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RAPID REPORT

Complete blockade of the vasorelaxant effects of angiotensin-(1–7) and bradykinin in murine microvessels by antagonists of the receptor Mas

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Key points

- Two distinct angiotensin-(1–7) [Ang-(1–7)] receptor blockers, A779 and D-Pro-Ang-(1–7), can completely prevent Ang-(1–7)-induced vasorelaxation.
- Genetic deficiency of Mas completely prevents vascular responses to Ang-(1–7).
- Genetic deficiency of Mas completely prevents vascular responses to other NO-dependent vasorelaxants (bradykinin).
- Mas plays a key role in NO-mediated vasodilatation by modulating vasorelaxant-mediated phosphorylation of endothelial nitric oxide synthase in endothelial cells.

Abstract The heptapeptide angiotensin-(1–7) is a biologically active metabolite of angiotensin II, the predominant peptide of the renin–angiotensin system. Recently, we have shown that the receptor Mas is associated with angiotensin-(1–7)-induced signalling and mediates, at least in part, the vasodilatory properties of angiotensin-(1–7). However, it remained controversial whether an additional receptor could account for angiotensin-(1–7)-induced vasorelaxation. Here, we used two different angiotensin-(1–7) antagonists, A779 and D-Pro-angiotensin-(1–7), to address this question and also to study their influence on the vasodilatation induced by bradykinin. Isolated mesenteric microvessels from both wild-type and Mas-deficient C57Bl/6 mice were pre-contracted with noradrenaline, and vascular reactivity to angiotensin-(1–7) and bradykinin was subsequently studied using a small-vessel myograph. Furthermore, mechanisms for Mas effects were investigated in primary human umbilical vein endothelial cells. Both angiotensin-(1–7) and bradykinin triggered a concentration-dependent vasodilatation in wild-type microvessels, which was absent in the presence of a nitric oxide synthase inhibitor. In these vessels, the pre-incubation with the Mas antagonists A779 or D-Pro-angiotensin-(1–7) totally abolished the vasodilatory capacity of both angiotensin-(1–7) and bradykinin, which was nitric oxide mediated. Accordingly,

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Mas-deficient microvessels lacked the capacity to relax in response to either angiotensin-(1–7) or bradykinin. Pre-incubation of human umbilical vein endothelial cells with A779 prevented bradykinin-mediated NO generation and NO synthase phosphorylation at serine 1177. The angiotensin-(1–7) antagonists A779 and D-Pro-angiotensin-(1–7) equally block Mas, which completely controls the angiotensin-(1–7)-induced vasodilatation in mesenteric microvessels. Importantly, Mas also appears to be a critical player in NO-mediated vasodilatation induced by renin–angiotensin system-independent agonists by altering phosphorylation of NO synthase.

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Abbreviations A779, D-Ala^7-angiotensin-(1–7); ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang I, angiotensin I; Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7); Ang-(1–9), angiotensin-(1–9); AT$_1$ receptor, angiotensin II type 1 receptor; AT$_2$ receptor, angiotensin II type 2 receptor; BK, bradykinin; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; NA, noradrenaline; NOS, nitric oxide synthase; RAS, renin–angiotensin system.

Introduction

The renin–angiotensin system (RAS) was initially identified as an important regulator of arterial blood pressure, electrolyte homeostasis and water and sodium intake (Weir & Dzau, 1999; Wilson *et al.* 2005), but the RAS is also involved in the regulation of tissue regeneration, cellular proliferation and growth factor release (Unger *et al.* 1996; Takeda *et al.* 2004), as well as haematopoietic recovery after myelosuppression and progenitor engraftment (Rodgers *et al.* 2003; Ellefson *et al.* 2004). Renin metabolizes angiotensinogen to form the biologically inactive decapeptide, angiotensin I (Ang I). Angiotensin I is then cleaved by angiotensin-converting enzyme (ACE) to yield the main active peptide of the RAS, angiotensin II (Ang II). Angiotensin II is well known as a potent regulator of vasoconstriction, water intake and salt metabolism and is well known to worsen cardiovascular function under pathological conditions. Recently, a homologue of ACE, termed angiotensin-converting enzyme 2 (ACE2), has been identified (Donoghue *et al.* 2000; Tipnis *et al.* 2000). ACE2 cleaves a single peptide from either Ang I or the octapeptide Ang II to form angiotensin-(1–9) [Ang-(1–9)] or angiotensin-(1–7) [Ang-(1–7)], respectively (Vickers *et al.* 2002). In the last decade, Ang-(1–7) has become a peptide of interest, because it can counter-regulate adverse effects of Ang II (Freeman *et al.* 1996; Strawn *et al.* 1999; Zhu *et al.* 2002). We identified the G protein-coupled receptor Mas to be associated with Ang-(1–7)-induced signalling, demonstrating that genetic deletion of Mas, encoded by the *Mas* proto-oncogene, abolishes the binding of Ang-(1–7) to mouse kidney (Santos *et al.* 2003). This binding could be blocked by cotreatment with D-Ala^7-angiotensin-(1–7) (A779), a specific Ang-(1–7) antagonist. Furthermore, Mas-deficient aortas lost their Ang-(1–7)-induced relaxation response (Santos *et al.* 2003).

We recently demonstrated that isolated microvessels lacking *Mas* showed significantly impaired dilatation not only in response to Ang-(1–7), but also in response to other endothelium-dependent vasodilators, including bradykinin (BK) and acetylcholine (Peiró *et al.* 2007). These results indicate that *Mas* deficiency may lead to microvascular endothelial dysfunction. We found that the concentration-dependent relaxation induced by Ang-(1–7) in vessels isolated from *Mas*-deficient mice was diminished by ∼40%. Similar to Mas-deficient vessels, the Ang-(1–7)-mediated relaxation was reduced by A779 in vessels from wild-type mice. Nevertheless, a residual relaxation in both wild-type vessels pretreated with A779 and *Mas*-deficient vessels was observed in response to Ang-(1–7) (Peiró *et al.* 2007). These data suggested that Ang-(1–7) may also interact with an additional specific receptor other than Mas, as speculated recently (Silva *et al.* 2007).

In order to investigate the presence of an additional receptor for Ang-(1–7) further, we examined in detail the response of isolated mesenteric vessels of *Mas*-deficient and wild-type mice to Ang-(1–7), BK and noradrenaline (NA), after a pretreatment with A779, D-Pro-Ang-(1–7), another specific Ang-(1–7) antagonist (Lemos *et al.* 2005), or the nitric oxide synthase (NOS) inhibitor L-NAME.

Methods

Animals

Six-month-old male C57Bl/6 mice or *Mas*-deficient mice on a C57Bl/6 background (Esteban *et al.* 2009) were used in the experiments. Animals were maintained

under standardized conditions with an artificial 12 h–12 h dark–light cycle, with free access to food and water. All animal studies were performed according to national guidelines and were approved by the institutional animal care and ethics committees.

Vascular reactivity experiments in mesenteric microvessels

Mice were anaesthetized with 70 mg kg^{-1} I.P. sodium pentobarbital and exsanguinated. Third-branch mesenteric arteries (mean internal diameter ranging between 150 and 200 μm) were mounted as ring preparations on a small-vessel myograph to measure isometric tension as described before (Vallejo *et al.* 2000; Peiró *et al.* 2007). Arteries were contracted with 10 μmol l^{-1} noradrenaline (NA; Sigma, St Louis, MO, USA), and then the vasoactive responses to Ang-(1–7) (Bachem, Bubendorf, Switzerland; 1 pmol l^{-1} to 1 μmol l^{-1}) or bradykinin (BK; Sigma; 1 nmol l^{-1} to 30 μmol l^{-1}) were tested by adding increasing concentrations of the drugs. In an acute experiment, the vasoactive response to a single concentration of Ang-(1–7) (1 μmol l^{-1}) was investigated in both wild-type and Mas-deficient vessels. In some cases, the mesenteric segments were preincubated for 15 min with the Mas antagonists A779 (Bachem; 1 μmol l^{-1}) or D-Pro-Ang-(1–7) (Biosyntan GmbH, Berlin, Germany; 1 μmol l^{-1}) or with the NOS inhibitor L-NAME (100 μmol l^{-1}) before addition of NA. In a parallel set of experiments, microvessels were preincubated with or without L-NAME (100 μmol l^{-1}), contracted with NA and then maintained without any additional treatment throughout the duration of the experiment (time control). In another set of experiments, concentration-dependent curves to NA (10 pmol l^{-1} to 100 μmol l^{-1}) were performed, either in control conditions or in the presence of L-NAME, A779, or both.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were prepared essentially as described previously (Peiró *et al.* 2007). In brief, HUVECs were obtained by collagenase digestion from umbilical cords provided by Hospital Universitario de Getafe, as approved by the Hospital Ethics Committee. The HUVECs were cultured in M199 medium supplemented with 10% fetal calf serum, 25 mg ml^{-1} endothelial cell growth supplement and 100 mg ml^{-1} heparin. For experiments, cultures between passages one and five were used. The cells were pretreated with either A779 (1 μmol l^{-1}) or saline for 15 min.

In order to investigate the impact of the pretreatment on NO generation and ligand–receptor-induced phosphorylation of endothelial nitric oxide synthase (eNOS), such pretreated cells were stimulated for 0, 1, 5, and 10 min with BK (10 μmol l^{-1}).

Measurement of NO production in HUVECs

Intracellular NO was monitored with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-2-FM diacetate; Molecular Probes, Invitrogen, Madrid, Spain), a fluorescence indicator of NO that emits fluorescence in response to a reaction with NO. To measure intracellular NO, HUVECs were seeded on 24-well plates. At 80% confluence, cells were loaded with 2.5 μmol l^{-1} DAF-2-FM in M199 without Phenol Red for 30 min, according to the manufacturer's instructions. After loading, cells were rinsed three times with culture medium and exposed to the desired treatments. To quantify the DAF-related fluorescence, the cells were observed under an inverted fluorescence Nikon Eclipse Ti-S microscope. Fluorescence from five different fields per well was measured (excitation wavelength 488 nm; emission wavelength 515 nm). Fluorescence signals were quantified using NIS-Elements 3.0 software (Nikon Izasa SA, Barcelona, Spain).

Western blotting

The activation of eNOS was assessed through phospho-eNOS (serine 1177) levels. The HUVECs were lysed in lysis buffer (10 mmol l^{-1} Tris, pH 7.4; 1% sodium dodecyl sulphate; 10 mmol l^{-1} sodium orthovanadate; 2 mmol l^{-1} phenylmethylsulphonyl fluoride; and 12.5 mg ml^{-1} aprotinin), and the protein concentration was determined with a BCA Protein Assay Kit (Perbio Science GmbH, Bonn, Germany) according to the manufacturer's instructions. Western blot was performed as described previously (Gembardt *et al.* 2005*a*). In brief, proteins and a prestained protein-weight marker (Amersham Biosciences GmbH, Freiburg, Germany) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were incubated with rabbit polyclonal antibodies against phospho-eNOS (serine 1177; dilution 1:1000; Cell Signalling, Cambridge, UK) and total eNOS (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution 1:5000; Bio-Rad Laboratories, Hercules, CA, USA), and bands were detected by enhanced chemiluminescence (Amersham Biosciences GmbH, Freiburg, Germany). The bands on the X-ray film were quantified by densitometry scanning with free ImageJ software (National Institute of Health, http://rsb.info.nih.gov/ij/). Normalized results were expressed as the phospho-eNOS (Ser 1177) to total eNOS ratio.

Statistical analysis

Results were expressed as means ± SEM. Deviations from the mean regarding the curves to drugs were statistically analysed using factorial two-way ANOVA. Western blot data were analysed by unpaired Student's t test. Significance was considered from a value of $P < 0.05$.

Results

Mas deletion or pharmacological blockade blunts the vasorelaxation in response to Ang-(1–7) and BK in mesenteric microvessels

The vasodilator effects of Ang-(1–7) and BK were studied in murine mesenteric microvessels, precontracted with NA. The vasoconstrictor responses to NA were similar in wild-type and Mas-deficient microvessels (4.18 ± 0.23 and 4.14 ± 0.23 mN, respectively; results from 13–15 animals). In wild-type microvessels, Ang-(1–7) elicited a concentration-dependent relaxation; this response was significantly impaired in microvessels lacking the receptor Mas, although a residual relaxing effect was still observed (Fig. 1*A*). Likewise, Ang-(1–7)-induced vasorelaxation was impaired in wild-type microvessels pretreated with the Mas receptor antagonist A779 (Fig. 1*A*). The impairment observed in Mas-deficient and wild-type microvessels pretreated with A779 was statistically equivalent.

Analogously, both Mas deficiency and pharmacological blockade with A779 resulted in an equal impairment of the concentration-dependent vasorelaxation triggered by the RAS-independent vasodilator BK (Fig. 1*B*). Again, a residual vasorelaxant response to BK after Mas deletion or blockade was still observed (Fig. 1*B*).

Murine mesenteric microvessels undergo a non-specific loss of tension mediated by NO

To understand the origin of the residual vasorelaxant responses to Ang-(1–7) or BK, parallel experiments were performed, in which arteries were contracted with NA and maintained without being subsequently exposed to Ang-(1–7) or BK. As shown in Fig. 1*C*, a spontaneous and gradual vasorelaxation was equally observed in both wild-type and Mas-deficient microvessels throughout the duration of the experiment. In fact, such spontaneous vasodilatation was not significantly different from the residual vasodilatation in response to Ang-(1–7) (Fig. 1*A*) and BK (Fig. 1*B*) observed after Mas deletion or blockade. The spontaneous loss of tension in both wild-type and Mas-deficient microvessels was dependent on NO release, because it was totally prevented by preincubation with the NOS-inhibitor L-NAME (Fig. 1*C*).

In order to provide further proof that there was no agonist-specific vasodilatation independent of Mas,

we investigated the vasorelaxant effect of a single concentration of Ang-(1–7) ($1\ \mu\mathrm{mol\ l^{-1}}$) on wild-type and Mas-deficient mesenteric arteries. The time course of this experiment did not allow for a significant spontaneous loss of tension to take place. While Ang-(1–7) induced a marked reduction of vascular tone in untreated wild-type mesenteric arteries (Fig. 1*D*), no significant vasoactive responses were observed in A779-pretreated or Mas-deficient vessels. Based on all of these observations, it appeared that the remaining vasorelaxant response to either Ang-(1–7) or BK observed in Mas-deficient or A779-pretreated wild-type microvessels in a concentration–response experiment (Fig. 1*A* and *B*) was unspecific and not mediated by the agonists themselves.

Effect of different Mas antagonists on Ang-(1–7)- and BK-induced relaxation in mesenteric microvessels

We next compared the ability of two different Mas antagonists, A779 and D-Pro-Ang-(1–7), to block the vasorelaxation specifically induced by Ang-(1–7) and BK. In both wild-type and Mas-deficient microvessels, the specific vasorelaxation was calculated as the difference between the total vasorelaxation observed in the presence of the agonist and the spontaneous vasorelaxation.

Figure 2*A* shows the effect of A779 and D-Pro-Ang-(1–7) on the vasodilatation induced by Ang-(1–7) in wild-type mesenteric microvessels. The pre-incubation with A779 completely blocked the vasorelaxation elicited by Ang-(1–7) at any concentration used. A similar effect was observed following pre-incubation with D-Pro-Ang-(1–7), either alone or in combination with A779 (Fig. 2*A*). The vasorelaxant response elicited by Ang-(1–7) in wild-type vessels was totally dependent on NO release, because it was blocked by pre-incubation with L-NAME (Fig. 2*A*). In microvessels lacking the receptor Mas, where Ang-(1–7) showed no vasorelaxant effects, vascular reactivity was not modified by L-NAME, A779 or D-Pro-Ang-(1–7) or by both antagonists in combination (Fig. 2*B*).

Importantly, the relaxation induced by BK in wild-type microvessels was also completely abolished by pre-incubation with A779 and D-Pro-Ang-(1–7), either alone or in combination (Fig. 3*A*). As observed for Ang-(1–7), the vasodilatation induced by BK in wild-type microvessels was completely prevented by pre-incubation with L-NAME (Fig. 3*A*). Again, in the absence of a vasorelaxant response to BK due to Mas deficiency, neither A779 nor D-Pro-Ang-(1–7), alone or in combination, exerted any vasoactive effect (Fig. 3*B*). L-NAME did not modify vascular reactivity in *Mas*-deficient mice stimulated with BK (Fig. 3*B*).

Effects of the Ang-(1–7) receptor antagonist A779 on NO production and eNOS phosphorylation in HUVECs

To evaluate whether the *ex vivo* findings correlate with *in vitro* effects, we investigated the impact of A779 on NO production in HUVECs. While A779 alone did not affect NO production over the 10 min of the experiments, BK increased the NO production in comparison to control cells. However, this significant increase was completely blunted by pretreatment with A779 (Fig. 4*A*). We then analysed eNOS phosphorylation in response to A779 treatment in HUVECs in order to characterize a potential mechanism responsible for both

the blockade of BK-stimulated NO production in cell culture and the endothelial dysfunction observed in mice with genetic deficiency in Mas or after pharmacological Mas blockade. Treatment of primary HUVECs with A779 did not influence phosphorylation at serine 1177, used as a marker of eNOS activation. Notably, the phosphorylation pattern did not change for the control cells and A779-treated cells over the time of the experiment (data not shown). Congruent to NO production, BK stimulated the phosphorylation at serine 1177 as early as at 1 min. Pretreatment with A779 completely prevented the BK-mediated serine 1177 phosphorylation (Fig. 4*B*).
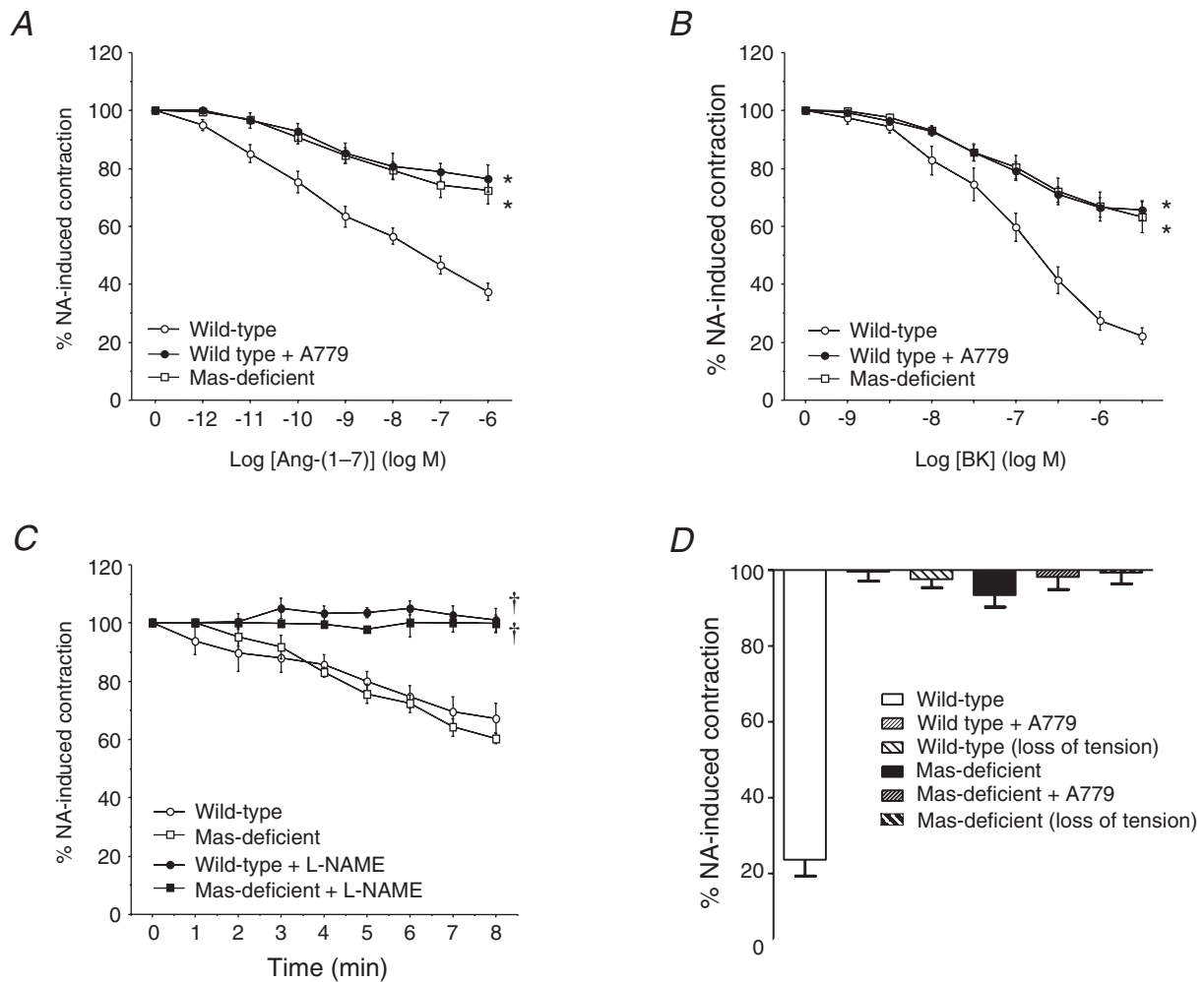

Figure 1. Effect of genetic Mas deficiency or its pharmacological blockade on vascular reactivity
Vasorelaxant responses to angiotensin-(1–7) [Ang-(1–7); 1 pmol l^{-1} to 1 mmol l^{-1}; *A*] and bradykinin (BK; 1 nmol l^{-1} to 30 μmol l^{-1}; *B*) in Mas-deficient or wild-type microvessels preincubated with the Mas antagonist A779 (1 μmol l^{-1}). *C*, after the initial contraction with noradrenaline (NA), the isolated microvessels undergo a spontaneous loss of tension over time, which is similar between wild-type and Mas-deficient segments. Some of the segments used for time control were preincubated with L-NAME (100 μmol l^{-1}). The vasoactive responses are expressed as a percentage of a previous contraction elicited by NA (10 μmol l^{-1}). For each curve, results are shown as means ± SEM of 10–15 mesenteric microvessels from at least 5 different animals. *D*, acute vasorelaxant effect of a single concentration of Ang-(1–7) (1 μmol l^{-1}) on wild-type and Mas-deficient mesenteric arteries, with and without A779 preincubation. *P < 0.05 vs. wild-type microvessels; †P < 0.05 vs. the respective time control without L-NAME.

None of the treatments changed the total eNOS levels over the 10 min of the experiment (data not shown).

Response to NA in Mas-deficient and wild-type microvessels pretreated with Ang-(1–7) receptor antagonists

In order to investigate whether the reduced eNOS activity in response to A779 might have an influence not only on vasorelaxant agents, but also on vasoconstrictor substances due to less counteracting NO release, we analysed the impact of the Ang-(1–7) receptor antagonist A779 and the NOS inhibitor L-NAME on the vascular effects of NA. In microvessels from wild-type animals, NA led to a concentration-dependent constriction of the mesenteric arteries (Fig. 5A). After pretreatment with the Ang-(1–7) receptor antagonist A779 or L-NAME, the wild-type vessels showed a significantly increased sensitivity to NA stimulation (Fig. 5A), whereas none of the pretreatments could influence the response to NA in Mas-deficient vessels (Fig. 5B). This difference is caused by the fact that the Mas-deficient vessels already showed a significantly higher susceptibility to NA in baseline conditions (Fig. 5C).

Discussion

Previous studies led to a controversial discussion about the receptor(s) mediating Ang-(1–7) effects. Some publications suggested an involvement of the classical Ang II receptors, AT$_1$ and AT$_2$ (Gironacci *et al.* 1994, 2004; Handa *et al.* 1996), while other groups discussed a role for the BK B$_2$ receptor in mediating Ang-(1–7)-stimulated effects (Heitsch *et al.* 2001). However, most reports indicated an independent Ang-(1–7) receptor, because defined actions of the heptapeptide could be completely blocked by A779, a specific Ang-(1–7) antagonist (Ambuhl *et al.* 1994; Santos *et al.* 1994). Our group identified the G protein-coupled receptor Mas to be associated with Ang-(1–7)-induced signalling, demonstrating that genetic deletion of Mas, encoded by the *Mas* proto-oncogene, abolished the binding of Ang-(1–7) to mouse kidney (Santos *et al.* 2003). Accordingly, Ang-(1–7) was able to bind to *Mas*-transfected cells and elicited arachidonic acid release, which was completely blocked by A779.

Previously, we generated data implying that part of the Ang-(1–7)-mediated vasorelaxant effects might be Mas independent (Peiró *et al.* 2007), because Ang-(1–7) induced a residual relaxation in both wild-type vessels pretreated with A779 and *Mas*-deficient vessels. These observations suggested that Ang-(1–7) may also interact with an additional receptor other than Mas to elicit vasodilatation, as speculated by others recently (Silva *et al.* 2007). In the present study, we wanted to investigate the mechanism of this Mas (A779)-independent vasorelaxant effect further. Interestingly, we have clearly demonstrated that the remaining vasorelaxant effect of Ang-(1–7) in
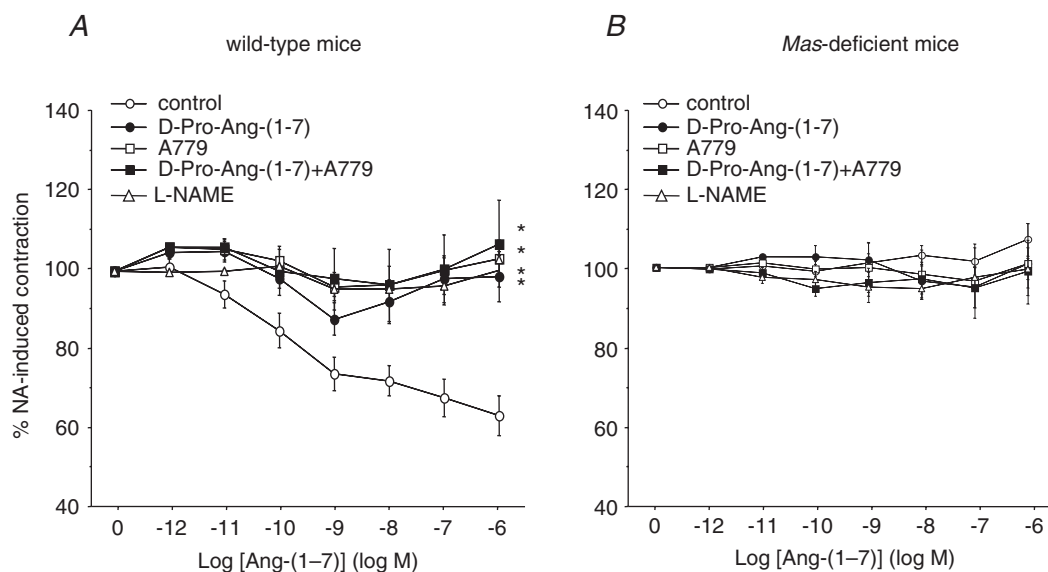

Figure 2. Effect of the Mas antagonists A779 and D-Pro-Ang-(1–7) on the vasodilatation elicited by Ang-(1–7)
Both wild-type (*A*) and Mas-deficient microvessels (*B*) were exposed to Ang-(1–7) (1 pmol l^{-1} to 1 μmol l^{-1}), after pre-incubation with or without A779 (1 μmol l^{-1}), D-Pro-Ang-(1–7) (1 μmol l^{-1}), both antagonists or L-NAME (100 μmol l^{-1}). The vasoactive responses are expressed as a percentage of a previous contraction elicited by NA (10 μmol l^{-1}). The curves are normalized to the loss of tension over time. For each curve, results are shown as means ± SEM of 10–15 mesenteric microvessels from at least 5 different animals. *P < 0.05 *vs.* wild-type microvessels.

Mas-deficient or A779-pretreated wild-type mesenteric arteries is due to a spontaneous loss of tension of the system over time that is completely independent of Ang-(1–7). Such relaxation, which is equivalent in both types of microvessels, depends on the release of NO and is not triggered by the heptapeptide. We could generate further proof that there is no remaining vasorelaxation that is independent of Mas (after subtracting the spontaneous loss of tension) by performing an acute experiment with a single concentration of Ang-(1–7) on wild-type and Mas-deficient mesenteric arteries. Given that no significant loss of tension occurred in this experimental setting, the marked reduction of vascular tone induced by a single concentration of Ang-(1–7) was completely blunted in A779-pretreated vessels or Mas-deficient vessels.

In a previous report, from a study performed in rat mesenteric microvessels, Neves *et al.* (2003) observed a similar spontaneous vasorelaxation over time, although the authors did not identify the vasodilator agent involved. Our finding that Ang-(1–7)-mediated vasorelaxation depends completely on the receptor Mas is congruent with data from our group, excluding an influence of the Ang II receptors (AT$_1$ and AT$_2$) on the Ang-(1–7)-stimulated vasorelaxation in an acute *in vivo* animal model (Gembardt *et al.* 2012).

In contrast to the work by Silva *et al.* (2007), we did not find different effects for A779 and D-Pro-Ang-(1–7), another specific Ang-(1–7) antagonist (Lemos *et al.*

2005). In our hands, both antagonists are equally potent, and they completely abolish the vasorelaxant effects specifically induced by the heptapeptide in our *ex vivo* experiments. The data suggest that both Ang-(1–7) antagonists target the same receptor, and thus indicate that there is only a single receptor (complex) triggering the Ang-(1–7)-induced vasorelaxant effects.

In the present study, we have also shown that the blockade of Mas with either A779 or D-Pro-Ang-(1–7) not only abolished Ang-(1–7)-induced vasodilatation, but also completely prevented the vasodilatation induced by a RAS-independent vasodilator peptide, namely BK. However, microvessels genetically deficient in the receptor Mas lose the capacity to respond to BK. This observation, together with previous results from our group (Peiró *et al.* 2007), strongly suggests that Mas has a broader vascular action beyond mediating the actions of Ang-(1–7). Indeed, the presence of functional Mas appears to be a critical element for the microvessels to respond to a series of endothelium-dependent vasodilators, including Ang-(1–7), BK, and ACh. All three vasodilators trigger the release of endothelial NO to induce relaxation of the underlying smooth muscle. In the present study, we found that the effect of pharmacological blockade of Mas with either A779 or D-Pro-Ang-(1–7) on Ang-(1–7)-induced vasodilatation was equivalent to that obtained with the NO synthase inhibitor L-NAME. In accordance, Silva *et al.* (2007), have
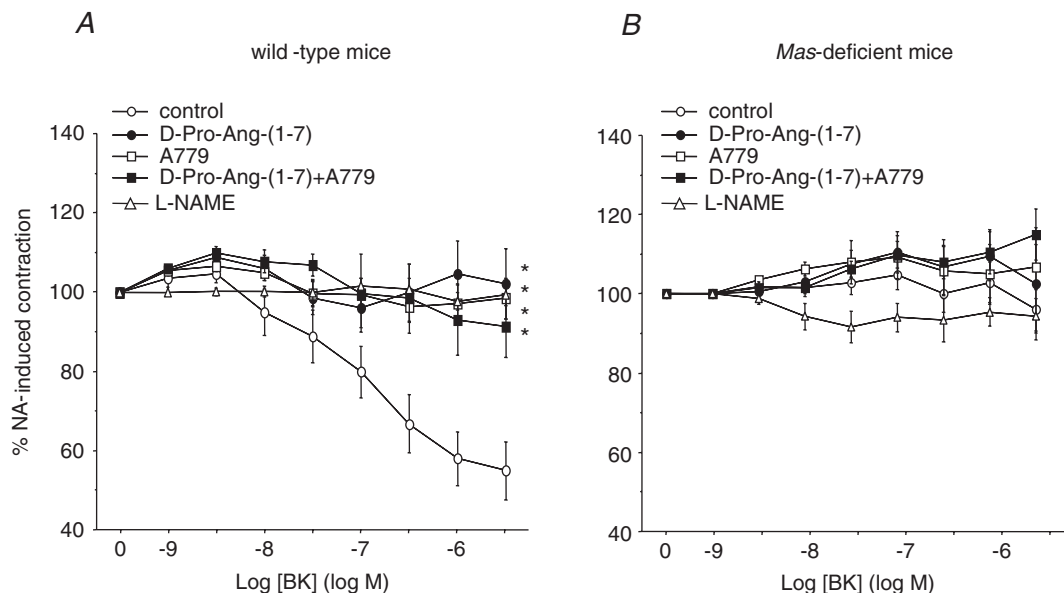
Figure 3. Effect of the Mas antagonists A779 and D-Pro-Ang-(1–7) on BK-induced vasodilatation
Both wild-type (*A*) and Mas-deficient microvessels (*B*) were preincubated with or without A779 (1 μmol l^{-1}), D-Pro-Ang-(1–7) (1 μmol l^{-1}), both antagonists or L-NAME (100 μmol l^{-1}), and subsequently exposed to increasing concentrations of BK (from 1 pmol l^{-1} to 1 μmol l^{-1}). The vasoactive responses are expressed as a percentage of a previous contraction elicited by NA (10 μmol l^{-1}). The curves are normalized to the loss of tension over time. For each curve, results are shown as means ± SEM of 10–15 mesenteric microvessels from at least 5 different animals. *$P < 0.05$ *vs.* wild-type microvessels.

previously demonstrated that Ang-(1–7)-induced vaso-dilatation in rat aorta is completely NO dependent, with no involvement of cyclo-oxygenase-derived products, as stated by others (Benter *et al.* 1993; Machado *et al.* 2002). Likewise, NO accounted for the entire vasorelaxation response induced by BK in murine microvessels. These data suggest that Mas blockade may specifically inter-fere with the synthesis of endothelial NO. In accordance, we have previously reported that cultured HUVECs preincubated with A779 lose the ability to generate and release NO after stimulation with BK (Peiró *et al.* 2007). Thus, Mas does not appear to be a third receptor for BK, in addition to B_1 and B_2 receptors, as previously suggested, but rather plays a broader and central role in endothelial NO-mediated vasorelaxation.

This central role of Mas for endothelial function is further accentuated by the demonstration that A779 did abrogate eNOS activation by BK. These results clearly demonstrate that in the absence of functioning Mas, the signal pathways leading to eNOS stimulation are affected, rather than the baseline eNOS phosphorylation. Such alterations in eNOS phosphorylation were paralleled by a similar pattern of NO synthesis in endothelial cells. Also here, A779 prevented the effect of BK, without changing total eNOS levels. In contrast to the unaltered total quantities of eNOS in our experiments, Stegbauer

et al. (2011) showed an effect of Ang-(1–7) on eNOS quantities. However, they demonstrated such regulation of the abundance of eNOS in pathological conditions after a long-lasting treatment, probably as an indirect result of a significant improvement of the pathophysiological condition in the investigated apoliprotein E-deficient mice. Given that we have used HUVECs from healthy donors and used the same short-term treatment with A779 as we used for our vessel preparations, we believe that the effects described by Stegbauer *et al.* (2011) are not related to our experimental settings, in which we explored an acute effect of Mas on eNOS phosphorylation, NO production and, consequently, vasorelaxation.

The spontaneous vasorelaxant effect in our micro-vessel preparation is not different between Mas-deficient and wild-type microvessels, probably because the base-line eNOS activity and NO release is less dependent on modulation of Mas than the ligand–receptor-mediated vasorelaxation. In fact, we have previously reported in HUVECs that Mas blockade reduces the agonist-induced but not the basal production of NO (Peiró *et al.* 2007). Furthermore, given that Mas has a central position in vascular function, the reactivity to vasoconstrictive agents stimulating both vascular smooth muscle cells and end-othelial cells should be altered in Mas-deficient vessels. In the present study, indeed, we have shown that Mas
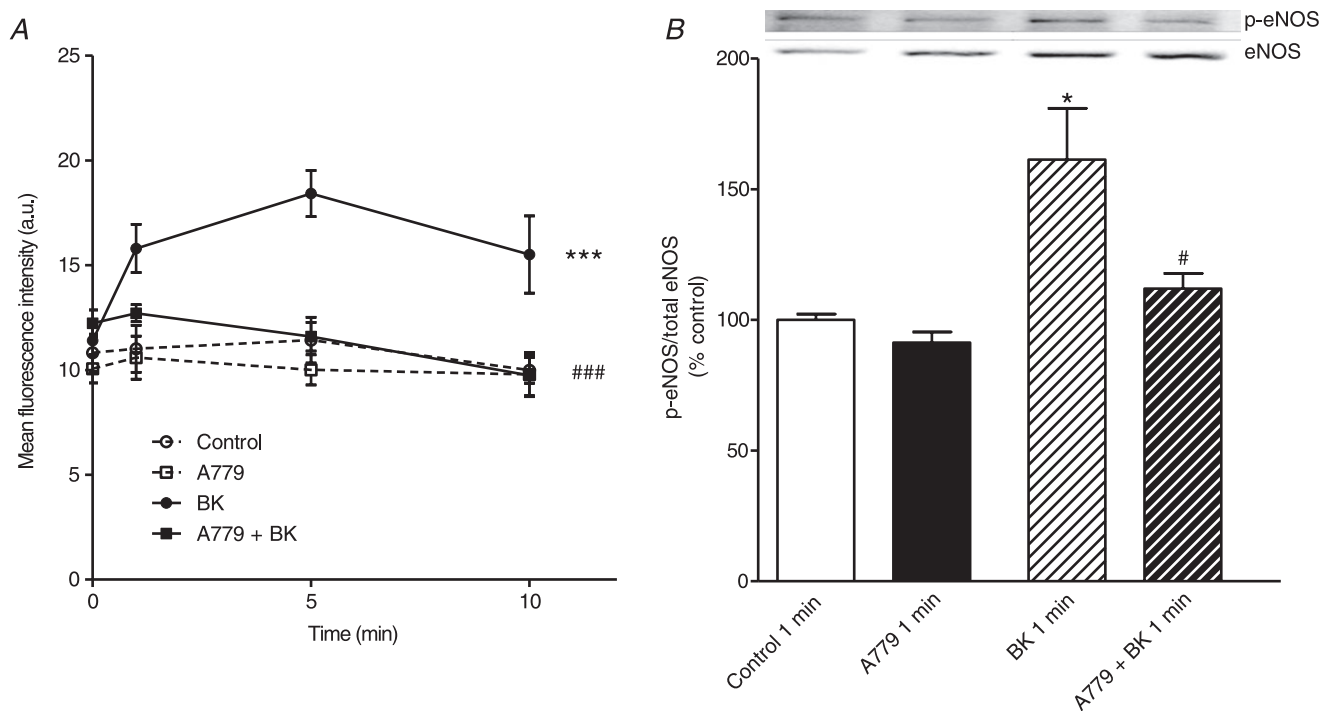

Figure 4. Effect of the Mas antagonist A779 on NO production and endothelial nitric oxide synthase (eNOS)-activating phosphorylation in human umbilical vein endothelial cells (HUVECs)
Cells were pretreated with or without A779 (1 μmol l^{-1}) for 15 min. *A*, NO production in HUVECs is shown for different time points over the 10 min of stimulation. *B*, levels of eNOS phosphorylation at serine 1177. The inset shows a representative Western blot. *$P < 0.05$, ***$P < 0.001$ *vs.* saline-treated cells; #$P < 0.05$, ###$P < 0.001$ *vs.* BK-treated cells. Each experiment was repeated 6 times using cells from 4 independent isolations.

deficiency and its pharmacological blockade generated an increased responsiveness to NA, while the inhibition of eNOS by L-NAME increased the vasoconstrictive potency of NA in the same manner. Therefore, it seems reasonable to suggest that the increased vasoconstrictive response to NA in Mas-deficient microvessels is caused by a reduced release of vasorelaxant NO in endothelial cells upon NA stimulation. Notably, at the NA concentration we used for preconstriction ($10 \, \mu\text{mol} \, l^{-1}$) in our experiments, Mas-deficient and wild-type vessels showed the same maximal constriction.

Using sodium nitroprusside, we have previously shown that smooth muscle function is not impaired in Mas-deficient vessels or in vessels pretreated with A779 (Peiró *et al.* 2007). Taken together, such results

and our present findings indicate that these effects are due to a reduced endothelial NO release and not to an increased constriction of the underlying smooth muscle. This clearly supports our hypothesis that the receptor Mas is a global regulator of endothelial function by regulating NO bioavailability and eNOS activity.

One additional possible mechanism for such a global position of Mas might be the peptide-independent intracellular signalling of the receptor. We and others previously described a constitutive activity of the receptor Mas (Gembardt *et al.* 2005*b*; Canals *et al.* 2006). The absence of this constitutive activity in vessels deficient for Mas might play a role in the lack of vasorelaxant capacity. However, this hypothesis requires further investigations to clarify the impact of this constitutive
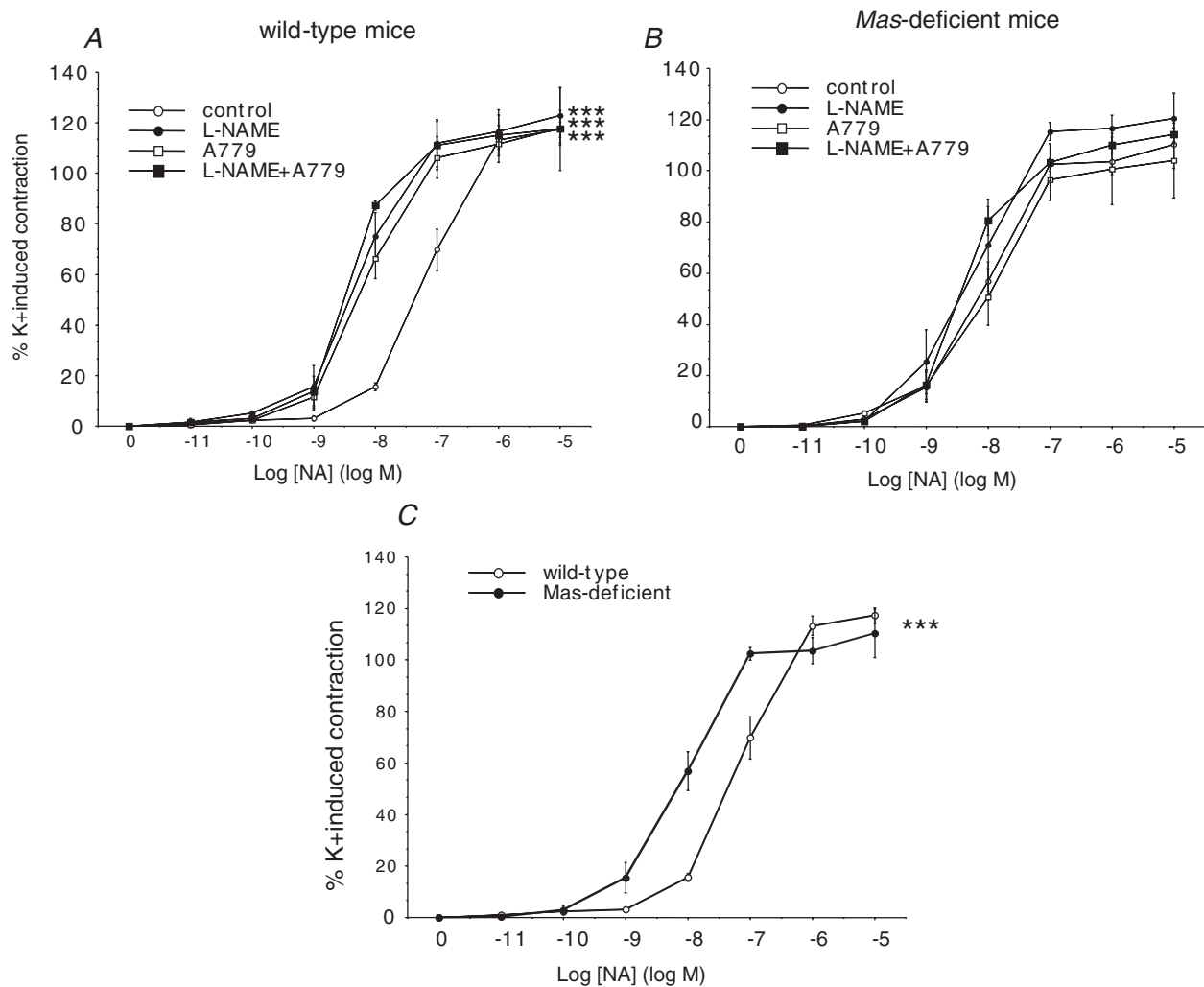

Figure 5. Concentration-dependent effects of NA in Mas-deficient and wild-type mesenteric arteries
Vascular response to NA ($10 \, \text{pmol} \, l^{-1}$ to $10 \, \mu\text{mol} \, l^{-1}$) in wild-type (*A*) and Mas-deficient microvessels (*B*) preincubated with the Mas antagonists A779 ($1 \, \mu\text{mol} \, l^{-1}$) or D-Pro-Ang-(1–7), the eNOS inhibitor L-NAME, the combination of Mas antagonists with L-NAME, or saline. *C*, the vasoconstrictive response to NA is altered in *Mas*-deficient animals compared with their wild-type controls. For each curve, results are shown as means ± SEM of 10–15 mesenteric microvessels from at least 5 different animals. ***$P < 0.0001$ *vs.* wild-type microvessels.

activity and to elucidate the possible cellular mechanisms involved.

In conclusion, we have shown that the Mas antagonists A779 and D-Pro-Ang-(1–7) equally block a single receptor, which mediates Ang-(1–7)-induced vasodilatation in mesenteric microvessels. Importantly, beyond mediating the vascular actions of Ang-(1–7), Mas appears to be a critical component required for NO-mediated vasodilatation induced by RAS-dependent and RAS-independent agonists. The receptor Mas therefore arises as a key pharmacological target to modulate endothelial function and might be an outstanding target for the treatment of diseases characterized by endothelial dysfunction.

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Author contributions

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