The Antithrombotic Effect of Angiotensin-(1-7) Involves Mas-Mediated NO Release from Platelets

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The antithrombotic effect of angiotensin(Ang)-(1-7) has been reported, but the mechanism of this effect is not known. We investigated the participation of platelets and receptor Mas-related mechanisms in this action. We used Western blotting to test for the presence of Mas protein in rat platelets and used fluorescent-labeled FAM-Ang-(1-7) to determine the specific binding for Ang-(1-7) and its displacement by the receptor Mas antagonist A-779 in rat platelets and in *Mas*$^{-/-}$ and *Mas*$^{+/+}$ mice platelets. To test whether Ang-(1-7) induces NO release from platelets, we used the NO indicator DAF-FM. In addition we examined the role of Mas in the Ang-(1-7) antithrombotic effect on induced thrombi in the vena cava of male *Mas*$^{-/-}$ and *Mas*$^{+/+}$ mice. The functional relevance of Mas in hemostasis was evaluated by determining bleeding time in *Mas*$^{+/+}$ and *Mas*$^{-/-}$ mice. We observed the presence of Mas protein in platelets, as indicated by Western Blot, and displacement of the binding of fluorescent Ang-(1-7) to rat platelets by A-779. Furthermore, in *Mas*$^{+/+}$ mouse platelets we found specific binding for Ang-(1-7), which was absent in *Mas*$^{-/-}$ mouse platelets. Ang-(1-7) released NO from rat and *Mas*$^{+/+}$ mouse platelets, and A-779 blocked this effect. The NO release stimulated by Ang-(1-7) was abolished in *Mas*$^{-/-}$ mouse platelets. Ang-(1-7) inhibited thrombus formation in *Mas*$^{+/+}$ mice. Strikingly, this effect was abolished in *Mas*$^{-/-}$ mice. Moreover, Mas deficiency resulted in a significant decrease in bleeding time (8.50 ± 1.47 vs. 4.28 ± 0.66 min). This study is the first to show the presence of Mas protein and specific binding for Ang-(1-7) in rat and mouse platelets. Our data also suggest that the Ang-(1-7) antithrombotic effect involves Mas-mediated NO release from platelets. More importantly, we showed that the antithrombotic effect of Ang-(1-7) *in vivo* is Mas dependent and that Mas is functionally important in hemostasis.

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INTRODUCTION

Platelets play a critical role in hemostasis and thrombosis, and drugs inhibiting platelet aggregation are valuable for preventing thrombotic events (1). Platelets, which are activated by a wide variety of conditions, adhere to the injured blood vessel wall and subsequently aggregate (2). Those processes may result in the formation of occlusive thrombi in the lumen of the injured vessel. These thrombi are the source of thromboembolic complications of atherosclerosis, such as heart attacks, stroke, and peripheral vascular disease. The platelet surface has receptors for various agents that can modulate its activity and signaling pathways (2,3).

An important platelet-modulating agent is nitric oxide (NO), which exerts antiplatelet actions and plays a crucial role in thrombotic events (4). The NO generated by the endothelium or platelets regulates platelet activation, causing inhibition of adhesion and aggregation (4).

The renin-angiotensin system (RAS) is a major hormonal regulator of blood pressure and hydroelectrolyte balance (5). It also participates in hemostasis (6). The vasoactive peptide angiotensin II (Ang II), considered the main active member of the RAS, has prothrombotic activity associated with increases in the production and secretion of plasminogen activator-inhibitor type 1 (PAI-1) from endothelial and smooth muscle cells and augmentation of tissue factor (TF) expression (7,8). Moreover, platelets also have receptors for Ang II (9), and Ang II potentiates platelet aggregation and activation (10,11).

Another bioactive member of the RAS is Ang-(1-7), now considered a counter-regulator of the cardiovascular effects of Ang II (12). We recently identified the G protein-coupled receptor (GPCR) Mas as a receptor for Ang-(1-7) (13).

Ang-(1-7) antithrombotic activity has been observed in rats, and the antithrombotic effect of ACE inhibitors (ACEi) and angiotensin type 1 (AT1) receptor antagonists may be mediated by Ang-(1-7) (14,15). This effect appears to involve NO and prostaglandin I_2 (PGI_2). Ang-(1-7) promotes NO release from endothelial cells (16-18), suggesting that these cells participate in the Ang-(1-7) antithrom-

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botic effect, but the roles of platelets and Mas in this effect are not known (6).

The aim of this study was to clarify the mechanism involved in the antithrombotic effect of Ang-(1-7). We hypothesized that Ang-(1-7) binds to Mas on the platelet surface and generates NO from these cells. Therefore, we tested for the presence of Ang-(1-7) receptor Mas protein in rat and mouse platelets and investigated Ang-(1-7) binding on these cells. As well, we investigated the effect of Ang-(1-7) on NO release from platelets. Additionally, we assessed whether the antithrombotic effect of Ang-(1-7) is dependent of Mas. Finally, We also evaluated the physiological relevance of Mas in hemostasis by determining the bleeding time in *Mas*$^{-/-}$ and *Mas*$^{+/+}$ mice.

MATERIALS AND METHODS

Animals

The following animals were used: male Wistar rats (270-290 g body weight) and male C57BL/6 mice (15-17 weeks old) bred at Biological Science Institute (CEBIO, Federal University of Minas Gerais); male *Mas* Knockout (*Mas*$^{-/-}$) mice (pure genetic background C57BL/6) and wild-type C57BL/6 control mice (*Mas*$^{+/+}$) (15-17 weeks old) produced at the Max-Delbrück Center for Molecular Medicine, Berlin, Germany, and maintained at the transgenic animal facility in the laboratory of hypertension, ICB-UFMG. Animals were kept in temperature-controlled rooms with a 12/12 hour light/dark cycle and had free access to water and food. The animal care committee from the Federal University of Minas Gerais, Brazil, approved all experimental protocols (number 155/06).

Western Blotting

Platelet preparation. Cardiac puncture was use to collect samples of rat blood into 1:10 anticoagulant buffer (20 mM EDTA in 0.9 % saline). Platelet-rich plasma (PRP) was obtained after centrifugation of blood at 140*g* for 15 min. The PRP was washed two times with a buffer containing 150 mM NaCl, 50 mM Tris-HCl and 20 mM EDTA (pH 7.5). The resulting platelet pellet was lysed with a hypotonic buffer containing 2% Nonidet-P (NP 40) and a protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA) by sonication three times for 3 s on ice. The supernatant obtained after centrifugation at 2000*g* for 15 min at 4°C was used for protein concentration determination by the Coomassie blue method (19) and for subsequent steps.

Electrophoresis and immunoreaction. Protein samples obtained from rat platelets were subjected to electrophoresis on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. Blots were blocked with TBS-T (Tris-base at 3%, Tween 20, pH 7.6) containing 5% nonfat skim milk and incubated overnight at 4°C with the specific polyclonal antibody anti-Mas (1:1000). After sequential incubation with horseradish peroxidade-conjugated goat antimouse, the blots were visualized using a chemiluminescence Western blotting detection reagent (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) on photographic film (Kodak Scientific Imaging Film; Kodak, Rochester, NY, USA).

Binding of Ang-(1-7) on Platelets

The procedure described above was used to obtain PRP from Wistar rats and *Mas*$^{+/+}$ and *Mas*$^{-/-}$ mice. For each experiment PRP was separated in two or three samples (200 µL each) and centrifuged at 1400g for 10 min. The pellet was resuspended in buffer A (150 mM NaCl, 50 mM Tris-HCl, 20 mM EDTA, pH 7.5) and after 10 min submitted to a new centrifugation. The pellet was resuspended in the assay buffer (buffer A plus 0.01% bacitracin, 0.002% phenylmethylsulfonyl fluoride, and 0.01% 1,10-phenanthroline).

Binding of FAM-Ang-(1-7) was performed at 4°C using 2×10^{-9} mol/L FAM-Ang-(1-7) in the presence (nonspecific binding) or absence of 10^{-5} mol/L Ang-(1-7) or 10^{-5} mol/L of the Mas antagonist D-Ala^{7}-Ang-(1-7) (A-779) (total binding) (13,17). During a 60-min incubation the samples were carefully shaken every 15 min. Afterward the samples were washed by centrifugation at 1400*g* for 10 min, resuspended in buffer A, and fixed in a gelatin chromo slide. Relative fluorescence measurements were performed on a Zeiss LSM 510 META laser-scanning confocal microscope excited at 488 nm with an argon-ion laser (oil-immersion objective lens, 63X) and the total fluorescence was quantified by use of Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA). Six images were captured in each slide.

Evaluation of NO Release from Platelets

PRP from rat and *Mas*$^{-/-}$and *Mas*$^{+/+}$ mouse blood samples were centrifuged at 1400*g* for 10 min and the pellet resuspended in calcium-free HEPES/Tyrode's buffer/BSA (20) (10 mM HEPES, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4, 0.1% BSA, and 10 mM EDTA) plus bacitracin at 0.01%, phenylmethylsulfonyl fluoride at 0.002% and 1,10-phenanthroline at 0.01%. This suspension was incubated with the NO indicator 4-amino-5 methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) at 2.5 µmol/L for 30 min and subsequently Ang-(1-7) at 10^{-10} and 10^{-9} mol/L, with or without A-779 at 10^{-7} mol/L, was added. After incubation for 30 min at 37°C, the samples were again centrifuged at 1400*g* for 10 min. The pellet was resuspended in the assay buffer, and the platelets were immobilized in a gelatin chromo slide for evaluation by confocal microscopy. In some experimental protocols, N^{G}-nitro-L-argininemethyl ester (L-NAME) (100 mM) was added 5 min after the incubation with DAF-FM. Fluorescent images were obtained using a Zeiss LSM 510 META laser scanning confocal microscope excited at 488 nm with argon-ion laser (oil-immersion objective lens 63X).

In Vivo Thrombus Induction

To examine the role of Mas in the antithrombotic effect of Ang-(1-7), thrombus formation was induced in male C57Bl/6 *Mas*$^{-/-}$ and *Mas*$^{+/+}$ mice, by a procedure based on two previous studies (14,21). Twelve hours before thrombosis

induction, the mice were deprived of food but had free access to water. After anesthesia (ketamine 100 mg/kg, xylazine 10 mg/kg), the vena cava was exposed and carefully ligated with a cotton thread just below the left renal vein. Filter paper (2 ×5 mm) steeped in a 35% ferric chloride solution was applied below the ligature (21). The C57Bl/6 mice were submitted to intravenous infusion of vehicle, Ang-(1-7) (1, 10, 100, or 1000 pmol/kg/min), A-779 (1000 pmol/kg/min) or Ang-(1-7) (10 pmol/kg/min), combined with A-779 (1000 pmol/kg/min) through the jugular vein, starting 15 min before thrombus induction and lasting for the entire experimental period [$Mas^{-/-}$ and $Mas^{+/+}$ mice received intravenous infusion of vehicle or Ang-(1-7) at 10 pmol/kg/min]. Thirty minutes after application of the ferric chloride solution the thrombus formed was carefully removed and dried at 37°C overnight. The thrombotic response was measured by the thrombus weight.

Bleeding Time

Bleeding times were determined as previously described (22). Briefly, animals were kept in a container and the terminal 5-mm portion of the tail was severed using a razor blade. Bleeding time was measured by a stopwatch to the nearest second using a filter paper carefully applied every 15 s to the adherent drops of blood.

Drugs and Reagents

The following drugs and reagents were used: FAM-Angiotensin I-(1-7) (Phoenix Phamaceutical, St. Joseph, MO, USA), DAF-FM (Molecular Probes, Eugene, OR, USA), Ang-(1-7) (Bachem, Torrance, CA, USA), A-779 (Bachem), N^G-nitro-L-argininemethyl ester (Sigma), Bacitracin (Calbiochem, San Diego, CA, USA), phenylmethylsulfonyl fluoride, (Sigma), 1,10-phenanthroline monohydrate (Sigma), ketamine (Vetanarcol-König, Avellaneda, Argentina), xylasine (Anasedan®, Vetbrands, Jacareí, Brazil), polyclonal anti-Mas antibody (18), secondary antibody goat anti-mouse IgG conjugated-horseradish peroxidase (Bio-Rad, Hercules, CA, USA).

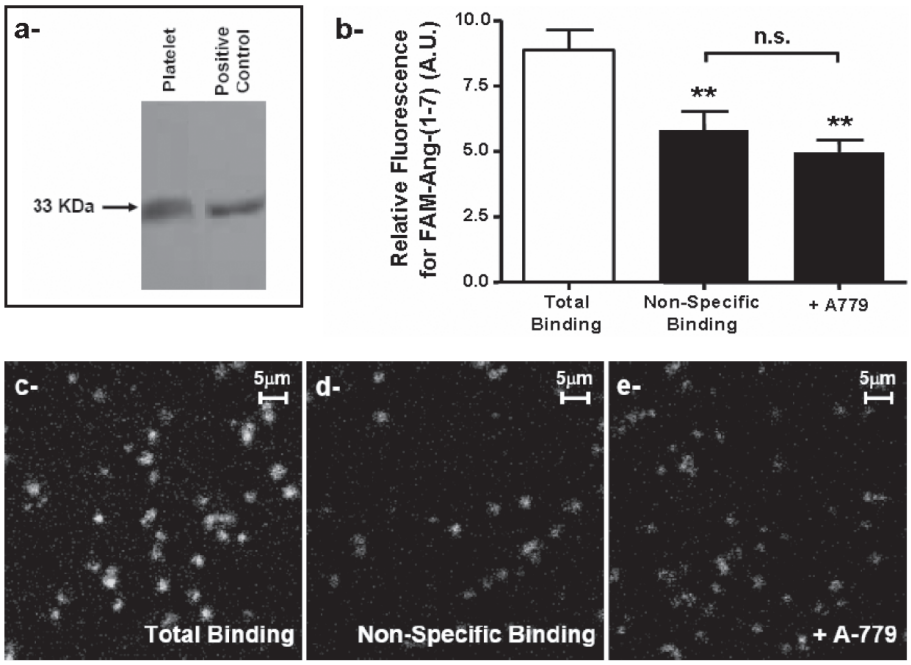

Figure 1. Identification of Mas protein in platelets. (A) Western blot of platelet lysates from Wistar rats were probed with a polyclonal anti-Mas mouse antibody, revealing a single band corresponding to the Mas protein (33 kDa). A positive control sample of Wistar rat testis was also applied to each lane of the the gel. Blots shown are representative of at least three independent experiments controlled for load and detectable protein. For each blot, a total of 30 μg of protein was applied to the gel. (B) Ang-(1-7) binding to platelets. Ang-(1-7) at 10^{-5} mol/L and A779 at 10^{-5} mol/L displaced FAM-Ang-(1-7) at 2×10^{-9} mol/L. Graphic shows the total fluorescence captured. (C,D,E) Confocal photomicrographs of the FAM-Ang(1-7) binding on rat platelets; (C) total binding, (D) nonspecific binding, and (E) Mas-antagonist A-779 displacement of the FAM-Ang-(1-7) binding. $^{**}P < 0.01$, significantly different from the respective control group (Mann Whitney test). Each column represents the mean ± SEM of relative fluorescence in arbitrary unity (A.U.) from four experiments.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis for FAM-Ang-(1-7) binding and bleeding time were performed using the unpaired Student t test. Statistical analysis for NO release and in vivo mouse thrombus formation were performed by one-way ANOVA followed by the Dunnett post-test.

RESULTS

Mas Protein is Present in Rat Platelets

Western-blotting revealed a single band corresponding to the Mas protein (approximately 33 kDa) in protein extracts prepared from isolated rat platelets. A similar band was detected in the testis-positive control sample (Figure 1a).

Ang-(1-7) Binding to Platelets

After Western blotting demonstrated the presence of Mas in platelets, we next tested the specific binding of Ang-(1-7) in these cells. We observed binding of FAM-Ang-(1-7) on rat platelets. As expected, this binding was displaced by Ang-(1-7) and A-779 (Figure 1b). No difference was observed between the binding displaced by Ang-(1-7) and A-779. Furthermore, specific binding of Ang-(1-7) was also observed in $Mas^{+/+}$ mouse platelets. However, no Ang-(1-7) binding was ob-

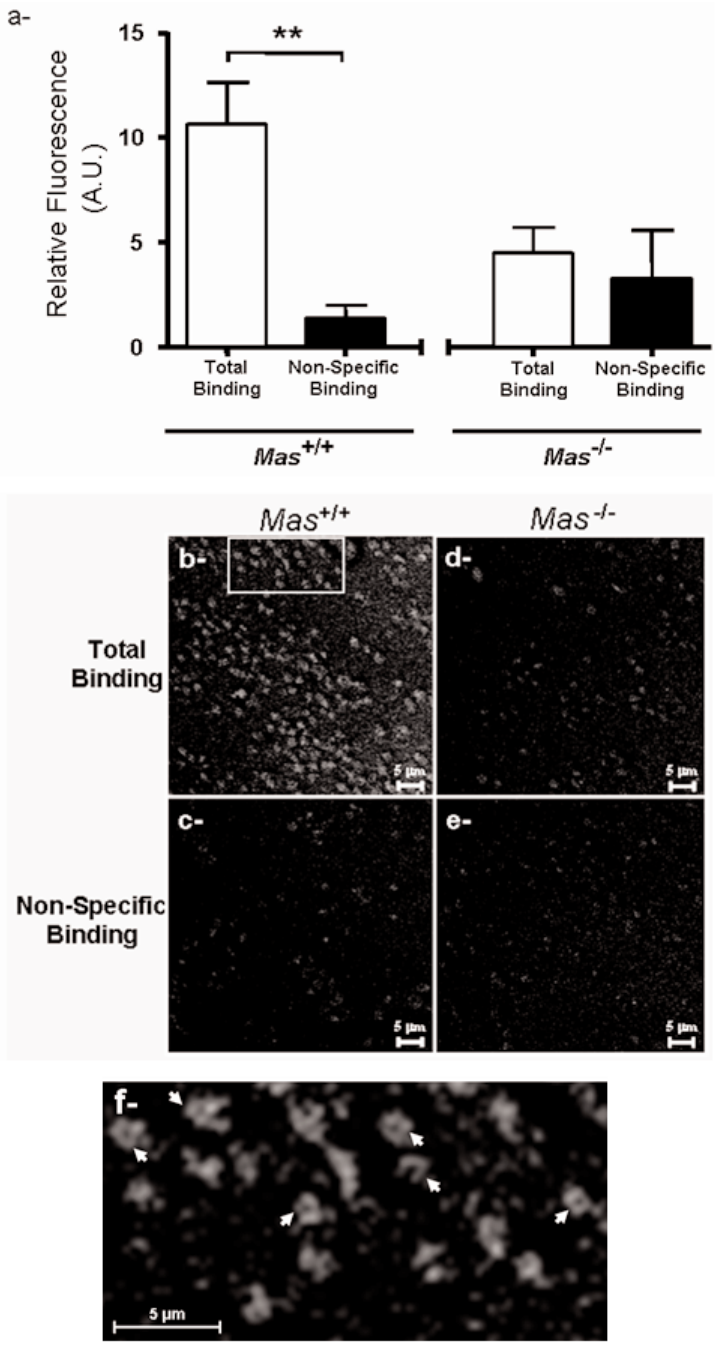

Figure 2. Ang-(1-7) binding on platelets is absent in *Mas*$^{-/-}$ mice. (A) Total fluorescence captured; Ang-(1-7) at 10^{-5} mol/L displaced FAM-Ang-(1-7) at 2×10^{-9} mol/L in *Mas*$^{+/+}$ mouse platelets. Ang-(1-7) binding was absent in *Mas*$^{-/-}$ mice. (B,C,D,E) Confocal photomicrographs of the FAM-Ang(1-7) binding on *Mas*$^{+/+}$ and *Mas*$^{-/-}$ mouse platelets. Total binding (B) and nonspecific binding (C) in *Mas*$^{+/+}$ mouse platelets. Total binding (D) and nonspecific binding (E) in *Mas*$^{-/-}$ mouse platelets. (F) In a digital magnification made from image B, focus planes show the inside layers from the middle or proximal to the surface of cells. The arrows shows FAM-Ang-(1-7) binding to the perimeter of platelets. Fluorescence was also observed inside platelets, suggesting internalization $^{**}P < 0.01$, significantly different from the respective control group (Mann Whitney test). Each column represents the mean ± SEM of relative fluorescence in arbitrary unity (A.U.) from four experiments.

served in *Mas*$^{-/-}$ mice (Figure 2). Under high magnification (digital magnification), FAM-Ang-(1-7) binding on mouse platelets perimeter as well as inside platelets was observed (Figure 2f).

Ang-(1-7) Stimulates NO Production in Platelets via Mas

As shown in Figure 3, Ang-(1-7) induced substantial NO production from rat platelets at 10^{-10} and 10^{-9} mol/L. Moreover the production of NO by Ang-(1-7) was completely blocked by the Ang-(1-7) antagonist A-779 at 10^{-7} mol/L (Figure 4). More important, the NO production stimulated by Ang-(1-7) was abolished in *Mas*$^{-/-}$ mouse platelets, contrasting with the significant NO production in *Mas*$^{+/+}$ mice. In addition, NO release was blocked by L-NAME in *Mas*$^{+/+}$ mice (Figure 5).

The Antithrombotic Effect of Ang-(1-7) Is Mas-Dependent

Infusion of Ang-(1-7) into the jugular vein of C57BL/6 mice at 1, 10, 10^2, or 10^3 pmol/kg/min starting 15 min before thrombus induction produced dose-dependent inhibition of thrombus formation with a maximal effect at 10 pmol/kg/min (0.34 ± 0.03 vs. 0.76 ± 0.12 mg). With higher doses the antithrombotic effect decreased (Figure 6a). The coadministration of A-779 at 1000 pmol/kg/min with Ang-(1-7) at 10 pmol/kg/min completely inhibited the antithrombotic effect of Ang-(1-7) (Figure 6b). To study the participation of Mas in the antithrombotic effect of Ang-(1-7), we administered a dose of 10 pmol/kg/min to male *Mas*$^{-/-}$ and *Mas*$^{+/+}$ mice. The antithrombotic effect of Ang-(1-7) was abolished in *Mas*$^{-/-}$ mice, whereas Ang-(1-7) produced a potent inhibition of thrombus formation in *Mas*$^{+/+}$ mice (*Mas*$^{-/-}$: 0.84 ± 0.09 vs. 0.76 ± 0.15 mg; *Mas*$^{+/+}$: 0.24 ± 0.04 vs. 0.62 ± 0.07 mg) (Figure 6c).

Mas$^{-/-}$ Mice had Decreased Bleeding Time

As shown in Figure 7, the bleeding time was decreased in *Mas*$^{-/-}$ mice (*Mas*$^{+/+}$: 8.50 ± 1.47 vs. *Mas*$^{-/-}$: 4.28 ±

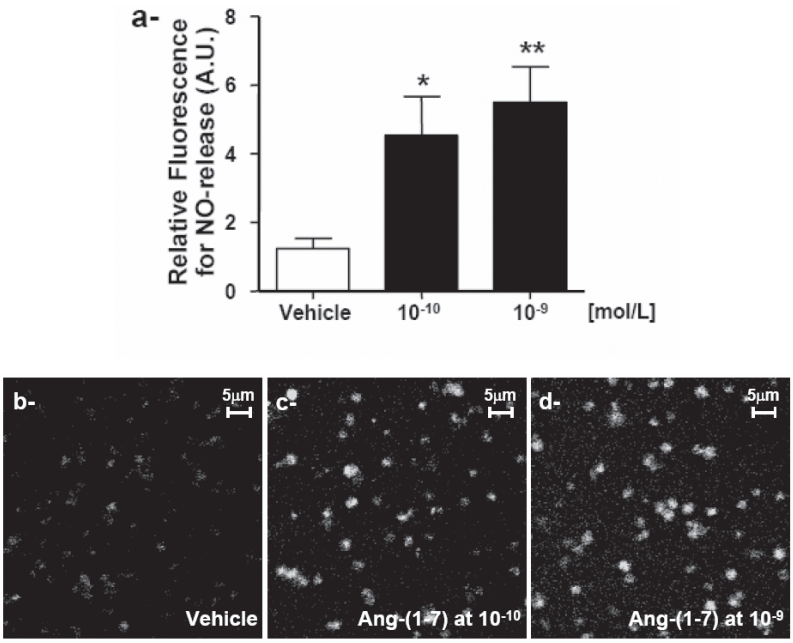

Figure 3. NO production in rat platelets induced by Ang-(1-7). (A) Ang-(1-7) stimulated NO release in isolated rat platelets. (B-D) Confocal photomicrographs showing representative images. (**$P < 0.01$,*$P < 0.05$: one-way ANOVA). Each column represents the mean ± SEM of relative fluorescence in arbitrary unity (A.U.) from four experiments.

0.66 min), suggesting a role of Mas in hemostasis.

DISCUSSION

Our study reports for the first time the presence of the GPCR receptor Mas in platelets and the dependence of the antithrombotic effect of Ang-(1-7) on Mas. Ang-(1-7) has been reported to enhance the antiaggregatory effects of the NO donor sodium nitroprusside (23). Here, we provide evidence that the antithrombotic effect of Ang-(1-7) also involves direct Mas-mediated NO release from platelets.

Many GPCRs present on the platelet surface modulate platelet activity (20). One of the pathways activated by these GPCRs is the phosphorylation of endothelial nitric oxide synthase (eNOS), the main isoform of NOS present in platelets (24). The results of this study showed by Western Blot that the GPCR Mas, identified as a target of Ang-(1-7) (13), is present in platelets. This observation is in keeping with the A-779 displaceable binding of FAM Ang-(1-7) and the absence of Ang-(1-7) binding in *Mas*$^{-/-}$ mouse platelets. This latter observation suggests that the fluorescence observed inside platelets from *Mas*$^{+/+}$ was probably due to internalization of the peptide/receptor complex rather than to non-receptor-mediated internalization of the fluorescent peptide or of products of its degradation.

We have also obtained evidence for the biological significance of the interaction between Mas and Ang-(1-7) in platelets: Ang-(1-7) at low nanomolar concentrations stimulates NO release from platelets, and this effect was completely blocked by the antagonist A-779. More importantly, the NO production stimulated by Ang-(1-7) was abolished in platelets from *Mas*$^{-/-}$, contrasting with the substantial NO production in platelets obtained from *Mas*$^{+/+}$ mice. This observation is in keeping with the identification of Mas in platelets.

NO production was evaluated by the dye DAF-FM, which has a high affinity for NO. One may argue that peroxynitrite

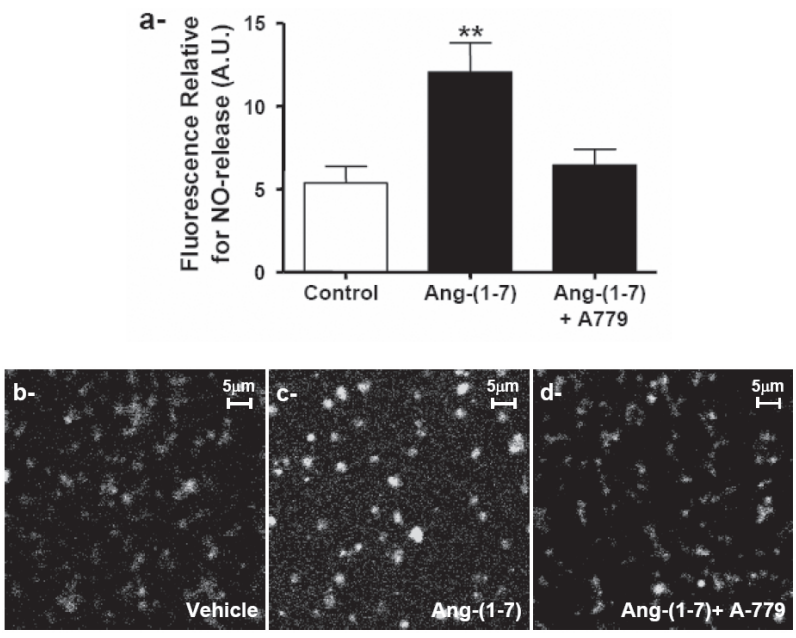

Figure 4. NO production in rat platelets induced by Ang-(1-7) was abolished by A-779. (A) Effect of A-779 on the Ang-(1-7)-stimulated NO release in isolated rat platelets. (B-D) Confocal photomicrographs showing representative images (**$P < 0.01$: one way ANOVA). Each column represents the mean ± SEM of relative fluorescence in arbitrary unity (A.U.) from four experiments.

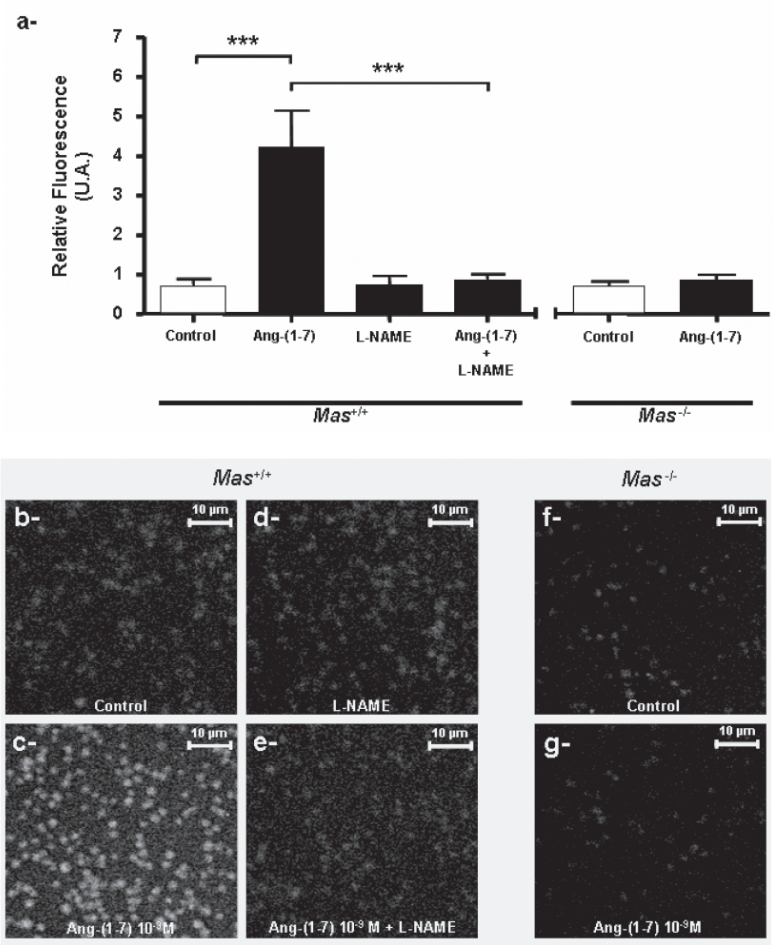

Figure 5. NO release induced by Ang-(1-7) was abolished in platelets from *Mas*$^{-/-}$. (A) Ang-(1-7) at 10^{-9} mol/L stimulates NO release in isolated *Mas*$^{+/+}$ mouse platelets. This effect was absent in *Mas*$^{-/-}$ mouse platelets. Furthermore, Ang-(1-7)-induced NO production was abolished by addition of L-NAME. (B-G) Confocal photomicrographs showing representative images (***$P < 0.001$: one-way ANOVA). Each column represents the mean ± SEM of relative fluorescence in arbitrary unity (A.U.) from four experiments.

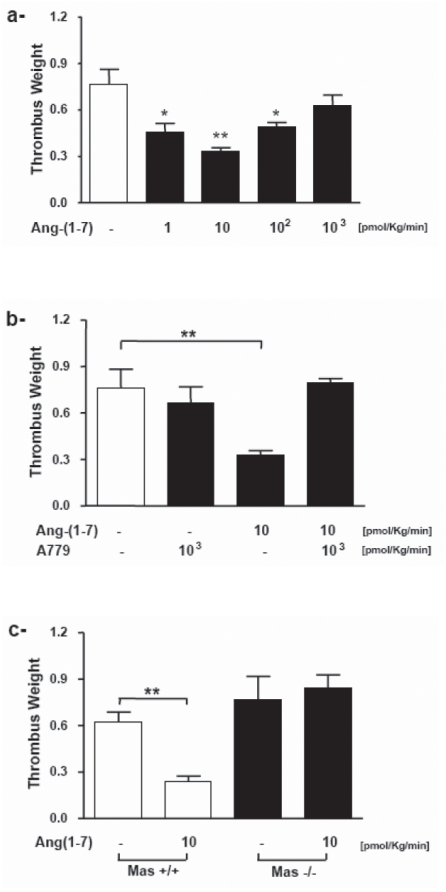

Figure 6. Effect of Ang-(1-7), A-779, and genetic deletion of Mas on the thrombosis induced by 35% ferric chloride solution, *in vivo*. (A) Ang-(1-7) produced a dose-dependent inhibition of thrombus formation in C57Bl/6 mice with a maximal effect at 10 pmol/kg/min (**$P < 0.01$, *$P < 0.05$; one way ANOVA). (B) The antithrombotic effect of Ang-(1-7) was blocked by the Mas antagonist A-779 (**$P < 0.01$; one-way ANOVA). (C) The antithrombotic effect of Ang-(1-7) was abolished in Mas$^{-/-}$ mice (*$P < 0.05$; one-way ANOVA). Each column represents the mean ± SEM from four to six experiments.

or other nitrogen species could also contribute to the fluorescence (25). However, the very weak fluorescence in the absence of Ang-(1-7) stimulation and the blockade of the increase in fluorescence induced by Ang-(1-7) in the presence of L-NAME or in *Mas*$^{-/-}$ platelets are strong evidence against a non-NO-nonspecific fluorescence in our experiment conditions.

We have not attempted to determine in more detail the mechanism of the NO release stimulated by Ang-(1-7). However, the blockade of its effect by L-NAME supports an essential role of NOS in this mechanism. It has been recently demonstrated that Ang-(1-7)-induced NO release in human aortic endothelial cells and in Mas-transfected CHO cells involves phosphorylation of Ser^{1177} of eNOS through a PI3K/Akt-dependent pathway (18). Whether a similar mechanism and/or a Ca^{+2}-dependent mechanism is involved in the NO release induced by Ang-(1-7) in platelets remain to be established.

An essential role of receptor Mas in the Ang-(1-7) antithrombotic effect was demonstrated *in vivo*. The potent antithrombotic effect of Ang-(1-7) in C57Bl/6 mice was completely blocked by A-779 and was absent in *Mas*$^{-/-}$ mice. These observations provided substantial evidence for a critical role of Mas in the antithrombotic effect of Ang-(1-7). Further evidence was provided by the fact that the antithrombotic effect of the heptapeptide in rats was also blocked by the Mas antagonist A-779 (13). Our results, however, do not allow us to ascertain whether the absence of the antithrombotic effect of Ang-(1-7) in Mas$^{-/-}$ is due mainly to the lack of endothelium-mediated mechanism, platelet-mediated NO release, or both.

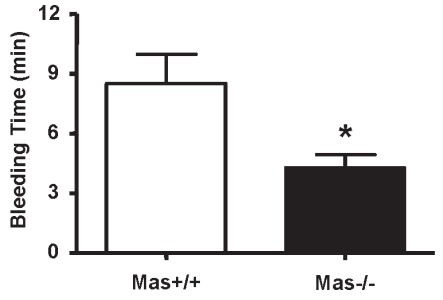

Figure 7. The bleeding time is decreased in *Mas*-deficient mice. The bleeding time was determined in $Mas^{-/-}$ and $Mas^{+/+}$ mice. $Mas^{-/-}$ mice showed a decrease in bleeding time ($Mas^{+/+}$: 8.50 ± 1.47 vs. $Mas^{-/-}$: 4.28 ± 0.66 min; $^{*}P < 0.05$; unpaired Student t test). Each column represents the mean ± SEM from 8 to 11 experiments.

The decrease in the bleeding time in $Mas^{-/-}$ mice suggests a role of Mas in the hemostasis process. However, considering that we did not observe a significant increase in the venous thrombus formation induced by ferric chloride in $Mas^{-/-}$ mice, the decrease in the bleeding time in Mas-deficient animals could be related to a higher local vasoconstriction or to other factors that await investigation.

The U-shaped format of the dose-response curve for the antithrombotic effect of Ang-(1-7) suggests that at higher concentrations the peptide may promote nonspecific effects such as stimulation of AT_1 receptors (26). A similar U-shaped dose-response curve was previously reported in rats (14).

It is well known that NO exerts antiplatelet actions and plays a crucial role in thrombotic events. The NO generated by either endothelium or platelets regulates platelet activation, causing inhibition of adhesion and aggregation (4). It has been demonstrated that Ang-(1-7) promotes NO release from endothelium (16). Furthermore, we have shown that the activation of Mas promotes NO-release in Mas-transfected CHO cells (17). Similar data were obtained for human aortic endothelial cells, which also express Mas (18). In these cells and in Mas-transfected CHO cells, Ang-(1-7)/Mas mediates eNOS stimulatory phosphorylation at Ser^{1177} and dephosphorylation of the inhibitory Thr^{485} residue. Thus, it appears that apart of the PGI_2 release from blood vessels (6), the antithrombotic effect of Ang-(1-7) is mediated by its dual effect on NO-release from both platelets and endothelial cells via interaction with Mas and by amplification of the antithrombotic action of NO (23).

It has been reported that Ang-(1-7) produces antithrombotic effects in renal hypertensive rats and that this heptapeptide appears to mediate the antithrombotic effects of ACEI and ARBs (14). We now provide evidence that the antithrombotic effect of Ang-(1-7) involves Mas-mediated NO release. These observations suggest that the Ang-(1-7)-Mas axis should be considered as a putative target for the development of a new class of drugs for the treatment of thrombotic diseases. Additional studies are needed to further address the significance of the Ang-(1-7)/Mas-pathway in platelets, such as studies assessing the effect of Ang-(1-7) on intraplatelet calcium levels, using $Mas^{-/-}$ platelets in $Mas^{+/+}$ mice or platelet aggregation assays.

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