, serotonin, and the thromboxane A2 mimetic U46619. These experiments were performed in all rabbits and dogs included in this arm of the study.

Fourteen milliliters of blood was drawn in a syringe containing 1.5 mL of 3.8% sodium citrate. Blood was centrifuged at 120g for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). PRP was removed and centrifuged at 1000g for 5 minutes to obtain platelet-poor plasma (PPP). Platelet aggregation was measured turbidimetrically on a Chronolog aggregometer.
Blood Flow Velocity

FIG 1. Schematic representation of the experimental preparations of intravascular platelet aggregation used in rabbits and in dogs. Either a carotid artery (in rabbits, left) or the left anterior descending coronary artery (in dogs, right) was exposed surgically under general anesthesia. Endothelial injury was produced by gently squeezing the artery between a pair of rubber-covered forceps. A plastic constrictor was then placed around the deendothelialized segment to create a stenosis of about 75% of the luminal diameter. Carotid or coronary blood flow velocity was measured continuously by a Doppler flow probe placed proximally to the constrictor. In both these models, cyclic flow variations (CFVs), characterized by gradual decreases in blood flow to almost zero values followed by restorations of flow to normal values, usually develop within few minutes after placement of the constrictor (bottom). These CFVs are caused by recurrent episodes of platelet aggregation and dislodgment of the thrombus at the site of the stenosis.

and recorded on a linear recorder. The aggregometer was calibrated with the use of PPP and the test was performed on 250 μL of PRP in a siliconized cuvette with continuous stirring. The platelet count in the PRP was adjusted to 300,000/μL by dilution with PPP as needed. Aggregation was induced in PRP in response to various concentrations of PAF, ADP, serotonin, and U46619. Since dog platelets do not usually respond to U46619 and serotonin alone, these agonists were tested (for the dog studies only) after platelets had been primed with subthreshold concentrations of epinephrine (10 μmol/L), according to experiments previously reported.8,9

Long-term Cyclic Flow Variations

This set of experiments was performed to determine whether PAF may become an important mediator of CFVs several hours after endothelial injury. Ten additional dogs were included in this arm of the study. CFVs were initiated in canine coronary arteries as previously described. Thirty minutes later, an intravenous bolus of BN52021 (0.6 mg/kg) was given and eventually was repeated up to 1.2 mg/kg, as previously described. Thereafter, the animals in which CFVs were abolished were followed for an additional 8 hours. After this time period, a second bolus of BN52021 was given intravenously, up to 1.2 mg/kg, and its effects on CFVs were recorded. During the whole experiment, CFV frequency and severity, heart rates, and arterial blood pressures were recorded continuously.

Ex vivo platelet aggregation. To ascertain whether the second administration of BN52021 was not additive with the first one, ie, that its effects had already disappeared at the time of the second administration (8 hours later), platelet aggregation in response to PAF was evaluated ex vivo under baseline conditions (no drug), after the first administration of BN52021 (30 minutes), and immediately before and after the last administration of the drug (8 hours). Platelets were collected, isolated, and aggregated ex vivo in response to PAF, ADP, serotonin, and U46619 as described above.

In Vitro PAF Production by Rabbit and Canine Platelets

Rabbit platelets synthesize and release significant amounts of PAF when stimulated by different agonists.17-19 In order to determine whether canine platelets also possess the ability of synthesizing this chemical mediator, PAF production in vitro was measured in canine washed platelets stimulated with thrombin and
was compared with the amount produced by rabbit platelets under the same conditions.

**Preparation and stimulation of platelets.** Rabbit and canine blood samples were collected in 3.8% sodium citrate (1:9 vol/vol) as described above. PRP was obtained by centrifuging whole blood at 120g for 20 minutes. PRP was removed and spun again at 1000g for 5 minutes, and PPP was discarded. The platelet pellet was suspended in a modified Tyrode's-bovine serum albumin (BSA) buffer with no calcium (KCl 195 mg; MgCl·6H₂O 212.5 mg; NaCl 8 g; NaHCO₃ 1.015 g; glucose 1 g; EGTA 76.07 mg; BSA 2.5 g; vol to 1 L; pH adjusted to 6.5 with 1N HCl). The platelets were washed twice with this buffer and then incubated with 2 mmol/L phenyl methyl sulfonyl fluoride (PMSF) at 37°C for 15 minutes to inhibit acetylhydrodrolase, the enzyme responsible for PAF catabolism.¹⁹ Platelets were then washed again twice and finally suspended in a Tyrode's-BSA buffer supplemented with calcium (KCl 195 mg; MgCl·6H₂O 212.5 mg; NaCl 8 g; CaCl₂·2H₂O 191 mg; Tris (hydroxymethyl)-aminomethane 1.21 g; glucose 1 g; BSA 2.5 g; vol to 1 L; pH adjusted to 7.4 with 1N HCl) to a final concentration of 300,000 cells/μL. Thereafter, 0.5−mL aliquots were stirred in the aggregometer and challenged with thrombin (1 U/mL final concentration). After 10 minutes, the reaction was terminated by the addition of 1.5 mL of chloroform/methanol (1:2 vol/vol).

**Extraction and bioassy of PAF.** Lipids were extracted by the method of Bligh and Dyer²⁰ and stored at −20°C. The organic phase was removed and evaporated to dryness under a continuous stream of nitrogen. Lipid extracts were redissolved in 50 μL of chloroform/methanol (1:1 vol/vol) and resolved by thin-layer chromatography on a silica gel G plate developed in chloroform/methanol/acetic acid/water (50:25:8:3 vol/vol). The area corresponding to PAF was identified by comigration with an authentic standard run in parallel and was scraped off and dissolved in 0.5 mL methanol/water (3:1 vol/vol). The solvent was then evaporated to dryness, and the samples were reconstituted in 100 μL of 0.9% NaCl supplemented with 0.25% BSA. PAF in the samples was detected by using washed rabbit platelets as a bioassay in the presence of aspirin (0.1 mmol/L) and phosphocreatine/creatine kinase (0.7 mmol/L to 13.9 U/mL) to inhibit arachidonic acid−dependent and ADP−dependent activation pathways, respectively. Under these conditions, washed rabbit platelets failed to aggregate in response to arachidonic acid (2 mmol/L) or ADP (100 μmol/L). The amount of PAF produced by rabbit and canine platelets was determined by comparing the extent of aggregation induced by the samples with that induced by known amounts of synthetic, exogenously added PAF.

**Histological Studies**

Circulating leukocytes are also capable of synthesizing significant amounts of PAF upon activation. Since in previous experiments we had noticed that a marked neutrophil infiltration occurs at the site of coronary stenosis and endothelial injury, we attempted to correlate the responsiveness of canine CFVs to BNS52021 with infiltration of leukocytes into the coronary arterial wall. Histological sections were obtained from the damaged LADs and the unmanipulated circumflex coronary arteries of 16 additional dogs that were killed 30 minutes (n=7) after and after 8 hours (n=9) after CFVs had been initiated. Dogs were given an overdose of sodium pentobarbital immediately after coronary flow was restored, i.e., when flow reached its maximum. The hearts were promptly excised and perfused through the aortas with 200 mL 10% buffered formalin at a constant pressure of 90 mm Hg. Segments of the coronary arteries were embedded in methacylate, and sections were obtained and stained with hematoxylin and eosin. Leukocyte infiltration was semiquantitatively evaluated by means of a score from 0 to +++; as follows: 0 was defined as no leukocyte attached to or infiltrating the arterial wall; + was defined as few leukocytes attached to the arterial wall; 2 to 3+ was defined as larger number of leukocytes attached to and infiltrating the arterial wall; and 4+ was defined as many leukocytes within the arterial wall. The extent of platelet accumulation and vessel wall injury was noted, including the extent of endothelial denudation. Histological examinations were performed without knowledge of the treatment groups.

**Statistical Analyses**

All values are expressed as mean±SEM. CFV frequency and severity, as well as hemodynamic variables, were compared within groups by a one-way ANOVA with a design for repeated measurements followed, when an F value was found to be significant, by a Student's t test for paired observations with the Bonferroni correction for comparison of different treatments. A two-way ANOVA with a design for repeated measurements was used to compare in vitro platelet aggregation data. When applicable, differences among groups were tested by a Student's t test for unpaired samples with the Bonferroni correction. A value of P<.05 defined significant differences between populations.

**Results**

**Short-term Cyclic Flow Variations**

**Rabbit studies.** Twelve rabbits were included in this arm of the study. After positioning the constrictor around the carotid artery, CFVs developed in all animals with a mean frequency of 18±3 cycles per hour and a severity, expressed as nadir carotid blood flow, of 5±1% of baseline values (Fig 2). After 30 minutes of CFVs, administration of BNS52021, a selective PAF receptor antagonist, resulted in a complete inhibition of CFVs (0 cycles per hour) in 10 of 12 animals. In these animals, carotid blood flow averaged 103±4% of baseline values (P=NS vs baseline, Fig 2). Rabbits were then followed for an additional 30 minutes after CFVs were abolished. During this period, CFVs returned in none of the animals. Heart rate and arterial blood pressure did not change throughout the study in these animals (Table).

**Dog studies.** Ten dogs were instrumented as described. CFVs were observed for 30 minutes in all animals. CFV frequency and nadir coronary blood flow averaged 10±1 cycles per hour and 4±2% of baseline values, respectively. However, BNS52021 at the same dose used in rabbits was ineffective in abolishing CFVs, as they were completely eliminated in only 2 of 10 animals, whereas in the remaining 8 dogs they were unchanged (Fig 3). In these same 8 animals, the severity of CFVs was also unaltered after administration of BNS52021 (Fig 3). Also in these animals, BNS52021 did
not cause significant changes in heart rate and blood pressure (Table).

These data indicate that PAF is not an important mediator of CFVs in this canine model at 30 minutes after endothelial injury and placement of the constrictor. Alternatively, dogs might be less responsive to BN52021 than rabbits.

Ex vivo platelet aggregation. Platelet aggregation was measured before and after administration of BN52021 in response to PAF, ADP, serotonin, and the thromboxane A2 analogue U46619 both in rabbits and dogs. In rabbits, according to the in vivo effects of BN52021 on CFVs, this compound inhibited PAF-induced aggregation, whereas it did not affect aggregation induced by the other agonists (Fig 4). These data confirm that BN52021 is a selective PAF antagonist. In dogs, a dissociation between the effects on CFVs and inhibition of ex vivo PAF-induced platelet aggregation was observed. Although CFVs were abolished in only 2 of 10 dogs in vivo, a marked inhibition of PAF-induced aggregation was observed in vitro after administration of BN52021 (Fig 5), thus excluding the possibility that the lack of effect on CFVs in these animals could be the consequence of a dose too low to achieve a sufficient degree of PAF receptor blockade. Also in dogs, BN52021 appeared to be selective for PAF receptors, as platelet aggregation in response to ADP, serotonin, and U46619 was not affected (Fig 5).

**Hemodynamic Variables in Rabbits and Dogs With Cyclic Flow Variations Receiving BN52021**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>CFVs</th>
<th>BN (I)</th>
<th>BN (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbits Mean arterial pressure</td>
<td>65±5</td>
<td>68±6</td>
<td>70±6</td>
<td>68±5</td>
</tr>
<tr>
<td>Heart rate</td>
<td>133±8</td>
<td>129±6</td>
<td>133±6</td>
<td>127±7</td>
</tr>
<tr>
<td>Dogs Mean arterial pressure</td>
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<td>110±6</td>
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<td>112±5</td>
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<tr>
<td>Heart rate</td>
<td>134±8</td>
<td>132±6</td>
<td>129±8</td>
<td>134±7</td>
</tr>
</tbody>
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CFVs indicates cyclic flow variations; BN (I), immediately after administration of BN52021; and BN (II), 30 minutes after administration of BN52021.

**FIG 2.** Graphs show effects of BN52021, a selective platelet activating factor receptor antagonist, on cyclic flow variations (CFVs) obtained in stenotic endothelially injured rabbit carotid arteries. Panel A shows that administration of BN52021 completely eliminated CFVs in 10 of 12 rabbits. Panel B shows that carotid blood flow velocity (as a percent of baseline values), an index of CFV severity, approached zero at the nadir of CFVs before administration of BN52021 and was significantly increased after administration of the compound.

**FIG 3.** Graphs show effects of platelet activating factor receptor antagonism achieved with BN52021 on cyclic flow variations (CFVs) in stenotic endothelially injured canine coronary arteries. Panel A shows that in contrast with rabbit CFVs, BN52021 eliminated CFVs in only 2 of 10 dogs with 30 minutes of CFVs, whereas in the remaining 8 animals they were unchanged. Panel B shows that as a result, coronary blood flow velocity did not increase significantly after administration of the compound with respect to baseline.

**Long-term Cyclic Flow Reductions**

Ten dogs were included in this group to test the hypothesis that PAF may become more important as a mediator of CFVs over time in dogs with coronary artery stenosis and endothelial injury. Accordingly, CFVs were initiated in 10 additional dogs, and they were observed for 30 minutes. At the end of this 30-minute period, CFV frequency and coronary blood flow averaged 12±3 cycles per hour and 7±3% of baseline values, respectively. Also in these animals, BN52021 appeared to be relatively ineffective in abolishing CFVs, as they were completely abolished in only
2 of 10 animals after 30 minutes of CFVS (Fig 6). The remaining 8 dogs in which CFVs were not abolished by BN52021 were followed for 8 additional hours. CFV frequency and severity remained relatively constant throughout the experimental period, 14±3 cycles per hour and 6±2%, respectively, at 8 hours (P=NS vs corresponding values measured at 30 minutes). Interestingly, administration of BNS2021 to the animals that did not respond initially to the compound resulted in elimination of CFVs in 6 of 8 animals (P<.05 vs 30 minutes by Fisher's exact test, Fig 6). These 6 dogs were followed for additional 30 minutes. None of them showed CFVs during this time period.

Ex vivo platelet aggregation. Platelet aggregation in response to PAF was evaluated in these animals at baseline, after the first administration of BN52021, and at 8 hours before and after the second administration of the drug. As previously described, BN52021 completely inhibited PAF-induced aggregation with respect to baseline (Fig 7). After 8 hours of CFVs, PAF-induced aggregation was no longer inhibited, thus demonstrating that at the time of administering the second dose of BN52021, the first dose was already metabolized. Again, the second dose of BN52021 resulted in a comparable inhibition of PAF-induced aggregation with respect to the first one (Fig 7).

In Vitro PAF Production by Rabbit and Canine Platelets

The bioassay method used in the present study to quantify the amounts of PAF produced by activated platelets is widely used, and it has been shown to be highly sensitive even to trace quantities of PAF. Under our experimental conditions, washed rabbit platelets were sensitive to exogenously added PAF up to a concentration of 2.5×10⁻¹¹ M (data not shown).

Previous studies by other groups of investigators have shown that rabbit platelets stimulated with thrombin synthetize significant amounts of PAF. Consistent with those studies, we have also observed a significant production of PAF by washed rabbit platelets incubated with PMSF, an inhibitor of acetylhydrolase, that averaged 4.9±1.2 pmol/mL at 10 minutes after the addition of thrombin. In contrast, canine platelets under the same conditions failed to synthetize PAF in amounts detectable with the bioassay used. Since the sensitivity of the method under our experimental conditions was 2.5×10⁻¹¹ M, it can be assumed that canine platelets do not synthetize PAF in quantities biologically relevant.

Histological Studies

Sixteen dogs were included in this section of the study. Seven animals were killed after 30 minutes of CFVs and the remaining 9 were killed after 8 hours of CFVs. Both groups of animals were killed when flow reached its maximum, ie, immediately after restoring it. Leukocyte infiltration, evaluated semiquantitatively on histological sections, was markedly increased in sections obtained from those animals killed at 8 hours as compared with dogs in which CFVs lasted for only 30
Platelet Activating Factor and Cyclic Flow Variations

FIG 5. Graphs show platelet aggregation ex vivo in response to platelet activating factor (PAF), serotonin, the thromboxane mimetic U46619, and adenosine diphosphate (ADP) in dogs before (baseline) and after administration of BN52021, a selective PAF receptor antagonist. Similar to the data obtained in rabbits, this compound prevented PAF-induced platelet aggregation, whereas the response to several other agonists was unchanged. Platelet aggregation in response to serotonin and U46619 was measured after priming platelets with subthreshold concentrations of epinephrine (10 μmol/L), since canine platelets are relatively unresponsive to these agonists.

minutes (Fig 8). No leukocyte infiltration was seen in the arterial segments obtained from the unmanipulated circumflex coronary arteries (data not shown).

Discussion

The data from the present study demonstrate that PAF may play an important role in mediating in vivo platelet aggregation at sites of arterial stenosis and

FIG 6. Graph shows differential effects of BN52021, a platelet activating factor receptor antagonist, on 30-minute and 8-hour cyclic flow variations (CFVs) obtained in stenotic endothelially injured canine coronary arteries. Similar to the first group of dogs, administration of BN52021 after 30 minutes of CFVs eliminated them in only few animals (2 of 10), whereas in the remaining 8 animals, CFV frequency did not change significantly. CFV frequency remained stable for 8 hours in these studies. Administration of BN52021 at 8 hours was effective in eliminating CFVs, as they were abolished in most animals (6 of 8).

FIG 7. Graph shows platelet aggregation ex vivo in response to platelet activating factor (PAF) in dogs subjected to 8 hours of cyclic flow variations (CFVs) measured at baseline, after administration of BN52021 at 30 minutes, at 8 hours, and at 8 hours after administration of BN52021. PAF-induced aggregation was completely blocked at concentrations up to 10^{-5} mol/L both at 30 minutes and at 8 hours after administration of the drug. Note that at 8 hours before administering the drug, platelet aggregation had returned to baseline values.
endothelial injury. In addition, our study also suggests that the influence of PAF on in vivo platelet aggregation seems more pronounced with the passage of time in the canine model. Thus, the importance of selected chemical mediators responsible for initiating and/or sustaining CFVs may change over time with different mediators exerting important effects at different times after development of CFVs.

Animal Models of Intravascular Platelet Aggregation

In 1976, a canine model of intracoronary platelet aggregation was described.6 This model involves placement of an external constrictor around a segment of a coronary artery with focal endothelial injury. After placement of the constrictor, the stenosed vessels develop cyclic changes in coronary blood flow characterized by progressive reductions in flow followed by sudden returns of flow to basal levels (CFVs). Histological studies21 and studies with radiolabeled platelets have convincingly demonstrated that CFVs are due to the recurrent formation of a platelet-rich thrombus followed by its dislodgment at the site of the stenosis. It has been shown that thromboxane A2 and serotonin are important mediators in initiating and/or sustaining CFVs, as interventions that interfere with these mediators usually abolish CFVs in the majority of the animals.6-9,22 Recently, it has been shown that other mediators, including thrombin10 and ADP,11 are also important in mediating CFVs in the canine model.

A similar model of intravascular platelet aggregation in rabbits has been described recently by our group.14 This model requires placement of a constrictor around deendothelialized segments of carotid arteries, and, similar to the data obtained in the canine coronary artery, we have also observed that thromboxane A2 and serotonin are important mediators in initiating and/or sustaining CFVs in stenotic rabbit carotid arteries with endothelial injury.14

One aspect of the present study deserves a particular comment. In 2 of 12 rabbits, CFVs were not abolished after administration of BN52021. This finding was not totally unexpected, as it has been reported previously in other studies using drugs that interfere with other chemical mediators. For example, thromboxane A2 synthesis inhibitors or receptor antagonists usually abolish CFVs in 70% to 80% of the animals, whereas they continue unchanged in the remaining animals.6-8,21,22

This occurs also when serotonin receptor antagonists,6,22 thrombin inhibitors,10 and interventions that remove the endogenous ADP released from activated platelets11 are used. Also in the present study, the possibility of too low a dose of BN52021 to completely block PAF receptors can be excluded, since PAF-induced platelet aggregation was completely inhibited also in those rabbits in which CFVs were not abolished. Thus, it is likely that, in those animals that do not respond to a single intervention, platelet aggregation can be sustained by the other mediators that are left free to exert their effects. This hypothesis is consistent with the observation that those animals that initially do not respond to a given intervention usually respond to addition of a second one in virtually all cases.22

More intriguing was the finding that in dogs, in the short-term studies, a small number of animals did respond to BN52021, whereas the large majority did not. Also in this case, the dose of BN52021 administered was appropriate, since PAF-induced aggregation was similarly inhibited in the responders and in the nonresponders. One possible explanation is that, since some dogs at 30 minutes already showed a significant infiltration of leukocytes in the arterial wall at the site of the damage, these leukocytes could have released PAF that contributed to platelet aggregation (see below). However, it should be outlined that the histological studies were performed in a separate group of dogs and, therefore, this hypothesis remains largely speculative.

PAF and Cyclic Flow Variations

PAF was originally recognized in experiments designed to characterize the cell-cell interaction after antigen stimulation of rabbit leukocytes containing IgE-sensitized basophils.13 During this immunologically initiated reaction, a soluble mediator was released, and it was characterized by its ability to induce the aggregation of, and initiate the release reaction from, isolated rabbit platelets. Because its chemical nature was not known, the term “platelet activating factor” was coined to describe its functional activity.13 Later, its physicochemical characteristics, including its phospholipid nature were discovered, and it is now accepted that PAF is 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine.23

Despite the fact that the first biological activity of PAF evidenced was its ability to activate platelets in vitro, the possible involvement of PAF in mediating in vivo platelet activation and thrombus formation has not received great attention. Apprill et al24 demonstrated that intracoronary infusions of PAF could restore CFVs that had been previously abolished by thromboxane A2 or serotonin receptor antagonists,24 thus suggesting a possible role for PAF in mediating CFVs. The study by Apprill et al, however, showed that exogenously infused PAF may restore CFVs but did not provide any information related to the possible involvement of endogenously synthesized PAF. A role for endogenous PAF in mediating CFVs has been suggested in a recent study by Torr et al25 in which oral administration of triazolam, a novel triazolobenzodiazepine with PAF antagonistic properties, abolished CFVs in dogs with coronary stenosis and endothelial injury.

Our study confirms and extends these previous observations by indicating that the role of PAF in mediating in vivo platelet aggregation is complex and not entirely
elucidated as yet. Indeed, we found that BN52021, a potent and selective PAF antagonist isolated from the leaves of the Ginkgo Biloba tree\textsuperscript{15,16} was very effective in abolishing CFVs at 30 minutes in rabbit carotid arteries with experimental stenosis and endothelial injury. On the other hand, BN52021, at the same dose used in rabbits, was relatively ineffective in abolishing CFVs in dogs at 30 minutes, whereas a second administration of the same dose of this compound after 8 hours of continuous CFVs resulted in elimination of CFVs in most animals. Since the possibility that administration of BN52021 at 8 hours might have resulted in a cumulative effect can be reasonably excluded on the ground of ex vivo platelet aggregation data, other reasons should account for this finding.

**Cellular Sources of PAF**

That in vivo platelet aggregation in dogs is relatively unresponsive to PAF receptor antagonists at 30 minutes is not an entirely surprising finding. In fact, PAF is a potent platelet agonist in most animal species, with the most notable exception being rat platelets, which lack PAF receptors.\textsuperscript{26} However, with respect to our study, the observation that canine platelets aggregate normally in vitro in response to exogenously added PAF can exclude the possibility that canine platelets also do not possess PAF receptors. Alternatively, the lack of responsiveness of canine CFVs to BN52021 at 30 minutes may be explained by a lack of significant release of PAF from canine platelets. Indeed, while it is well established that both rabbit and human platelets produce significant amounts of PAF when challenged in vitro with a variety of agonists such as collagen, thrombin, and the calcium ionophore A23187,\textsuperscript{17,18} no data are available as to the production of PAF by canine platelets. For instance, although PAF is synthesized by a number of cells including neutrophils, basophils, macrophages, mast cells, endothelial cells, and platelets, important differences have been noted in the same cell types isolated from different species.\textsuperscript{12} For example, rabbit basophils are a major source of PAF,\textsuperscript{13} whereas human basophils produce only little or no PAF.\textsuperscript{27} Similarly, human lung mast cells\textsuperscript{28} but not rat mast cells\textsuperscript{29} are able to synthesize PAF. Furthermore, not all of the PAF that is synthesized by a given cell is subsequently released. A typical example is human endothelial cells\textsuperscript{30} that release little if any PAF despite the fact that they are capable of synthesizing it. To test the hypothesis that canine platelets do not synthesize biologically relevant quantities of PAF, we have stimulated canine platelets with thrombin, an agonist known to cause PAF synthesis from rabbit platelets. Furthermore, canine platelets were preincubated with PMSF, a substance that inhibits acetylhydrolase, the enzyme responsible for PAF degradation.\textsuperscript{39} Under these conditions, canine platelets failed to release detectable amounts of PAF, whereas rabbit platelets did produce an average of 4.9 pmol/mL of PAF. Since the bioassay used in our experiments is among the most sensitive techniques to detect PAF (the sensitivity under our experimental conditions was $2.5 \times 10^{-11}$ M), it can be affirmed that canine platelets are unable to produce biologically relevant quantities of PAF. This is compatible with and may explain the finding that CFVs in dogs were not abolished by BN52021 when administered at 30 minutes, whereas the same intervention was effective in rabbits at the same time period.

A novel observation of our study is that CFVs in the dog became responsive to PAF receptor antagonism after 8 hours. This suggests that the chemical mediators responsible for the occurrence of CFVs may change over time and that some of these substances (such as PAF) may become more important as the process of arterial damage/platelet activation/inflammation proceeds. However, if canine platelets are unable of synthesizing PAF at 30 minutes, logic holds that these cells probably do not acquire this ability after only few hours. As a consequence, other cellular sources of PAF should be involved in the generation of PAF during late CFVs.

Endothelial cells are possible candidates, as they are capable of synthesizing biologically relevant quantities of PAF upon adequate stimulation.\textsuperscript{30} However, the fact that the animal models used in the present study are associated with a deep vascular injury with a complete disruption of the endothelial lining would argue against this hypothesis.

Activated leukocytes are other possible candidates. Indeed, these cells are able to produce large amounts of PAF that can be released into the medium.\textsuperscript{31} Furthermore, while on one hand it has been reported that canine neutrophils possess acetyltransferase, the enzyme responsible for the synthesis of PAF,\textsuperscript{32} on the other, it is well known that platelets and leukocytes may interact with each other and influence one another’s activity.\textsuperscript{33,34} For instance, degradation of basophils sensitized by IgE antibodies triggers activation of platelets, which leads to immune complex deposition in acute serum sickness in rabbits, a disease that involves renal and peripheral arteries.\textsuperscript{33} The interactions of leukocytes with platelets in various experimental preparations and in human pathological states are not well understood, but it has been shown that activated neutrophils release a variety of substances, among which thromboxane A\textsubscript{2} and PAF are potent platelet agonists. Thus, it is conceivable that leukocytes might progressively accumulate over time at the site of the arterial damage and that, once activated, they release several chemical mediators, including PAF, the concentration of which may become locally quite high. This leukocyte-derived PAF may in turn contribute to the late sustaining of CFVs. This hypothesis is supported by the observation in our study that the marked leukocyte infiltration at the damaged coronary arterial site observed at 8 hours is accompanied by the responsiveness of CFVs to PAF receptor antagonism. Indeed, a maximal degree of leukocyte infiltration was observed in histological sections obtained from animals killed after 8 hours of CFVs, whereas those obtained from dogs killed after 30 minutes of CFVs showed only little or no infiltration.

**Conclusions**

The present study demonstrates that PAF plays an important albeit complex role in mediating the in vivo platelet activation in canine models with endothelial injury and coronary artery stenosis sufficient to lead to CFVs and suggests for the first time that leukocytes may contribute to the formation of arterial thrombi by releasing one or more factors, such as PAF at the site of arterial stenosis and endothelial injury. Since human platelets produce PAF,\textsuperscript{17,18} it can be speculated that PAF is a short-term mediator of in vivo platelet aggre-
gation in humans similar to that observed in rabbits. In addition, because PAF is formed by and acts on both platelets and leukocytes, this substance may represent one of the critical links in cell to cell interactions, thus providing the cellular basis for potently amplifying mechanisms of injury. The present study also outlines the usefulness of PAF receptor antagonists as possible antithrombotic interventions. Whether these drugs will be effective in reducing the inflammatory component associated with the formation of arterial thrombi is not known, but this should be evaluated in future studies.

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