Effects of a Novel Platelet Nitric Oxide Donor (LA816), Aspirin, Clopidogrel, and Combined Therapy in Inhibiting Flow- and Lesion-Dependent Thrombosis in the Porcine Ex Vivo Model

Gemma Vilahur, MS; E. Segalés, MS; E. Salas, MD, PhD; L. Badimon, PhD, FESC

Background—Acetylsalicylic acid (ASA), or aspirin, plus clopidogrel is becoming the standard antithrombotic treatment in people with coronary disease. Novel approaches such as the use of platelet-selective nitric oxide (NO) donors may provide additional protection against thrombosis. We evaluated the antithrombotic properties of a novel platelet-selective NO donor (LA816) administered alone and in combination with ASA, clopidogrel, or ASA+clopidogrel.

Methods and Results—Thrombogenicity was measured in the porcine experimental model and assessed as platelet-thrombus formation in the ex vivo Badimon perfusion chamber. Pigs were randomly divided into 4 groups: (1) placebo control, (2) clopidogrel, (3) ASA, and (4) ASA+clopidogrel (ASA and clopidogrel were given orally, 10 mg · kg⁻¹ · d⁻¹ for 3 d). The animals were anesthetized, heparinized, and catheterized, and the Badimon perfusion chamber was placed in an extracorporeal shunt. After baseline perfusions, all animal groups received the intravenous infusion of LA816 for 2 hours. Platelet aggregation, blood pressure, and heart rate also were evaluated during the experiments. LA816, clopidogrel, and ASA+clopidogrel produced a reduction of ∼45% on thrombus mass versus placebo control perfusions (P<0.05). Combined treatment of oral ASA+clopidogrel and intravenous LA816 produced a significant further reduction of 25% in platelet deposition (70% from placebo controls; P<0.0001). LA816 intravenous treatment did not modify blood pressure or heart rate.

Conclusions—Acute NO donation with LA816, without modifying hemodynamic parameters, provides the same inhibitory effect as that obtained with chronic treatment with clopidogrel+ASA. Moreover, LA816 provides platelet inhibitory effects in addition to those of the combined blockade of cyclooxygenase and P2y(12) receptor. (Circulation. 2004;110:1686-1693.)

Key Words: platelets ■ thrombosis ■ nitric oxide

Thrombosis plays an important role in the pathogenesis of acute coronary syndromes. Rupture of the atherosclerotic plaque is the initiating event, which occurs spontaneously or is induced by percutaneous coronary intervention (PCI).¹ Vessel wall injury leads to the adherence of platelets and subsequent platelet activation. Once activated, platelets further stimulate thrombus formation and recruit additional platelets by releasing thromboxane A₂ (TXA₂), adenosine diphosphate (ADP), and cytokines, which produce and promote surface thrombin generation and release vasoconstrictor substances.² The effect of all of these platelet-active substances is magnified under certain pathological conditions (eg, coronary artery disease, diabetes, hyperlipidemia), which are characterized by endothelial dysfunction leading to the reduced synthesis of vasodilatory and antiaggregant factors (ie, nitric oxide [NO] and prostacyclin). Therefore, the blockade of platelet aggregation in addition to antiischemic properties may exert beneficial effects on coronary vascular function. Among the antiplatelet drugs, acetylsalicylic acid (ASA), or aspirin, remains the standard therapy with a clear clinical benefit.³ ASA irreversibly acetylates platelet cyclooxygenase (COX) and thereby blocks the formation of TXA₂, a potent vasoconstrictor and platelet aggregant⁴; however, despite its use, recurrent events remain high.² Therefore, considerable recent progress has been made in the development of new strategies involving drugs acting on ADP receptors, fibrinogen receptors (the GPIIb/IIIa complex), specific thrombin inhibitors, and NO donors.⁵ Clopidogrel, a thienopyridine, covalently binds to the P2y(12)⁶ platelet-ADP receptor and inhibits ADP-induced platelet aggregation. The results of the Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial⁷ showed a modest difference in effectiveness between ASA and clopidogrel (a relative risk [RR] reduction of 8.7%; P=0.043). This difference suggests that the clinical efficacy of the 2 drugs is comparable. Furthermore, recently the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) trial⁸ investigators concluded that the combination of ASA and clopidogrel

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From the Cardiovascular Research Center, Institut Català de Ciències Cardiovasculars-Consejo Superior de Investigaciones Científicas, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain (G.V., E. Segalés, L.B.); and Lacer, SA, Barcelona (E. Salas).
Correspondence to Prof L. Badimon, Cardiovascular Research Center, C/Jordi Girona 18-26, 08034 Barcelona, Spain. E-mail lbmucv@cid.csic.es © 2004 American Heart Association, Inc.
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dramatically reduces platelet activation as compared with ASA alone.

Although NO donors are not specifically antiplatelet agents, they do possess antiplatelet and vasodilator properties. The antiplatelet effects of organic nitrates remain controversial because of the suprapharmacological doses required to inhibit platelet aggregation and the subsequent hypotensive side effects of the available formulations. Contrarily, S-nitrosothiols (ie, S-nitrosoglutathione [GSNO]) possess significant antiplatelet actions at doses that cause fewer hemodynamic effects. It has been reported that GSNO releases NO by the action of enzymes associated with platelet membranes; therefore, GSNO seems to exhibit platelet selectivity and to inhibit platelet adhesion and aggregation to a greater extent than its effects on vascular tone.

In patients with acute coronary syndrome, endothelial- and platelet-derived NO are impaired probably because of a decreased synthesis of S-nitrosothiols. Considering the antithrombotic activities of GSNO and the effectiveness of antiplatelet therapy in preventing serious vascular events, inhibiting platelet activity in patients undergoing PCI, and inhibiting vasospasm in coronary arteries, a new S-nitrosothiol with antithrombotic activity has been synthesized by Lacer, SA. Such encouraging progress in discovering new and improved drugs for antiplatelet therapy also incites efforts to test new associations between antithrombotic drugs.

The present study was designed to (1) assess the antithrombotic/antiplatelet efficacy of a new platelet-selective NO donor (LA816, a nonnatural amino acid that is S-nitrosylated); (2) compare its antithrombotic effects with those achieved by the administration of conventional antiplatelet and antithrombotic agents, ASA, and clopidogrel; and (3) evaluate a possible synergistic effect of LA816 with those antiplatelet agents. The study was carried out under conditions that mimic vessel wall injury and the flow conditions that are typical of patent and stenosed coronary arteries in a porcine arteriovenous shunt model. We hypothesized that NO donation with LA816 would not only exhibit antithrombotic properties but also provide additional benefits to platelet passivation and the inhibition of thrombosis to the combined blockade of COX and P2y(12) without modifying blood pressure.

**Methods**

**Animal Model**

Normal pigs (Large White X Landrace; body weight ~36 kg) were individually caged in a light-, temperature- (22±2°C), and humidity-controlled environment with free feeding and access to water. All procedures in this study were performed in accordance with National Institutes of Health guidelines and followed American Physiological Society guidelines for animal research.

**Experimental Design**

**Radioactive Labeling of Platelets**

Thrombic risk was assessed by exposing the blood of studied animals to a thrombus-triggering damaged artery in the previously validated thrombic risk model. This study was performed in 16 pigs (data not shown).

**Ex Vivo Thrombosis**

Thrombic risk was assessed by exposing the blood of studied animals to a thrombus-triggering damaged artery in the previously validated and standardized Badimon perfusion chamber. After overnight fasting, the animals were sedated with azoperone (8 mg/kg IM, Stresssil, Janssen Pharmaceuticals), anesthetized (10 mg/kg pento-barbital, B. Braun Melsungen AG), and a carotid artery–jugular vein shunt established to place the Badimon perfusion chamber. Blood was perfused through the chamber for 5 minutes at 2 different shear rates of 212/s and 1690/s, which are typical of patent or mildly stenotic coronary arteries. Homologous porcine vessel walls with 2 types of damage, severe (ruptured vessel wall) and mild (eroded vessel wall), were used as substrates.

**Drug Therapy**

This study was performed in 16 pigs (average of 30 perfusions per pig) that were randomly distributed in 4 groups: (1) placebo-control (nontreated); (2) oral administration of clopidogrel (Sanofi-BMS; 10 mg/kg); (3) oral administration of ASA (Bayer; 10 mg/kg); (4) combined oral administration of ASA + clopidogrel (10 mg/kg each). A scheme of the experimental protocol is shown in Figure 1. Briefly, a daily dose of platelet inhibitors (ASA, clopidogrel, or ASA + clopidogrel) was administered starting 2 days before and on
the day of the experiment. The last oral dose of the drug (day 3) was given 1 hour before starting the perfusions. The nontreated group was kept 3 days under the same conditions. After baseline perfusions (1 hour), the 4 animal groups were given an intravenous infusion of LA816 (Lacer, SA)18 in the mammary vein at an infusion rate of 6.6 mmol · kg⁻¹ · min⁻¹ for 2 hours.

The dose regimen of LA816 was selected from previous data obtained from humans treated with GSNO,13 whereas the ASA-clopidogrel dose was based on previous work by other investigators.23 LA816 was diluted in physiological serum saline and kept refrigerated (4°C) until administration. No chamber perfusions were performed during the first 30 minutes of the NO donor infusion to allow blood biodistribution of the drug.

Whole Blood and Platelet-Rich Plasma Aggregation

Whole blood (WB) and platelet-rich plasma (PRP) aggregation triggered by collagen (3, 5, 10 μg/mL; Chrono-log) was measured as previously reported.22 The effects of ASA, clopidogrel, or ASA-clopidogrel on platelet aggregation were evaluated before the perfusion experiment started. The effects of LA816 on platelet aggregation were evaluated during the intravenous infusion. Optical platelet aggregation induced by ADP (3, 5, 10 μmol/L) was measured in PRP (Menarini Group) as previously described.23

In a different series of experiments, the antiaggregatory actions of the NO donor drug were examined in the presence or absence of the selective guanylate cyclase inhibitor ODQ ([1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-1-on3]; 1 μmol/L) and carboxy-PTIO ([2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], a NO radical scavenger (50 μmol/L). Platelets were incubated with ODQ and carboxy-PTIO for 10 minutes at 37°C before adding LA816 (0.1 μmol/L). Maximal platelet aggregation was measured for 5 minutes after addition of the agonist (collagen, 3 μg/mL) and expressed as a percentage of control aggregation.

Hemodynamic and Hematologic Parameters

Systemic blood pressure and heart rate were monitored via a pressure transducer (Letica) that was attached to the cannulated femoral artery in all of the experiments. The determination of blood cells, hematocrit, platelet number, and size distribution were performed with a Sysmex K-1000 automated cell counter (Sysmex, Kobe, Japan). White blood cells, platelets, and hematocrit were measured in PRP (Menarini Group) as previously described.21

RhoA Protein Expression in Platelets

Platelets were obtained from blood collected in acid citrate dextrose that was gently dropped into plastic tubes. The platelet number was adjusted to 4 × 10⁶ platelets/mL and 750 μL lysis buffer (50 mmol/L Tris/HCl, pH 7.4; 1 mmol/L ethylenediaminetetraacetic acid; 1% Triton X-100; 1 mmol/L phenylmethyl sulfonyl fluoride) were added. Platelet subfractionation was performed as described.21 The protein concentration was measured using the bicinchoninic acid (BCA) method (Pierce Biotechnology). Equal amounts of protein (25 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were then incubated with an appropriate dilution (1:250) of monoclonal antibody anti-RhoA (Santa Cruz Biotechnology). After incubation with peroxidase-labeled antimmunoglobulin (1:10,000), the antibodies anti-RhoA were visualized by the chemiluminescent method SuperSignal (Pierce Biotechnology) and evaluated by densitometry.

Statistical Analysis

Results are reported as mean±SEM. The statistical significance of the difference between group means was evaluated by an analysis of variance, and when significant, the Student t test was used to assess intergroup differences. StatView software (Abacus Concepts) was used for all of the statistical tests, and a P<0.05 was considered significant.

Results

Effects of the Antiplatelet Therapies on Thrombus Formation in the Flow Chamber

Denuded Vessel Wall (Erosion Model)

The effects of the different antiplatelet therapies on thrombus formation are shown in Figure 2. Under low–shear rate conditions (low thrombotic risk) (Figure 2A), platelet deposition (PD) is mild (see control in Figure 2A and 2B) and none of the studied agents showed any inhibitory effect on PD. In contrast, when increasing the thrombotic risk by increasing shear rate (1690/s) (Figure 2C), PD was significantly increased (see placebo-control in Figure 2C) and a significant reduction was obtained after a 3-day oral pretreatment with ASA (40% reduction versus the placebo-control group) or clopidogrel (59% reduction) or after intravenous infusion of LA816 (52% reduction) (P<0.05 versus control). Similar changes were observed with the combinations between ASA, clopidogrel, and LA816 (Figure 2D), indicating that the mechanism of deposition under these conditions (platelet adhesion) was not further reduced by simultaneously blocking the different pathways targeted by the drug treatments at the doses used.

Disrupted Vessel Wall

Under high thrombotic risk and a shear rate typical of patent coronary arteries (low shear rate, 212/s) (Figure 3A), the ASA-treated group showed nonsignificant changes in PD (43±5 versus 45±10×10⁶ platelets/cm²). In contrast, either LA816 or clopidogrel produced a significantly reduction on mural thrombus formation of about 45% and 58%, respectively (Figure 3A), versus the placebo-control group. Combination of clopidogrel or LA816 with ASA did not further decrease PD. The combined treatment of oral
ASA + clopidogrel and intravenous LA816 produced a further reduction of 12% (P > 0.05) in PD (70% from placebo-controls; P < 0.0001) (Figure 3B). When analyzing the most thrombogenic situation, severely damaged vessel wall and high shear rate (Figure 3C and 3D), we found that ASA again produced minimal inhibition on mural thrombus formation (62 ± 10 versus 47 ± 4 × 10^6 platelets/cm²; P > 0.05). LA816 and clopidogrel showed a significant inhibitory effect (45% and 58% versus placebo-control, respectively; P < 0.0001) (Figure 3C). The combination of ASA with either of these 2 antiplatelet compounds did not produce an inhibitory synergistic effect (Figure 3D). Interestingly, the combined treatment of both ASA + clopidogrel and clopidogrel alone with intravenous LA816 produced a significant further reduction (20%; P < 0.05) in PD (70% from placebo-controls; P < 0.0001), indicating a synergistic antithrombotic effect and a more powerful platelet passivation (Figure 3D).

Platelet Studies

Effect on Platelet Aggregation

ADP-induced PRP aggregation was significantly inhibited by clopidogrel ex vivo at any dose of the agonist (percentage of inhibition ≈ 30% versus nontreated platelet aggregation; P < 0.05) (Figure 4A and 4B). The combined oral treatment of ASA + clopidogrel produced a much higher inhibitory effect (P < 0.001). LA816 and ASA alone did not inhibit the platelet aggregation challenged by ADP in either individual or combined treatment (Figure 4A and 4B). All effects seemed to result from clopidogrel, an ADP receptor antagonist.

Collagen-induced platelet aggregation in PRP was significantly inhibited by all treatment regimens when challenged by low doses (3 µg/mL) of the agonist (Figure 4C and 4D). Moreover, combined oral treatment of ASA + clopidogrel produced a much higher inhibitory effect that also was observed at doses of 10 µg/mL collagen.

Collagen-induced platelet aggregation in WB was not reduced by clopidogrel, LA816, and ASA (Figure 4E). When combining ASA and clopidogrel, we found that the inhibitory results were significant. Furthermore, the administration of the intravenous infusion of LA816 to ASA + clopidogrel-treated animals further potentiated the reduction on 10 µg/mL collagen-induced platelet aggregation (Figure 4F).

Platelet Inhibitory Effects of LA816 Are NO Dependent

Blood was collected from control animals and added with LA816 in vitro, with or without the guanylate cyclase inhibitor and the NO radical scavenger as described in Methods. Inhibiting guanylate cyclase or scavenging NO radicals abolished the platelet inhibitory effects of LA816 (Figure 5).

Platelet RhoA Protein Expression

We analyzed the effect of LA816 on the level of expression of membrane RhoA using procedures that have been published previously. We compared RhoA protein expression established previously. We compared RhoA protein expression levels before and after LA816 intravenous administration in the control animals (Figure 6). Interestingly, LA816 treatment significantly decreased membrane RhoA protein expression (P < 0.05).

Follow-Up Hematologic, Hemostatic, and Physiological Parameters

Effect of Treatment on Hematologic and Clotting Parameters

The hematologic parameters (Table) were similar among the groups and were within the normal intervals for pigs. In all of the animal groups, the aPTT mean ratio was within the normal range for a low level of anticoagulation (mean aPTT ratio 1.5 to 2 with 50 U/kg heparin). PT was mildly prolonged in animals treated with LA816 either alone or combined with clopidogrel or ASA + clopidogrel.

Blood Pressure and Heart Rate

Treatment with LA816 produced only a mild, reversible, and nonsignificant reduction of blood pressure of 6 mm Hg (~9% reduction versus basal value) (Figure 7A), but no hypotensive episodes (defined as a fall in mean arterial pressure of 10 mm Hg) were detected. No significant effects in heart rate after intravenous treatment with the NO donor compound were noted (Figure 7B). Combining LA816 with any oral antithrombotic regimen did not produce any change in mean blood pressure and heart rate. Additionally, the most efficacious antithrombotic regime tested (ASA + clopidogrel + LA816) showed a constant blood pressure (Figure 7C) and heart rate (Figure 7D) in all of the experiments.

Discussion

The current study used the Badimon perfusion chamber to differentiate the relative antithrombotic properties of a new platelet-selective NO donor (LA816) in comparison with ASA, clopidogrel, and combined therapy on mural thrombosis. We have demonstrated that short-term administration of LA816, a platelet-selective NO donor drug, has antithrombotic properties that are similar to those of clopidogrel given
long term. In addition, we have shown that the combined treatment of clopidogrel and LA816 is more effective than either agent alone under high-thrombotic risk conditions.

In the presence of denuded vessels and under low-shear rate conditions, the thrombotic stimulus is mild and induces mainly platelet adhesion ($<5 \times 10^7$/cm$^2$), a platelet monolayer that does not progress to mural thrombosis. Consequently, these deposition levels were not affected by the antiaggregatory drugs used in the present study as we and others have previously shown. When increasing the shear rate, which produced further thrombogenesis, we found that the antithrombotic effects of LA816 on denuded vessel walls were similar to those of clopidogrel and ASA. The antithrombotic properties of either LA816 or clopidogrel on mural thrombosis induced by disrupted substrates were far superior to ASA in this model. In agreement with our results, several studies have demonstrated that the incidence of mural thrombosis was unchanged with ASA, although (also in agreement with our results) ASA markedly reduced platelet aggregation in vitro under Ca$^{2+}$-depleted conditions.

Under the highest thrombogenic stimulus (ie, disrupted vessel and high shear rate) typical of high-cardiovascular risk situations, the combined treatment of clopidogrel and LA816 was far superior to clopidogrel alone, suggesting that LA816 and clopidogrel exert synergistic effects in inhibiting thrombus formation. The synergy between these 2 antiplatelet agents may have originated from complementing the different platelet activation pathways inhibited by these drugs. Inter-

Figure 4. Platelet aggregation in vitro in animal groups. A, B, ADP-induced PRP aggregation. C, D, Collagen-induced platelet aggregation in PRP and (A, E, F) in whole blood. (*P<0.05 vs placebo control; †P<0.001 vs placebo control).
estingly, the blocking of COX-1 did not change the beneficial antithrombotic profile.

As previously reported, clopidogrel (10 mg/kg) produced no anticoagulant effects, as assessed by activated clotting time, which was essentially unchanged. LA816 caused a significant elongation in PT, suggesting that LA816 acts in the hemostatic system not only to inhibit platelets but also to reduce blood coagulation. It was reported that the NO precursor L-arginine inhibits the activation of hemostasis and thereby stabilizes coagulation and platelet function. Furthermore, the elongation in PT in LA816-tested animals also may reflect the suppression of tissue factor expression by NO as described by others.

We also have obtained information on the effect of LA816 on RhoA. RhoA is a small guanosine triphosphatase involved in many cell functions, including cell-shape changes. In accordance with our previous report, NO donation with nitrosothiols decreased RhoA expression in its active form (membrane), confirming that NO regulates RhoA activation, subsequent cytoskeleton organization, and GPIIb/IIIa activation. Experimental studies are needed to further characterize the effects of LA816 on RhoA activity and the tissue factor pathway because our results suggest that the effects of LA816 on these signal pathways could partly explain its pharmacological activity.

In addition, NO synergizes with thrombolytic agents, improving outcomes. ASA has been demonstrated to inhibit vessel wall NO and prostacyclin synthesis, which results in vasoconstriction, platelet aggregation, and reduction on thrombolysis; therefore, providing an exogenous source of NO could abrogate these adverse effects and improve the environmental milieu of the atherothrombotic process.

Hypotension is probably the most important cardiovascular side effect of NO donors. In a previous study, we observed...
that GSNO given in equimolar doses caused a significant reduction in mean arterial pressure of 14 mm Hg (∼30% reduction versus basal value) throughout the perfusion period. In contrast, LA816 intravenous treatment did not cause any significant variation in blood pressure throughout the experiment, indicating no concomitant negative hemodynamic effect. Thus, we observe that combining LA816 and LA816 + clopidogrel + ASA greatly reduces mural thrombus formation, which correlates with its inhibition on platelet aggregation without significant hemostatic or hemodynamic side effects.

According to the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events in patients undergoing Percutaneous Coronary Intervention (PCI-CURE) study and the Clopidogrel for Reduction of Events During Observation (CREDO) trial, the strategy of clopidogrel pretreatment is beneficial in reducing major cardiovascular events in patients with unstable angina and non-ST-segment—elevation myocardial infarction (NSTEMI) who have been given ASA and have undergone PCI. Short-term administration of LA816 seems to provide the same antithrombotic protection as that of 3-d treatment with clopidogrel. Therefore, short-term administration of LA816 could be as beneficial as pretreatment with clopidogrel in reducing major cardiovascular events. This hypothesis will require testing in humans.

Because clopidogrel, when added to ASA, increases the risk of bleeding during major surgery in patients who are scheduled for coronary bypass grafting, clopidogrel should be withheld for at least 5 d and preferably for 7 d before surgery. The short-term effects of intravenously administering LA816 could be modulated as required and free of this disadvantage. Interestingly, the triple treatment of ASA + clopidogrel + LA816 was not associated with a higher risk of bleeding because neither hematocrit nor blood pressure was modified and surgical wound blood oozing was not detected. Nevertheless, because bleeding assessment was not the objective of our study, the design we used does not allow any conclusions on this matter.

**Conclusion**

Our data provide evidence of the antithrombotic properties of the new platelet-selective NO donor and indicates its potential beneficial effects in combination therapy with ADP antagonists and ASA, the treatments of choice against the thrombotic events that cause ischemic syndromes.

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**Figure 7.** Hemodynamic values throughout experiments. A, Treatment with LA816 produced only mild, reversible, and nonsignificant reduction (6 mm Hg; ∼9% reduction vs basal value) in blood pressure. B, No significant effects noted on heart rate after IV treatment with LA816. Most efficacious antithrombotic regime tested (ASA + clopidogrel + LA816) showed constant blood pressure (C) and heart rate (D) in all experiments. Data are ±SEM. Between parentheses are normal pig values. *P<0.05.
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