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Inhibition of thrombosis by a novel platelet selective *S*-nitrosothiol compound without hemodynamic side effects☆

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Abstract

Platelet and endothelial production of bioactive nitric oxide (NO) is known to be impaired in acute coronary syndromes, thus compounds that release NO are useful candidates to restore NO-vascular functions. **Objective:** We have studied whether donation of NO with a novel platelet-selective *S*-nitrosothiol compound (LA810) at a systemic level can inhibit thrombosis elicited by damaged vessel wall (eroded and disrupted vessel wall) at hemodynamic conditions typical of patent and stenotic coronary arteries. **Methods:** Thrombogenicity was measured in the porcine experimental model and assessed as platelet–thrombus formation in the ex vivo Badimon perfusion chamber. After baseline perfusions, female pigs (Large White × Landrace) were given intravenous infusion of LA810 or GSNO standard *S*-nitrosothiol during 2 h. Changes in blood pressure, heart rate and in vitro platelet aggregation were measured. **Results:** LA810 significantly decreased thrombus formation at any degree of vascular damage and shear rate ($p < 0.001$) without hypotensive side-effects or heart rate variations. In contrast, inhibition of thrombus formation by GSNO required high doses associated to hypotensive episodes. Platelet aggregation induced by collagen was inhibited after nitrosothiol infusion in whole blood (LA810) and platelet rich plasma (LA810 and GSNO). In addition, there was a drug-dependent rise in platelet guanosine 3′,5′-cyclic monophosphate (cGMP) levels. **Conclusions:** This new anti-ischemic NO-donor (NOd) LA810 that inhibits platelet function without hypotensive side-effects seems a highly efficacious strategy to reduce acute thrombosis triggered by coronary artery disease.

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Keywords: Platelets; Nitric oxide; Thrombosis; Pig

1. Introduction

Thrombus formation within coronary vessels is the precipitating event of myocardial infarction and unstable angina, as documented by angiographic and pathologic studies [1]. Rupture of the atherosclerotic plaque is believed to be the responsible event for most of the coronary syndromes in a process mainly mediated by platelet adhesion, activation and aggregation [2]. Thrombus may partially or completely occlude the arterial lumen producing ischemia or infarction [3]. Antithrombotic and antiischemic agents are, therefore, employed in the treatment of acute coronary artery disease. Anti-platelet treatment with aspirin is effective in reducing platelet aggregation but there is evidence that platelet activation persists despite aspirin treatment [4]. Glycoprotein IIb/IIIa (GP IIb/IIIa) receptor antagonists [5] have provided remarkable achievements in the acute treatment of acute coronary artery disease; however, they still have some limitations [6].

Platelet and endothelial production of bioactive nitric oxide (NO) seems to be impaired in acute coronary syndromes [7]. Under physiological conditions, NO is released from the endothelium, platelets, and leukocytes in response to a number of circulating agonists and hemodynamic factors [8]. Among its diverse functions, endothelial-derived NO has been involved in neurotransmission [9], immune regulation [10], vascular smooth muscle relaxation [11], and inhibition of platelet adhesion and aggregation [12]. Platelet-derived NO modestly reduces primary aggregation response, whereas NO released from aggregating platelets markedly inhibits platelet recruitment [13] and thus limits

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progression of intra-arterial thrombosis. NO effects in platelet signalling in vitro suggests that NO activates guanylate cyclase leading to an increase of cGMP [14] and suppresses intracellular calcium flux reducing P-selectin and the active conformation of GP IIb/IIIa expression. In consideration to the physiological regulations on NO and to the well-established pathological implications of the decrease of NO bioavailability, there is a great interest in developing NO donors able to restore NO levels.

S-nitrosothiols are good candidates as new nitric oxide donor drugs. Indeed, although sufficient evidence shows that EDRF is NO, the argument has been advanced that EDRF might be a more chemically stable adduct of NO, such as an *S*-nitrosothiol [15]. In this sense, *S*-nitrosoglutathation (GSNO) has been detected in plasma at concentration of 0.1 μmol/l [15], and GSNO has been implicated in the intracellular transfer of NO from NO synthase to the guanylate cyclase. Moreover, some *S*-nitrosothiols have demonstrated some cell selectivity [15].

GSNO is a *S*-nitrosothiol from which NO is released by the action of enzymes associated with platelet membranes [16]. GSNO seems to have, therefore, platelet selectivity and to inhibit platelet adhesion and aggregation to a greater extent than its effects on vascular tone [16]. GSNO has shown to reduce asymptomatic embolizations after carotid angioplasty without hypotensive episodes [17] and also to reduce markers of platelet activation such as P-selectin (CD62/GMP140) and fibrinogen GPIIb/IIIa receptor density without altering blood pressure in patients following percutaneous transluminal coronary angioplasty [18].

Considering the antithrombotic activities of GSNO and the effectiveness of antiplatelet therapy in preventing serious vascular events [19], arterial occlusion [20], and venous thromboembolism [21] among a wide range of patients at high risk of occlusive vascular events, Lacer (Barcelona, Spain) has synthesised new *S*-nitrosothiols with antithrombotic activity [22].

It has been our objective to evaluate the antithrombotic/antiplatelet effects of a new platelet selective *S*-nitrosothiol compound [22] (LA810, *N*[*N*-γ-L-glutamyl-2-amino-2-(-4-(4-*S*-nitrosomercapto-1-methyl-piperidin))acetyl]glycine) in conditions mimicking vessel wall injury and flow shear rates typical of coronary arteries. We hypothesized that NO donation with LA810 would significantly inhibit thrombosis triggered by both superficial and deep vessel wall damage at different shear rates without significantly modifying blood pressure or heart rate.

2. Methods

2.1. Experimental conditions

Female pigs (Large White × Landrace) obtained from a local single farm ($n = 13$; body weight: 39.5 ± 2.5 kg; age: 1.5 months old) were individually caged in light, temperature, and humidity controlled environment with free access to water and feeding. All procedures in this study were performed in accordance with NIH guidelines and followed the American Physiological Society guidelines for animal research.

After overnight fasting, blood was withdrawn and platelets were labeled with ^{111}In-oxine (^{111}In) (Amersham Biosciences, London, UK) as previously described [23]. An average of $8.7 \times 10^6 \pm 0.1 \times 10^6$ ^{111}In-labeled platelets/μl were injected in a final volume of 4 ml of autologous plasma. Efficiency was $93.0 \pm 1.6\%$ and the injected activity was 250 ± 12 μCi. Twenty hours later, pigs were sedated with an intramuscular injection of 8 mg/kg of Azoperona (Stressnil®, Esteve, Barcelona, Spain), deeply anesthetized by intravascular infusion of pentobarbital sodium solution (10 mg/kg, B.Braum Medical, Barcelona, Spain) and then intubated and ventilated (Dog ventilator, Ugo basile, Italy). It has been previously described that barbiturates inhibit platelet activity at plasma levels of 10^{-4} mol/l [24]. These levels are often achieved in animals administered 30 mg/kg of sodium pentobarbital [24]. Therefore, to minimize the circulating plasma levels of pentobarbital, we administered 10 mg/kg intravenous bolus of pentobarbital sodium (deep anesthesia) followed by a continuous infusion of 10 mg/kg/h until all the experiment had been performed. This procedure produced a consistent anesthetic state with a minimal variation in hemodynamic parameters. Through a neck incision the common carotid artery (distal portion) and contralateral jugular vein were cannulated. Then, pigs were intravenously heparinized with a bolus (50 U/kg) followed by an infusion (50 U/kg/h) (Liquemine®, Roche, Basel, Switzerland). The catheterized carotid artery was connected by polyethylene tubing to the input of the Badimon perfusion chamber and the output of the chamber was connected to a peristaltic pump (Masterflex, Model 7518-10, Cole Parmer Instrument, Vernon Hills, USA). Blood that passed through the chamber was recirculated back into the animal by the contralateral jugular vein.

Post-mortem ^{111}In-biodistribution indicated a correct platelet distribution with maximal accumulation in blood ($47 \pm 4\%$ in blood, $28 \pm 3\%$ in liver, $14 \pm 2\%$ in spleen, $4.0 \pm 0.5\%$ in lungs, $0.2 \pm 0.03\%$ in kidneys, and $0.11 \pm 0.02\%$ in heart tissue). Serum levels of creatinine, protein, glucose, AST and ALT were measured in both treated groups by routine analytical chemistry assays.

2.2. Perfusion chamber and substrates

We have used the Badimon perfusion chamber that mimics the cylindrical shape of the blood vessels as reported [23,25]. Pig aortas were obtained fresh in a local slaughterhouse, transported in phosphate-buffered saline (PBS) and immediately cleaned from adventitia, cut in long pieces and frozen at −80 °C until needed. Before the experiments, the aortas were thawed in PBS at

4 °C, opened longitudinally, and cut into 30 × 10 mm segments. Segments of pig aorta were denuded (model of erosion) or mechanically disrupted (model of disruption) by peeling off the intimal layer with a thin portion of subjacent media, starting from a corner of the arterial segment as previously described [25]. We have selected a flow rate of 10 ml/min in the small (0.1 cm diameter) and large (0.2 cm diameter) chamber. This flow gives theoretically calculated average blood velocities of 21.2 and 5.3 cm/s, respectively [23]. Shear conditions at the vessel wall were calculated from the expression for shear rate given for a Newtonian fluid in the tube flow [26]. These shear rates correspond to values encountered in the arterial circulation (1690/s and 212/s) [23]. The substrates were mounted in the chamber and PBS solution at 37 °C was perfused for 60 s. After the preperfusion period, blood entered the chamber at a preselected flow rate of 10 ml/min for 5 min. At the end of blood flow, buffer was again passed for 30 s through the chamber under identical flow conditions. The perfused segments were fixed in 4% paraformaldehyde in PBS and counted in a gamma counter (Wizard Wallac, Boston, USA) for quantization of deposited platelets. Values were normalized by blood ^{111}In activity (counts), platelets counts in blood, and area of exposed surface.

2.3. Drug administration

After baseline perfusions (1 h), pigs were given an i.v. infusion (mammary vein) either of LA810 (stable analogue of GSNO, Lacer®, Barcelona, Spain) [22] or GSNO (a physiological platelet specific NOd) at the same infusion rate of 6.6 nmol/kg/min during 2 h. Both compounds were diluted in physiological serum saline to avoid any possible interaction effects. This dose regime was selected from previous data obtained in humans treated with GSNO [17]. Each animal served as its own control. No perfusions were performed during the first 30 min of the NOd infusion to allow blood distribution of the drug.

2.4. Whole blood and platelet rich plasma (PRP) platelet aggregation

Whole blood impedance platelet aggregation triggered by collagen (3, 5, 10 μg/ml) (Chrono-Log model 530; ChronoLog, Yzasa SL, Havertown, USA) was measured as previously reported [27] at baseline conditions and during *S*-nitrosothiol intravenous infusion (30 and 120 min). Optical platelet aggregation was measured in PRP as previously described [27] in the same time periods as whole blood. Collagen (3, 5, 10, 15 μg/ml) was used as platelet agonist.

Additionally, following previously described procedures [28], we evaluated the collagen (0.5–1 μg/ml)-induced platelet aggregation effects of LA810 (0.1μmol/l) with or without ODQ ([1H-[1,2,4]Oxadiazole[4,3-*a*]quinoxalin-1-on3]), a potent and selective inhibitor of nitric oxide-sensitive guanylyl cyclase (1 μmol/l) and Carboxy-PTIO ([2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], a nitric oxide radical scavenger (50 μmol/l).

2.5. Physiological and hematological parameters

Systemic blood pressure (systolic arterial pressure, diastolic arterial pressure, and mean arterial pressure) and heart rate were monitored via a pressure transducer (Letica, Rochester, USA) attached to the cannulated femoral artery throughout all the experiments.

Determination of blood cells, hematocrit, platelet number, and size distribution were determined (System 9000, Serono-Baker Diagnostics, Allentown, USA). Levels of prothrombin time (PT) and activated partial tromboplastina time (aPTT) were monitored with an ST4 automated clotter (Diagnostica Stago, Asnières, France) and the corresponding specific kits (American Diagnostica, Stanford, USA) according to the manufacturer's instructions.

2.6. Determination of platelet cGMP levels

Blood was withdrawn in EDTA at different time periods (pre-treatment and 30–120 min during treatment), centrifuged at 250 × *g* 10 min at room temperature (RT) and incubated with IBMX and PGI2 to avoid phosphodiesterases activity. Samples were centrifuged at 1.400 × *g* 15 min RT to obtain platelet pellets which were stored deep frozen (− 80 °C) until measurements. To determine cGMP levels a commercially available cGMP enzyme immunoassay (EIA) kit (Amersham, Chicago, USA) was used with the addition of an acetylating step to increase sensitivity.

2.7. Immunohistochemistry

Perfused arterial segments were fixed in 4% paraformaldehyde solution, cryoprotected with 2.3 mol/l sucrose and frozen over dry ice in OCT (Tissue-Tek OCT Compound 4583, Germany). Serially cut 4 to 5 μm sections in the blood flow direction were obtained on a cryostat (Jung CM 300, Leica) mounted on gelatinized slides. Immunohistochemical analysis was performed in sections stained with an antifibrinogen polyclonal antibody (DAKO A080, Glosatrup, Denmark) and an anti-platelet polyclonal antibody (pabBP19) produced in our laboratory and previously described [29] as primary antibodies. Secondary antibodies were FITC-conjugated F(ab')2 fragment of anti-rabbit polyclonal (Sigma, F1262, New York, USA) and TRITC-conjugated swine anti-rabbit immunoglobulins (DAKO R156, Glosatrup, Denmark). Results were evaluated with a fluorescence microscope (Vanox AHBT3, Olympus, Melville, USA). The images were digitalized with a Sony 3CCD camera [30]. Controls of primary and secondary antibody staining were always performed in parallel in serial cuts on the same axial segment (central piece). To

Table 1
Hematological, coagulation (A) and biochemical (B) parameters in both nitrosothiol-treated groups

(A)

Group	Before LA810 i.v. treatment	LA810 (6.6 nmol/kg/min)	Before GSNO i.v. treatment	GSNO (6.6 nmol/kg/min)
RBC ($\times 10^6$ μl^{-1}) (5–7)	6.15 ± 0.3	5.8 ± 0.1	4.5 ± 0.1	4.5 ± 0.1
PLT ($\times 10^3$ μl^{-1}) (250–450)	404.0 ± 29.1	380.0 ± 20.2	505 ± 15.7	499 ± 14.9
HCT (%) (25–35)	34.7 ± 1.7	30.9 ± 1.0	25.5 ± 0.1	25.4 ± 0.1
APTT (s)	237.0 ± 35.0	232.0 ± 27.0	222.0 ± 23.0	227.0 ± 30.0
PT (s)	13.6 ± 0.5	15.9 ± 0.7*	12.9 ± 0.1	12.8 ± 0.1

(B)

	Pigs treated with LA810	Pigs treated with GSNO
AST ($U\,l^{-1}$) (15.3–55.3)	21.8 ± 7.0	36.0 ± 1.0
ALT ($U\,l^{-1}$) (9–43)	42.0 ± 2.4	38.5 ± 3.1
Crea. (mg dl^{-1}) (0.8–1.4)	1.0 ± 0.03	1.2 ± 0.08
TP (g dl^{-1}) (3.4–4.4)	5.3 ± 0.06	5.0 ± 0.5
GLU (mg dl^{-1}) (77–99)	96.6 ± 0.9	99.0 ± 30.4

Red blood cells (RBC); platelets (PLT); hematocrit (HCT); activated partial tromboplastin time (aPTT); prothrombin time (PT); PT and aPTT are values expressed in seconds. Values are expressed as mean ± standard error. Between brackets normal values for 1.5-month-old pigs.
* $p < 0.05$.

avoid interference of location in comparative analysis all stained sections were taken from the same central piece of the specimens.

2.8. RhoA in platelets

Platelets were obtained from blood collected in acid citrate dextrose gently dropped into plastic tubes. Platelet number was adjusted to 4×10^8 platelets/ml and 750 μl lysis buffer (50 mmols/l Tris/HCl pH 7.4, 1 mmols/l EDTA, 1% Triton-X 100, 1 mmols/l PMSF) were added. Samples were then mixed for 10 min, sonicated for 10 s and centrifuged at $1600 \times g$ for 15 min at 4 °C. Platelet subfraccionation was performed as described [31]. Total protein concentration in platelet extracts was measured using the Pierce BCA (bicinchoninic acid) protein assay method (Pierce, Rockford, IL, USA) [32]. Equal amounts of protein (25 μg) were subjected to SDS/PAGE and transferred onto nitrocellulose membranes (Bio-Rad, California, USA). The membranes were then incubated with an appropriate dilution (1:250) of monoclonal antibody anti-Rho-A (Santa Cruz Biotechnology, California, USA). After incubation with peroxidase-labeled antimouse immunoglobulins (1:10000) antibody visualization was performed by the chemiluminiscent method SuperSignal® (Pierce, New York, USA) and autoradiography was performed at RT using AGFA CURIX-RP2 films.

2.9. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical analysis was performed by the Student's *t*-test for paired data when groups had equal variances (*F* test) and by Mann–Whitney *U*-test for groups with unequal variances (*F* test). When experimental design consisted of repeated measures an analysis of variance for repeated measures (ANOVA) and a Dunnett's test were applied. A Power Macintosh computer equipped with Statview™ software (Abacus) was used for

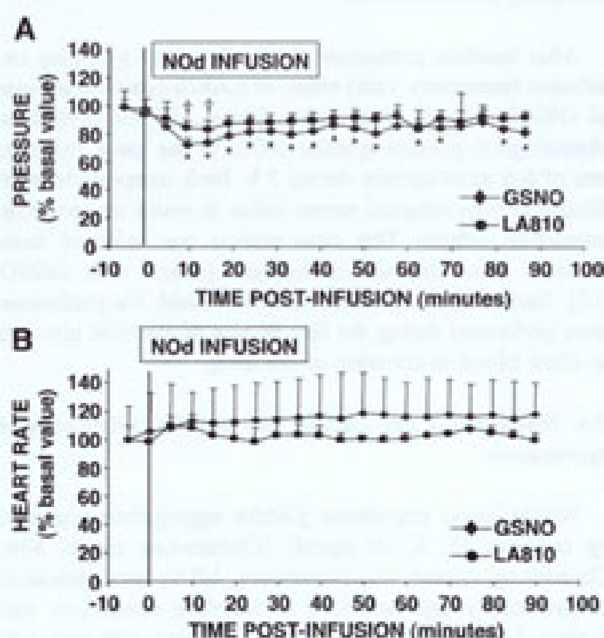

Fig. 1. Haemodynamic values throughout the experiments. (A) A mild and rapidly reversible reduction of blood pressure that never reaches values of fall in mean arterial pressure higher than 10 mm Hg is observed after LA810 drug infusion. GSNO-treated animals show a maintained and significant reduction in blood pressure throughout the experiment (≈ 30% reduction vs. basal value). (B) No changes in heart rate are observed after LA810 or GSNO infusion. (MANOVA + Dunnett's test; †$p < 0.05$ LA810 vs. basal value; *$p < 0.05$ GSNO vs. basal value). Data are given as percentage of basal value (mean value: 73 ± 5 mm Hg, $n = 13$).

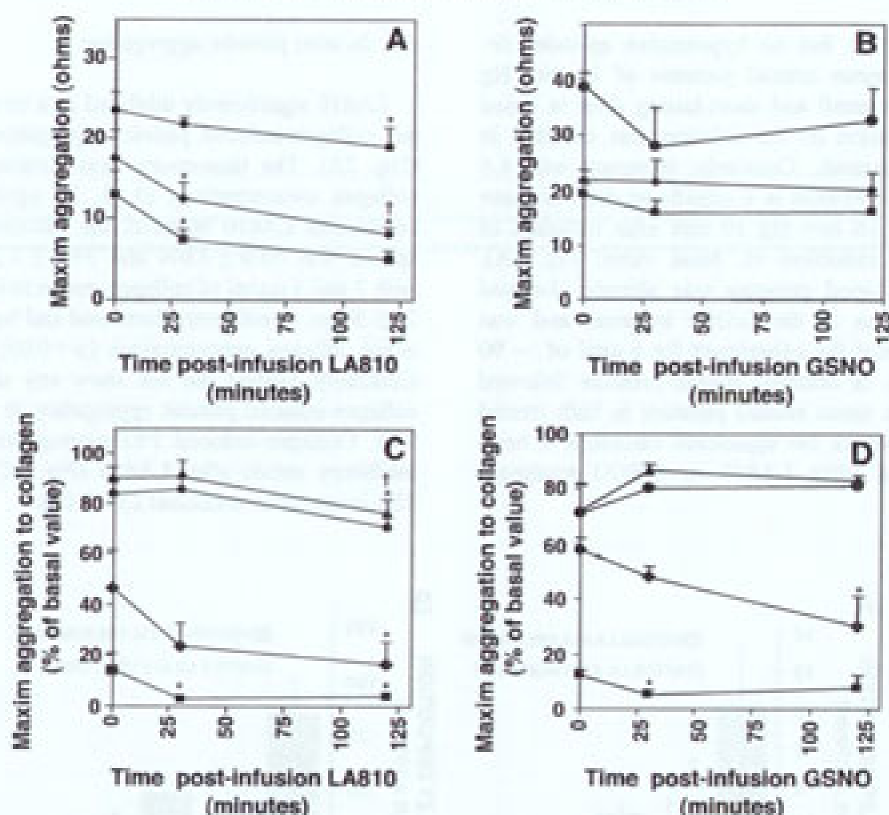

Fig. 2. Platelet aggregation in vitro before (control; $t=0$) and after ($t=30$ and $t=120$ min) LA810 (A, C) and GSNO (B, D) intravenous treatment. Collagen-induced platelet aggregation was evaluated in whole blood (A, B) and PRP (C, D). (*$p<0.05$, significant vs. $t=0$, and †$p<0.05$ vs. previous measured time). -■- 3 μg/ml; -◆- 5 μg/ml; -●- 10 μg/ml; -▲- 15 μg/ml.

all analysis. Statistical significance was considered when $p<0.05$.

3. Results

3.1. Evolution of hematological levels and coagulation

Hematological and coagulation follow up parameters are summarized in Table 1A. No significant differences were found in hematological parameters before and after LA810 and/or GSNO intravenous infusion. All values were also within the normal range for 1.5-month-old pigs. PT showed a small but significant increase (13.6 ± 0.5 vs. 15.9 ± 0.7 s; $p<0.05$) after LA810 infusion. No significant differences were found in aPTT before and after any *S*-nitrosothiol treatment. Biochemical parameters in both treated groups were always within the normal pig range (Table 1B).

3.2. Blood pressure and heart rate

Treatment with LA810 caused only a mild and reversible reduction of blood pressure 10 min after initiation of

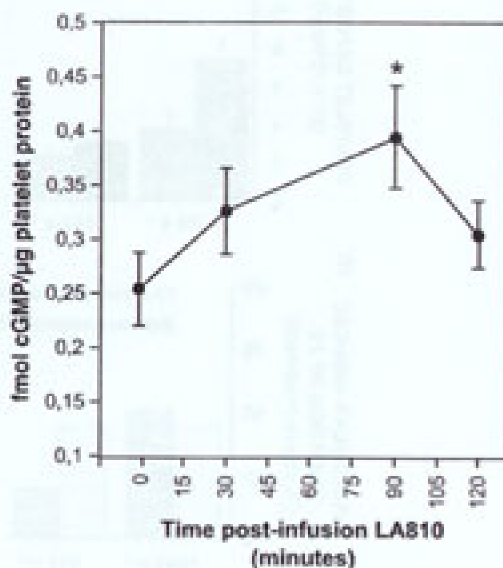

Fig. 3. Platelet cGMP concentrations before ($t=0$) and after ($t=30$, 90, 120 min) drug infusion. The increase in intraplatelet cGMP levels reached significance 90 min after continuous LA810 infusion (*$p<0.05$).

the infusion (Fig. 1A), but no hypotensive episodes defined as a fall in mean arterial pressure of 10 mm Hg were detected. That small and short-lasting drop in blood pressure upon initiation of the infusion was detected in only 60% of the animals. Contrarily, treatment with 6.6 nmol/kg/min GSNO resulted in a significant drop of mean arterial pressure of 16 mm Hg 10 min after initiation of the infusion (30% reduction vs. basal value; Fig. 1A). This reduction of blood pressure was already observed after 1 min initiation of the GSNO infusion, and was maintained throughout the experiment for a total of ∼ 90 min. Either systolic or diastolic arterial pressure followed the same pattern as mean arterial pressure in both treated groups (data not shown). No significant variations in heart rate were detected after LA810 or GSNO treatment (Fig. 1B).

3.3. In vitro platelet aggregation

LA810 significantly inhibited in a time dependent manner collagen-induced platelet aggregation in whole blood (Fig. 2A). The time-course was similar for the different collagen concentrations (3, 5, 10 μg/ml). After 2 h of continuous LA810 infusion, the reduction of platelet response was $48.0 \pm 3.0\%$ and $39.8 \pm 1.2\%$ when induced with 3 and 5 μg/ml of collagen, respectively ($p < 0.001$; Fig. 2A). Slope significantly decreased and lag time increased at equal collagen concentrations ($p < 0.05$; data not shown). Contrarily, GSNO did not show any inhibitory effect in collagen-induced platelet aggregation in whole blood (Fig. 2B). Collagen-induced PRP-aggregation showed similar inhibitory trends after LA810 (Fig. 2C) or GSNO (Fig. 2D) intravenous treatment ($p < 0.05$).

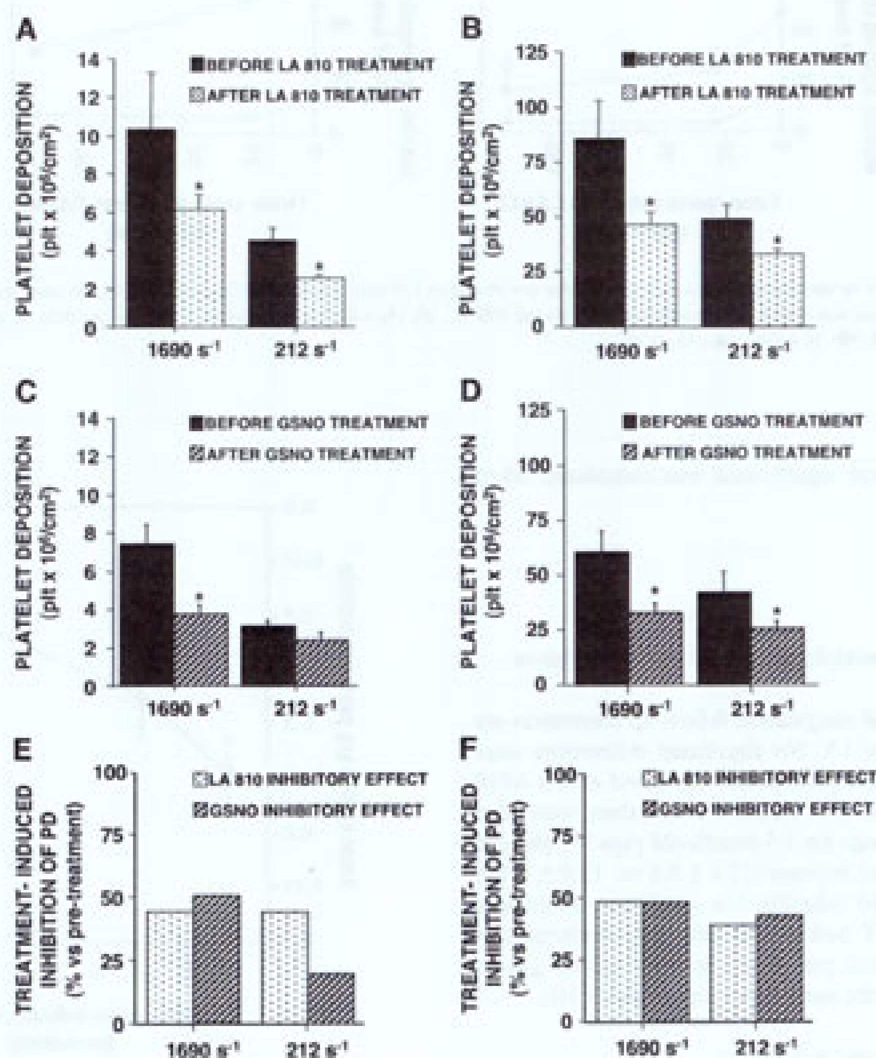

Fig. 4. Bar graph of platelet–vessel wall interaction as determined by the radioisotopic (^{111}In-labeled platelets) method. Results are expressed as mean values of Platelet Deposition (PD) ($\times 10^6$/cm^2) ± S.E. (A–B) Effect of LA810 on PD. (C–D) Effect of GSNO on PD. Platelet deposition triggered by mildly (A, C) or severely (B, D) damaged vessel wall at low (212/s) and high (1690/s) shear rate (*$p < 0.001$). (E–F) Percentage of treatment-induced inhibition on PD.

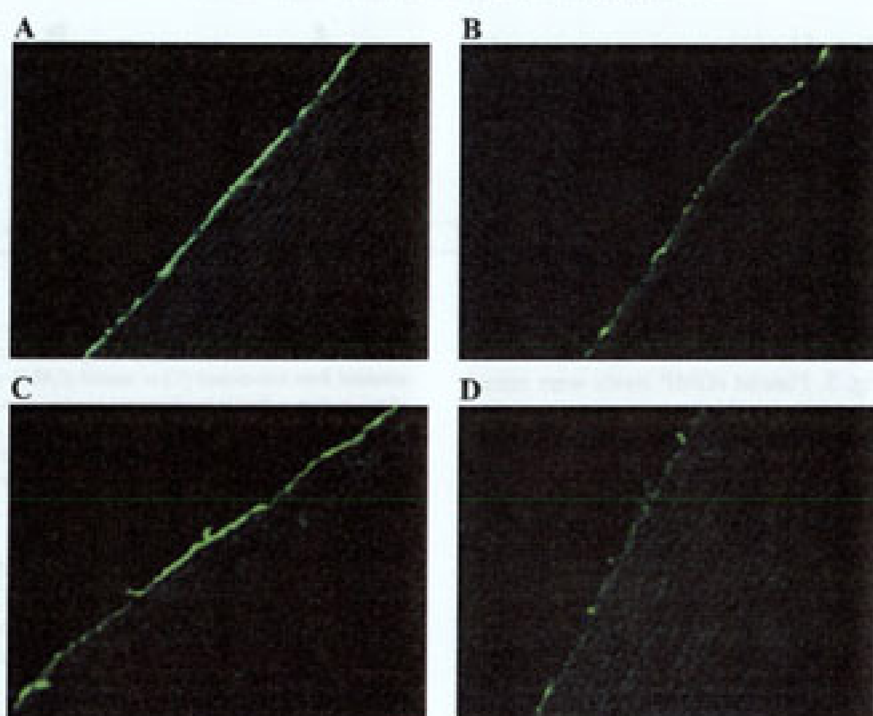

Fig. 5. Representative immunophotomicrographs of mildly damaged vessel wall perfused at low shear rate (A, B: 212/s) and high shear rate (C, D: 1700/s) before (A, C) and after the LA810 treatment (B, D). Note reduction on fibrin (shown in green) deposition in LA810-treated substrates.

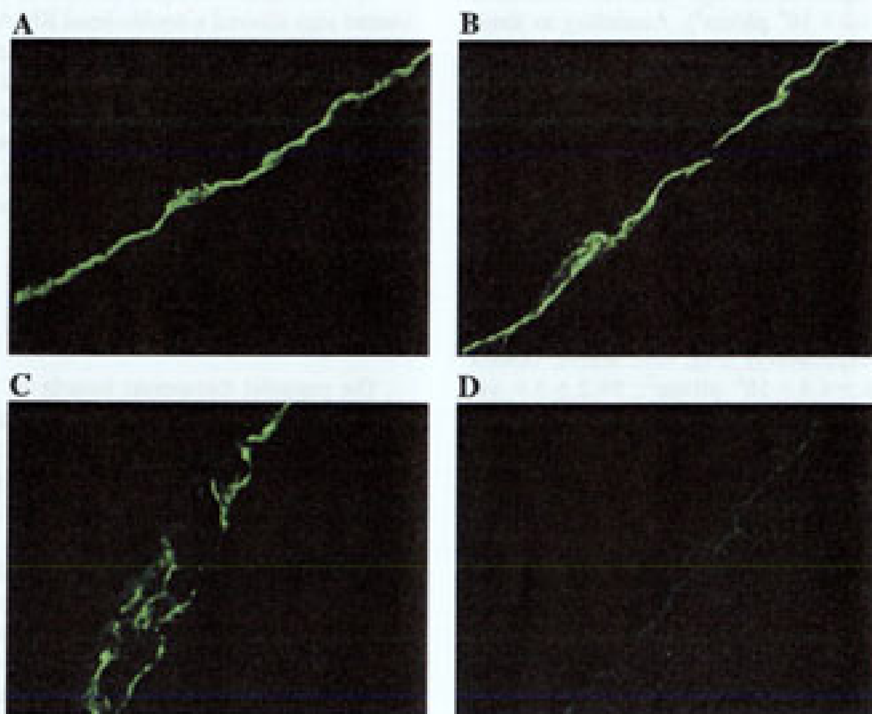

Fig. 6. Representative immunophotomicrographs of severely damaged vessel wall perfused at low shear rate (A, B; 212/s) and high shear rate (C, D; 1700/s) before (A, C) and after (B, D) the LA810 treatment. Note the significant reduction on fibrin deposition (shown in green) in LA810-treated substrates, especially at high shear rate conditions.

The inhibitory effect of LA810 was more pronounced 2 h after intravenous drug administration than at only 30 min of infusion ($p < 0.002$).

In a further set of experiments we observed that collagen-induced platelet aggregation was not inhibited by LA810 when platelets had been incubated (10 min) with ODQ and Carboxy PTIO (data not shown).

3.4. Platelets cGMP levels

The time-course of LA810 effects in intraplatelet cGMP levels is shown in Fig. 3. Platelet cGMP levels were raised upon initiation of NOd infusion and remained rose during the whole infusion period. The increase reached statistical significance after 90 min of drug infusion (basal vs. treated: 0.25 ± 0.04 vs. 0.39 ± 0.10 fmol cGMP/μg platelet protein, $p < 0.05$).

3.5. Platelet deposition

3.5.1. Mildly damaged vessel wall

Platelet deposition (PD) on eroded vessel wall was significantly decreased ($p < 0.001$) after LA810 6.6 nmol/kg/min infusion, both, at low (4.5 ± 0.9 vs. $2.5 \pm 0.4 \times 10^6$ plt/cm^2) and high (10.3 ± 3.4 vs. $6.0 \pm 0.9 \times 10^6$ plt/cm^2) local shear rate conditions (Fig. 4A). In contrast, although inhibitory effects of GSNO were not significant at low (3.0 ± 0.2 vs. $2.4 \pm 0.4 \times 10^6$ plt/cm^2) shear rate conditions (Fig. 4C), they were significant at high shear rate conditions (7.4 ± 1.1 vs. $3.7 \pm 1.0 \times 10^6$ plt/cm^2). According to these results, treatment-induced inhibition of PD (% inhibition vs. pre-treatment) was approximately 50% with both nitrosothiol compounds at high shear rate conditions while at low shear rate conditions, LA810 caused a reduction of about 50% and GSNO only reduced a 20% (Fig. 4E).

3.5.2. Severely damaged vessel wall

PD on severely damaged vessel wall was significantly reduced at low and high shear rates after LA810 (49.6 ± 7.9 vs. $32.2 \pm 4.0 \times 10^6$ plt/cm^2; 86.2 ± 19.0 vs. $46.1 \pm 7.4 \times 10^6$ plt/cm^2, respectively) (Fig. 4B) and/or GSNO (43.9 ± 4.0 vs. $25.8 \pm 4.4 \times 10^6$ plt/cm^2; 59.2 ± 3.9 vs. $31.8 \pm 5.3 \times 10^6$ plt/cm^2, respectively) intravenous infusion (Fig. 4D) ($p < 0.001$). GSNO or LA810 caused a similar inhibition of PD, of about 50%, at both local shear rate conditions (Fig. 4F).

3.6. Immunohistochemical analysis

Immunohistochemical staining of perfused substrates showed different amounts of fibrin and PD according to the vascular wall damage. Eroded vessels demonstrated fibrin formation depending on shear conditions while severely damaged vessel wall induced significantly higher amounts of fibrin deposition (mildly damaged vessel wall, Fig. 5; severely damaged vessel wall, Fig. 6). Interestingly, LA810 significantly reduced fibrin deposition suggesting effects on the tissue factor-thrombin (TF-thrombin) pathway. PD was highly reduced and followed the pattern already seen in the radioisotopic quantitative analysis (data not shown).

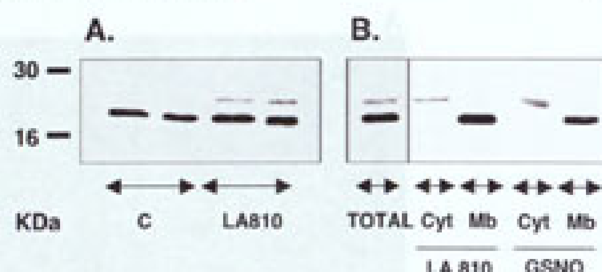

Fig. 7. RhoA protein expression in platelets. (A) Representative immunoblot showing RhoA expression in extracts of whole platelets obtained from non-treated (C) or treated (LA810 and GSNO) animals. Pig platelets show a double-band protein expression when incubated with anti-RhoA antibody showing the cytoplasmatic (inactive) protein. (B) Representative immunoblot showing RhoA distribution in the cytoplasm (Cyt) or membrane (Mb) relative to total protein expression in LA810- and GSNO-treated platelets.

Platelet inhibitory effects were independent of shear and degree of vessel damage. We observed that in both vascular lesions, treatment with LA810 reduces fibrin and PD.

3.7. RhoA protein expression

By Western blot analysis extracts of whole platelets from treated pigs showed a double-band RhoA protein expression (Fig. 7A). After subcellular platelet fractionation (Fig. 7B), cytoplasmatic RhoA expression (inactive form) corresponded to the higher molecular weight (MW) band while membrane RhoA expression (active form) corresponded to the lower MW band. Interestingly, LA810 and GSNO treatment increased expression of the inactive cytoplasmatic form of RhoA.

4. Discussion

The potential therapeutic benefit of NO agents, in particular *S*-nitrosothiols, especially as antiischemic agents in their own right and as anti-hypertensive agents has been the focus of extensive research [33]. Our objective was to study whether a novel *S*-nitrosothiol (LA810) could have an inhibitory effect on platelet deposition and thrombus formation on mildly and severely damaged vessel wall at wall shear rates typical of coronary arteries with no hypotensive episodes. Here, we demonstrate that mural thrombosis is significantly reduced when animals are treated with LA810 without hypotensive side effects. We have used a physiological NO-donor with described platelet specificity, GSNO, as positive control for the potential antiplatelet effect of LA810. GSNO given at equimolar dose showed inhibition in platelet mural thrombosis; however, the concomitant drop

in blood pressure maintained during GSNO infusion limits its therapeutic use.

The beneficial roles ascribed to NO-donors in the vasculature are numerous [14], but they also exhibit some cardiovascular side effects including hypotensive episodes [34] and negative inotropic effects [35]. Several studies have been done in animals, healthy volunteers, and in patients studying the antithrombotic activity and platelet selectivity of GSNO. In healthy volunteers [36] and in some clinical conditions [37] GSNO has proven to inhibit platelet activation and/or aggregation with no concomitant effect on blood pressure. However, proper dose dependent studies to fully study the degree and selectiveness of the antithrombotic activity of GSNO have not been done in patients due to the apprehension of the unwanted and dangerous potential hypotension. Moreover, in some clinical conditions no significant platelet inhibition by GSNO has been observed [38]. This may represent that in these clinical conditions a greater stimulus for platelet activation was present and probably no inhibition could be obtained without affecting blood pressure. In this regard, it would be convenient to have in vivo models able to mimic clinical situations to study the antithrombotic activity of new NO-donors. Only in such way, we could enter clinical development with highly efficient and safer compounds.

The Badimon's chamber seems to be an optimal experimental set up for this since it allows controlling the different variables that regulate thrombus formation, degree of lesion, and local hemodynamics. Thus, it has been useful in this study to differentiate the relative anti-thrombotic and haemodynamic effects of two platelet selective *S*-nitrosothiols (GSNO and LA810) given at equimolar doses.

In our study, LA810 significantly decreased thrombus formation at any degree of vascular damage and shear rate without hypotensive side-effects or heart rate variation. In contrast, as described by Vodovotz et al. [39] in a study in swine following balloon injury, GSNO decreased thrombus formation (except in mildly damaged vessel wall under low shear rate conditions) but with hypotensive side-effects.

Antiplatelet activity of LA810 was also detected in the study of ex vivo platelet aggregation. Platelet aggregation induced by collagen in whole blood was significantly inhibited by LA810 that showed a positive time dependent effect. From these results, we can conclude that the platelet inhibitory properties of LA810 were resistant to the scavenging of NO by haemoglobin in blood, which limits the therapeutic application of most *S*-nitrosothiol compounds as antiplatelet agents, including GSNO [40].

The early and sustained increase on intraplatelet cGMP by LA810 is compatible with the release of NO from LA810, the activation of guanylyl cyclase, and subsequently cGMP formation [14]. On the other hand, it has been previously reported that LA810 at 0.1 μmol/l completely inhibits collagen (0.5–1 μg/ml)-induced human platelet aggregation (washed platelet suspension) [41]. In the present study, this LA810 inhibitory effect was completely prevented by both, a selective inhibitor of NO-sensitive guanylyl cyclase and a NO radical scavenger. These results strongly support that the antiplatelet effect of LA810 is mediated by NO donation.

Additionally, we have also obtained information on the effect of LA810 and GSNO on RhoA. RhoA is a small GTPase involved in many cell functions including cell shape changes [42]. Interestingly, we demonstrated that LA810 and GSNO increased RhoA expression in its inactive form (cytoplasmatic) suggesting that NO regulates RhoA activation, subsequent cytosqueleton organization and platelet passivation. Therefore, RhoA could be an important target for the NO/cGMP inhibitory signalling pathway turned on by NOd and deserves further investigation. The inhibitory effect of the NO/cGMP pathway on RhoA in endothelial cell cultures has also been proposed [43].

We have also shown by immunohistochemical analysis that LA810 decreases not only platelet deposition but the deposition of fibrin that typically forms on injured vessel wall. Although there is not yet a clear explanation for this new finding, it has been described that NO regulates transglutaminases [44], among them FXIII. Because FXIII catalyses cross-linking of fibrin monomers during blood coagulation and stabilizes the blood clot, its inhibition could reduce thrombosis. On the other hand, an effect on the TF-cascade cannot be overlooked because of the importance of the TF-pathway in thrombosis triggered by severely injured vessels [45]. Our results strongly support the view that LA810 not only acts in the haemostatic system inhibiting platelet aggregation and adhesion but also reducing blood coagulation. Indeed, the elongation in prothrombin time in LA810 tested animals may reflect the suppression of TF expression by NO described by others [46].

More experimental studies are needed to further characterize the effects of LA810 on RhoA activity and on TF-pathway since from our results the effects of LA810 on these signal pathways could explain in part its pharmacological activity.

In conclusion, thrombosis is important in the pathogenesis of unstable angina and acute myocardial infarction and here we show that donation of NO by a new *S*-nitrosothiols (LA810) is a good mechanism to reduce mural thrombosis triggered by damaged vessel wall (superficial and deep damage) with effects that are shear-rate independent without hypotensive side effects.

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ARTÍCULO CUARTO

Effects of a novel platelet NO-donor (LA816), aspirin, clopidogrel and combined therapy in inhibiting flow and lesion-dependent thrombosis in the porcine ex vivo model

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Effects of a novel platelet NO-donor (LA816), aspirin, clopidogrel and combined therapy in inhibiting flow and lesion-dependent thrombosis in the porcine *ex vivo* model

Running Title: *Antiplatelet agents, LA816, and thrombosis*

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Abstract

Background: Aspirin (ASA) plus clopidogrel is becoming standard antithrombotic treatment in coronary disease. However, novel approaches such as the use of platelet selective nitric oxide (NO)-donors may also provide additional protection against thrombosis. We evaluated the antithrombotic properties of a novel platelet selective NO-donor (LA816) when 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: I) placebo-control; II) clopidogrel; III) ASA; IV) ASA+clopidogrel (ASA and clopidogrel were given orally, 10 mg/kg day, 3 days). Animals were anesthetized, heparinized, catheterized and the Badimon perfusion chamber was placed in an extracorporeal shunt. After baseline perfusions, all group of animals received the intravenous infusion of LA816 during 2 hours. Platelet aggregation, blood pressure and heart rate were also evaluated throughout the experiments. Either LA816, clopidogrel, and/or ASA+clopidogrel produced a reduction of about 45% on thrombus mass vs 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 nor heart rate.

Conclusions: Acute NO donation with LA816, without modifying hemodynamic parameters, provides the same inhibitory effect to that obtained with chronic treatment with clopidogrel+ASA. Moreover, LA816 provides additional platelet inhibitory effects to those of the combined blockade of cyclooxygenase and P2Y (12) receptor.

Condensed abstract

Antithrombotic properties of a novel NO-donor (LA816) administered alone or in combination with ASA, clopidogrel or ASA+clopidogrel were evaluated. Acute treatment with the NO-donor (LA816), without modifying blood pressure, provided the same platelet inhibitory effects to those of chronic treatment with ASA and clopidogrel.

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, occurring spontaneously or 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 A_2 (TXA_2), ADP , cytokines, producing and promoting surface thrombin generation and releasing vasoconstrictor substances.[2] The effect of all these platelet-active substances is magnified under certain pathological conditions (CAD, diabetes, hiperlipidemia, etc) characterized by endothelial dysfunction leading to reduced synthesis of vasodilatory and antiaggregant factors (NO and prostacyclin). Therefore, blockade of platelet aggregation in addition to anti-ischemic properties may have beneficial effects on coronary vascular function. Among antiplatelet drugs, ASA remains as the standard therapy with a clear clinical benefit.[3] ASA irreversibly acetylates platelet COX and thereby blocks the formation of TXA_2 (a potent vasoconstrictor and platelet aggregant).[4] However, despite its use, recurrent events remain high.[2] Therefore, there has been considerable recent progress in the development of new strategies involving drugs acting on ADP receptors, fibrinogen receptor (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 CAPRIE trial[7] showed overall a modest difference in effectiveness between ASA and clopidogrel (a RR reduction of 8.7%; P=0.043). This suggests that the two drugs have a comparable clinical efficacy. Furthermore, recently the CURE trial[8] concluded that combination of ASA and clopidogrel dramatically reduces platelet activation compared with ASA alone.

Although NO-donors are not specifically antiplatelet agents,[9] they do possess antiplatelet[10] and vasodilator properties.[11] However, the antiplatelet effects of organic nitrates remain controversial because of the suprapharmacological doses required to inhibit platelet aggregation and the subsequent hypotensive side-effects[12] of the available formulations. Contrarily, S-nitrosothiols (S-nitroso-glutathione; GSNO) have significant antiplatelet actions at doses that causes less hemodynamic effects.[13] It has been previously reported that GSNO releases NO by the action of enzymes associated with platelet membranes,[10] therefore, it seems to have platelet selectivity and to inhibit platelet adhesion and aggregation to a greater extent than its effects on vascular tone. In patients with ACS, endothelial and platelet[14] derived NO are impaired probably due to a decreased synthesis of S-Nitrosothiols.[15] Considering the antithrombotic activities

of GSNO and the effectiveness of antiplatelet therapy in preventing serious vascular events,[2] in the inhibition of platelets activity in patients undergoing PCI,[16] and in the inhibition of vasospasm in coronary arteries,[17] a new S-nitrosothiol with antithrombotic activity has been synthesized by Lacer S.A. (Barcelona, Spain).[18] Such encouraging progress to find new and improved drugs for anti-platelet therapy also incites efforts to test new associations between anti-platelet drugs. The present study was designed to: a) assess the antithrombotic/antiplatelet efficacy of a new platelet selective NO-donor (LA816, a non-natural aminoacid S-nitrosilated)[18]; b) to compare its antithrombotic effects to those achieved by the administration of conventional antiplatelet and antithrombotic agents, ASA and clopidogrel; and c) evaluate a possible synergistic effect of LA816 with the former antiplatelet agents. All of three in conditions mimicking vessel wall injury and flow conditions typical of patent and stenosed coronary arteries in a porcine arteriovenous shunt model. We hypothesized that NO donation with LA816 will not only exhibit antithrombotic properties but also provide additional benefits in platelet passivation and 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±2ºC), and humidity-controlled environment with free feeding and access to water. All procedures in this study were performed in accordance with NIH guidelines and followed the American Physiological Society guidelines for animal research.

Experimental Design

Radioactive Labelling of Platelets

After overnight fasting, blood was withdrawn and platelets were labelled with ^{111}In-oxine (^{111}In) (Amersham Biosciences) as previously described.[19] Efficiency was 94.0±1.0% and the injected activity was 252±10 μCi. Post-mortem ^{111}In-biodistribution indicated a correct platelet distribution with maximal accumulation in blood. Serum levels of creatinine, protein, glucose, AST and ALT were measured in both treated groups by routine analytical chemistry assays. Values were within the normal pig range (data not shown).

Ex Vivo Thrombosis

Thrombic risk was assessed by exposure of blood from studied animals, to a thrombus triggering damaged artery in the previously validated and standardized Badimon perfusion chamber. After an overnight fasting, animals were sedated with azoperona (8 mg/kg IM, Stressnil®), anesthetized (10 mg/kg Pentobarbital®, B.Braum) and a carotid artery-jugular vein shunt established to place the Badimon perfusion chamber. Blood was perfused through the chamber for 5 minutes and at two different shear rates of 212s-1 and 1690s-1 typical of patent or mildly stenotic coronary arteries. Homologous porcine vessel wall with two types of damage, severe (ruptured vessel wall) and mild (eroded vessel wall) were used as substrate.

Drug Therapy

This study was performed in 16 pigs (average of 30 perfusions/pig) which were randomly distributed in 4 groups; I) placebo-control (non-treated) group; II) oral administration of clopidogrel (Sanofi-BMS) (10 mg/kg); III) oral administration of ASA (Bayer) (10 mg/kg); IV) combined oral administration of ASA+clopiogrel (10 mg/kg each). A scheme of the experimental protocol is shown in Figure 1 ***(Página 121 de la Tesis).*** Briefly, a daily dosage 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 drug (3rd day) was given 1 hour before starting the perfusions. The non-treated group was kept 3 days under the same conditions. After baseline perfusions (1 h), the four groups of animals were given an intravenous infusion (mammary vein) of LA816 (Lacer®)[18] at an infusion rate of 6.6 nmols/kg/min during 2 hours.

The dose regime of LA816 was selected from previous data obtained in humans treated with GSNO[13] while ASA and clopidogrel dose was based on previous work by other investigators.[21] LA816 was diluted in physiological serum saline and kept refrigerated (4ºC) until administration. No chamber perfusions were performed during the first 30 minutes of the NOd infusion to allow blood biodistribution of the drug

Whole Blood (WB) and Platelet Rich Plasma (PRP) Platelet Aggregation.

WB and PRP impedance platelet aggregation triggered by collagen (3, 5, 10 µg/mL) (Chrono-Log) was measured as previously reported.[22] ASA, clopidogrel, or ASA+clopidogrel effects on platelet aggregation were evaluated before the perfusion experiment started. LA816 effects on platelet aggregation were evaluated during the intravenous infusion.Optical platelet aggregation induced by ADP (3, 5, 10 µmols/L) was measured in PRP (Menarini) as previously described.[21]

In a different series of experiments, the anti-aggregatory actions of the NO-donor drug were examined in the presence or absence of a selective guanylate cyclase inhibitor ODQ ([1 H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-on3]) (1 µmol/L) and Carboxy-PTIO ([2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], a nitric oxide radical scavenger (50 µmols/L). Platelets were incubated with ODQ and Carboxy-PTIO for 10 min at 37ºC prior to addition of LA816 (0.1 µmols/L). Maximal platelet aggregation was measured for 5 min following agonist addition (collagen 3 µg/mL), and expressed as a percentage of control aggregation.

Hemodynamic and Hematological Parameters

Systemic blood pressure and heart rate were monitored via a pressure transducer (Letica) attached to the cannulated femoral artery throughout all the experiments.

Determination of blood cells, hematocrit, platelet number, and size distribution were performed with as System 9000 Serono cell analyzer. Levels of PT and aPTT were monitored with an ST4 automated clotter (Diagnostica Stago) and the corresponding specific kits (American Diagnostica) according to the manufacturer's instructions.

Rho-A Protein Expression in Platelets

Platelets were obtained from blood collected in acid citrate dextrose gently dropped into plastic tubes. Platelet number was adjusted to $4x10^8$ platelets/mL and 750 µL lysis buffer (50 mmols/L Tris/HCl pH 7.4, 1 mmols/L EDTA, 1% Triton-X 100, 1 mmols/L PMSF) were added. Platelet subfraccionation was performed as described.[23] Protein concentration was measured using the bicinchoninic acid method (BCA-Pierce). Equal amounts of protein (25 µg) were subjected to SDS/PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with an appropriate

dilution (1:250) of monoclonal antibody anti-Rho-A (Santa Cruz Biotechnology). After incubation with peroxidase-labelled antimouse immunoglobulin (1:10.000) antibody visualization was performed by the chemiluminiscent method SuperSignal® (Pierce) and evaluated by densitometry.

Statistical Analysis

Results are reported as means ± SEM. The statistical significance of the difference between group means was evaluated by an analysis of variance, and when significant, Student's *t* test was used to assess intergroup differences. Statview software (Abacus Concepts) was used for all statistical tests. $P<0.05$ was considered significant.

Results

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

a) Denuded vessel wall (model of erosion): Effects of the different antiplatelet therapies on thrombus formation are shown in Figure 2 (***Página 123 de la Tesis***). Under low shear rate conditions (low thrombotic risk) (Figure 2A) platelet deposition (PD) is very mild (see control in Fig 2A-B) 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 three-day orally pre-treatment with ASA (40% reduction vs the placebo-control group), clopidogrel (59%), or after intravenous infusion of LA816 (52%) ($P<0.05$ vs control). Similar changes were observed with the possible combinations between ASA/clopidogrel/LA816 (Figure 2D) indicating that the mechanism of deposition in these conditions (platelet adhesion) is not further reduced by simultaneously blocking the different pathways targeted by the drug treatments at the doses used.

b) Disrupted vessel wall: Under high thrombotic risk and shear rate typical of patent coronary arteries (low shear rate; 212/s) (Figure 3A) (***Página 125 de la Tesis***) the ASA-treated group showed non-significant changes in PD (43±5 vs 45±10 $x10^6$ plt/cm^2). In contrast, either LA816 or clopidogrel produced a significant reduction on

mural thrombus formation of about 45% and 58%, respectively (Figure 3A) vs the placebo-control group. Combination of clopidogrel or LA816 with ASA did not further decrease platelet deposition. The combined treatment of oral ASA+clopidogrel and intravenous LA816 produced a non-significant further reduction of 12% 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-D), ASA again produced minimal inhibition on mural thrombus formation (62 ± 10 vs 47 ± 4 $x10^6$ plt/cm^2; $P>0.05$). However, LA816 and clopidogrel showed significant inhibitory effect (45 and 58% vs placebo-control, respectively; $P<0.0001$) (Figure 3C). Combination of ASA with either of these two antiplatelet compounds did not produce an inhibitory synergistic effect (Figure 3D). However, interestingly, the combined treatment of both ASA+clopidogrel and clopidogrel alone with intravenous LA816 produced a significant further reduction of 20% ($P<0.05$) in platelet deposition (70% from placebo-controls; $P<0.0001$), indicating a synergistic antithrombotic effect and a more powerful platelet passivation (Figure 3D).

II. Platelet Studies

a) Effect on Platelet Aggregation

ADP-induced PRP-aggregation was significantly inhibited by clopidogrel ex vivo at any dose of agonist (percentage of inhibition, ≈30% vs non-treated platelet aggregation; $P<0.05$) (Figure 4A-B) (***Página 127 de la Tesis***). Combined oral treatment of ASA+clopidogrel produced a much higher inhibitory effect ($P<0.001$). LA816 and ASA alone did not inhibit platelet aggregation challenged by ADP either in individual or combined treatment (Figure 4A-B). All effects seemed to be due to clopidogrel, an ADP-receptor antagonist.

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

Collagen-induced platelet aggregation in WB was not reduced by clopidogrel, LA816, and ASA (Figure 4E). However, when combining ASA and clopidogrel, inhibitory results were significant. Furthermore, administration of the intravenous infusion of LA816 to

ASA+clopidogrel treated animals further potentiated the reduction on collagen (10 µg/mL)-induced platelet aggregation (Figure 4F).

b) Platelet Inhibitory Effects of LA816 are NO-dependent

Blood was collected from control animals and in vitro added with LA816, with or without the guanylate cyclase inhibitor and the NO radical scavenger as described in methods. The inhibition of guanylate cyclase or the scavenging of NO radicals, abolished the platelet inhibitory effects of LA816 (Figure 5) (***Página 129 de la Tesis***).

c) Platelet Rho-A Protein Expression

We analyzed the effect of LA816 on the level of expression of membrane Rho-A following previously published procedures.[24] We compared Rho-A protein expression levels before and after LA816 intravenous administration in the controls animals (Figure 6) (***Página 131 de la Tesis***). Interestingly, LA816 treatment significantly decreased membrane Rho-A protein expression ($P<0.05$).

III. Follow up Hematological, Hemostatic and Physiological Parameters

a) Effect of Treatment on Hematological and Clotting Parameters

Hematological parameters (Table 1) (***Página 120 de la Tesis***) were similar among the different groups and they were within pig normal intervals. In all animal groups aPTT mean ratio was within the normal range for low level of anticoagulation (mean aPTT ratio: 1.5-2 with 50 U/kg heparin). PT was mildly prolonged in animals treated with LA816 either alone, or combined with ASA and/or clopidogrel.

b) Blood Pressure and Heart rate

Treatment with LA816 only produced a mild, reversible and non-significant reduction of blood pressure of 6 mm Hg (≈9% reduction vs basal value) (Figure 7A) (***Página 133 de la Tesis***) but no hypotensive episodes were detected defined as a fall in mean

arterial pressure of 10 mm Hg. No significant effects were noted on heart rate with after the intravenous treatment with the NOd compound (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) also showed a constant blood pressure (Figure 7C) and heart rate (Figure 7D) throughout all the experiments.

Discussion

This study uses the Badimon perfusion chamber to differentiate the relative anti-thrombotic properties of a new platelet selective NOd (LA816), in comparison to ASA, clopidogrel and combined therapy on mural thrombosis. We have demonstrated that acute administration of LA816, a platelet selective NO-donor drug, has antithrombotic properties that are similar to those of clopidogrel when given chronically. 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 very mild and induces mainly platelet adhesion (less than $5x10^6/cm^2$), a platelet monolayer without growth to mural thrombosis.[25] Consequently, these deposition levels were not affected by the antiaggregatory drugs used in this study as we and others have previously shown.[26] When increasing the shear rate producing a more thrombogenic situation, the antithrombotic effects of LA816 on denuded vessel wall were similar to those of clopidogrel and ASA. 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;[27] although, also in agreement with previous reports, ASA markedly reduced platelet aggregation *in vitro* in Ca^{2+} depleted conditions (aggregometer).[28]

Under the highest thrombogenic stimulus (disrupted vessel and high shear rate) typical of high cardiovascular risk situations, combined treatment of clopidogrel and LA816 was far superior to clopidogrel alone suggesting that LA816 and clopidogrel have synergistic effects in inhibiting thrombus formation. The synergy between these two antiplatelet agents may have been originated from the complementation of the

different platelet activation pathways inhibited by these drugs. Interestingly the blocking of Cox-1 did not change the beneficial antithrombotic profile.

As previously reported,[21] clopidogrel (10 mg/kg) had no anticoagulant effects, as assessed by activated clotting time, which was essentially unchanged.[29] Besides, LA816 caused a significant elongation in prothrombin time suggesting that LA816 not only acts in the haemostatic system inhibiting platelets but also reducing blood coagulation. It was reported that the NO precursor L-arginine inhibits the activation of hemostasis and thereby stabilizes coagulation and platelet function.[30] Furthermore, the elongation in prothrombin time in LA816 tested animals may also reflect the suppression of TF expression by NO described by others.[31]
We have also obtained information on the effect of LA816 on Rho-A. Rho-A is a small GTPase involved in many cell functions including cell shape changes.[32] In accordance with our previous report,[24] NO-donation with nitrosothiols decreased Rho-A expression in its active form (membrane) confirming that NO regulates Rho A activation, subsequent cytosqueleton organization and GPIIb/IIIa activation. More experimental studies are needed to further characterize the effects of LA816 on Rho-A activity and on TF-pathway since from our results the effects of LA816 on these signal pathways could explain in part its pharmacological activity.

In addition, NO also synergizes with thrombolytic agents improving outcomes.[33] ASA has demonstrated inhibition of vessel wall NO and prostacyclin synthesis resulting in vasoconstriction, platelet aggregation and a reduction on thrombolysis;[34] therefore, providing an exogenous source of NO could abrogate these adverse effects improving the environmental milieu of the atherothrombotic process.

Hypotension is probably the most important cardiovascular side effect of NO-donors.[11] In a previous study[24] we observed that GSNO given at equimolar doses caused a significant reduction of mean arterial pressure of 14 mm Hg (≈30% reduction vs basal value) during all the perfusion period. In contrast, LA816 intravenous treatment did not cause any significant variation of blood pressure throughout all the experiment, indicating no concomitant negative hemodynamic effect. Thus, we observe that combining LA816 plus clopidogrel and LA816 plus clopidogrel plus aspirin highly reduces mural thrombus formation, which correlates with inhibition on platelet aggregation, without significant hemostatic or hemodynamic side effects.

According to PCI-CURE and the CREDO trial, in patients with unstable angina and non-ST-segment elevation myocardial infarction (NSTEMI) who are given ASA and are

undergoing percutaneous coronary intervention (PCI), a strategy of clopidogrel pre-treatment is beneficial in reducing major cardiovascular events. Since, in this paper we communicate that acute administration of LA816 provides the same anti-thrombotic protection than 3 days treatment with clopidogrel, acute administration of LA816 could be as beneficial as pre-treatment with clopidogrel in reducing major cardiovascular events.

Because clopidogrel, when added to ASA, increases the risk of bleeding during major surgery in patients who are scheduled for coronary bypass grafting (CABG), clopidogrel should be withheld for at least 5 days and preferably for 7 days before surgery. The acute and intravenous administration of LA816 should be free of this disadvantage.

In summary, our data provides evidence of the antithrombotic properties of this new platelet selective NO-donor and indicates its potential beneficial effects in combination therapy with ADP antagonists and aspirin, treatments of indication against the thrombotic events causing ischemic syndromes.

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