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Evidence for a Functional Interaction of the Angiotensin-(1–7) Receptor Mas With AT₁ and AT₂ Receptors in the Mouse Heart

Carlos Henrique de Castro, Robson Augusto Souza dos Santos, Anderson José Ferreira, Michael Bader, Natalia Alenina, Alvaír Pinto de Almeida

Abstract—The aim of this study was to evaluate the angiotensin (Ang)-(1–7) effects in isolated mouse hearts. The hearts of male C57BL/6J and knockout mice for the Ang-(1–7) receptor Mas were perfused by the Langendorff method. After a basal period, the hearts were perfused for 20 minutes with Krebs-Ringer solution (KRS) alone (control) or KRS containing Ang-(1–7) (0.22 pmol/L), the Mas antagonist A-779 (115 nmol/L), the angiotensin type 1 receptor antagonist losartan (2.2 μ mol/L), or the angiotensin type 2 receptor antagonist PD123319 (130 nmol/L). To evaluate the involvement of Ang receptors, prostaglandins, and nitric oxide in the Ang-(1–7) effects, the hearts were perfused for 20 to 30 minutes with KRS containing either A-779, losartan, PD123319, indomethacin, or N^G-nitro-L-arginine methyl ester (L-NAME) alone or in association with subsequent Ang-(1–7) perfusion. In addition, hearts from Mas-knockout mice were perfused for 20 minutes with KRS containing Ang-(1–7) (0.22 pmol/L) and losartan. Ang-(1–7) alone did not change the perfusion pressure. Strikingly, in the presence of losartan, 0.22 pmol/L Ang-(1–7) induced a significant decrease in perfusion pressure, which was blocked by A-779, indomethacin, and L-NAME. Furthermore, this effect was not observed in Mas-knockout mice. In contrast, in the presence of PD123319, Ang-(1–7) produced a significant increase in perfusion pressure. This change was not modified by the addition of A-779. Losartan reduced but did not abolish this effect. Our results suggest that Ang-(1–7) produces complex vascular effects in isolated, perfused mouse hearts involving interaction of its receptor with angiotensin type 1- and type 2-related mechanisms, leading to the release of prostaglandins and nitric oxide. (*Hypertension*. 2005;46[part 2]:1-6.)

Key Words: receptors, angiotensin ■ cardiac function ■ heart ■ angiotensin antagonist ■ prostaglandins

In the last decade, the classic renin-angiotensin system (RAS) concept has undergone important changes.^{1,2} Many novel biologically active components were described, such as angiotensin (Ang)-(1–7), Ang III, and Ang IV. Ang-(1–7) is now considered an important component of the RAS, with actions similar to or even opposite those displayed by Ang II.^{1,3,4} Moreover, chronic treatment with Ang-converting enzyme inhibitors and/or angiotensin type 1 (AT₁) receptor blockers increases plasma Ang-(1–7) levels up to 25-fold,^{5–7} suggesting that this heptapeptide could be involved in the beneficial effects observed with these therapies.^{5,8} In addition, many studies have observed that Ang-(1–7) has a bradykinin-potentiating activity in several vascular beds and species.^{9–13}

Recently, using mice with targeted disruption of the *Mas* proto-oncogene¹⁴ and *Mas*-transfected cells, Santos et al¹⁵ identified Ang-(1–7) as an endogenous ligand for the G protein-coupled receptor encoded by *Mas*.¹⁵ Furthermore, the novel Ang-(1–7)-forming enzyme ACE2^{16,17} has been

reported to be an important regulator of the RAS.¹⁸ This enzyme can form Ang-(1–7) by at least 2 different pathways: directly from Ang II¹⁹ and indirectly from Ang I.¹⁷ Considering that the heart has been identified as the main target for Ang-(1–7) actions^{20–22} and that ACE2 and *Mas* are present in the heart, the ACE2–Ang-(1–7)–*Mas* axis assumes a key role for understanding the actions of cardiac RAS.

It has been found that Ang-(1–7) has a cardioprotective effect in the rat. We have shown that Ang-(1–7) decreases the incidence and duration of ischemia/reperfusion arrhythmias²⁰ and improves postischemic function in isolated rat heart.²³ Ang-(1–7) was also effective in preventing the development of heart failure after myocardial ischemia induced by left coronary artery ligation.²¹ In addition, TGR(A1–7)3292 transgenic rats, which have a 2.5-fold increase in plasma Ang-(1–7) concentrations, were more resistant than control animals to induction of cardiac hypertrophy by isoproterenol and had a reduced duration of reperfusion arrhythmias and improved postischemic function in an isolated perfused heart

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Figure 1. Diagrammatic representation of the experimental protocols used in this study. Hearts were randomized into 3 groups: A, control (KRS alone); B, perfusion with KRS (basal period) followed by Ang-(1-7) or antagonist perfusion (experimental period); and C, perfusion with KRS plus antagonists (basal period) followed by Ang-(1-7) perfusion (experimental period). Abbreviations are as defined in text.

model.²⁴ A key role for the Ang-(1-7)-forming enzyme ACE2 in heart function has been suggested on the basis of the dysfunction observed in ACE2-deficient mice.²⁵ However, there are no available data concerning Ang-(1-7) effects in the mouse heart. The growing use of mice as an experimental model for cardiovascular studies led us to evaluate the effects of Ang-(1-7) in isolated mouse hearts. This model was used to evaluate the role of the receptor Mas in the coronary vessels and the possible interaction of Mas-mediated with AT₁- and AT₂-related mechanisms.

Methods

Male C57BL/6J and Mas-knockout mice weighing 25 to 30 g were used in this study. Male C57BL/6J mice were obtained from Cebio (Centro de Bioterismo), Biological Sciences Institute, Federal University of Minas Gerais and wild-type and Mas-knockout mice were obtained from the transgenic animal facilities at Laboratory of Hypertension, Federal University of Minas Gerais, Brazil. All experimental protocols were performed in accordance with guidelines for the humane use of laboratory animals at our institute and were approved by local authorities. The mice were decapitated 10 to 15 minutes after intraperitoneal injection of 200 IU heparin. The thorax was opened, and the heart was carefully dissected and perfused through an aortic stump with Krebs-Ringer solution (KRS) containing (in mmol/L) NaCl (118.4), KCl (4.7), KH₂PO₄ (1.2), MgSO₄·7 H₂O (1.2), CaCl₂·2 H₂O (2.5), glucose (11.7), and NaHCO₃ (26.5). The perfusion flow was maintained constant (2 to 2.5 mL/min) at 37±1°C and constant oxygenation (5% CO₂ and 95% O₂). Coronary perfusion pressure was measured by means of a pressure transducer connected to the aortic cannula and coupled to a data-acquisition system (Biopac System, Inc).

After a basal period (20 to 30 minutes), the hearts were perfused for an additional period of ≈20 minutes with (1) KRS (control, n=5)

or KRS containing (2) Ang-(1-7) (0.22 pmol/L, n=4); (3) the receptor Mas antagonist A-779¹⁵ (115 nmol/L, n=4); (4) the AT₁ receptor antagonist losartan (2.2 μmol/L, n=5); or (5) the AT₂ receptor antagonist PD123319 (130 nmol/L, n=4) (Figure 1). To evaluate the role of Ang receptors, cyclooxygenase products and nitric oxide (NO) in the Ang-(1-7) effects, the hearts were perfused for a period of 20 to 30 minutes with KRS containing (1) A-779 (115 nmol/L, n=5); (2) losartan (2.2 μmol/L, n=4); (3) PD123319 (130 nmol/L, n=4); (4) A-779 (115 nmol/L) plus losartan (2.2 μmol/L, n=5); (5) A-779 (115 nmol/L) plus PD123319 (130 nmol/L, n=4); (6) losartan (2.2 μmol/L) plus PD123319 (130 nmol/L, n=4); (7) A-779 (115 nmol/L) plus losartan (2.2 μmol/L) plus PD123319 (130 nmol/L, n=4); (8) losartan (2.2 μmol/L, n=4) plus indomethacin (1 μmol/L); or (9) losartan (2.2 μmol/L, n=4) plus N^G-nitro-L-arginine methyl ester(L-NAME; 10 μmol/L). After this period, Ang-(1-7) (0.22 nmol/L) was added to the perfusion solution containing the antagonists and/or inhibitors, and the hearts were perfused for an additional period of ≈20 minutes (Figure 1). In addition, to further evaluate the involvement of the Ang-(1-7) receptor Mas in the Ang-(1-7)-induced vasodepressor effects, isolated hearts from Mas-knockout mice and their controls were perfused for a period of 20 to 30 minutes with KRS containing losartan (2.2 μmol/L, n=4) followed by addition of Ang-(1-7) (0.22 pmol/L) in the KRS. The doses of Ang antagonists used in this study were based on previous studies.^{26–28} Data are reported as mean±SEM. Statistical analysis was performed by ANOVA followed by a Bonferroni test or Student *t* test. A value of *P*<0.05 was considered significant.

Results

As shown in Figure 2, Ang-(1-7) at 0.22 pmol/L had no effect in isolated, perfused mouse hearts. Because an AT₁-related mechanism could be masking the Ang-(1-7)-induced vasodilation, in the next set of experiment we tested the effect of

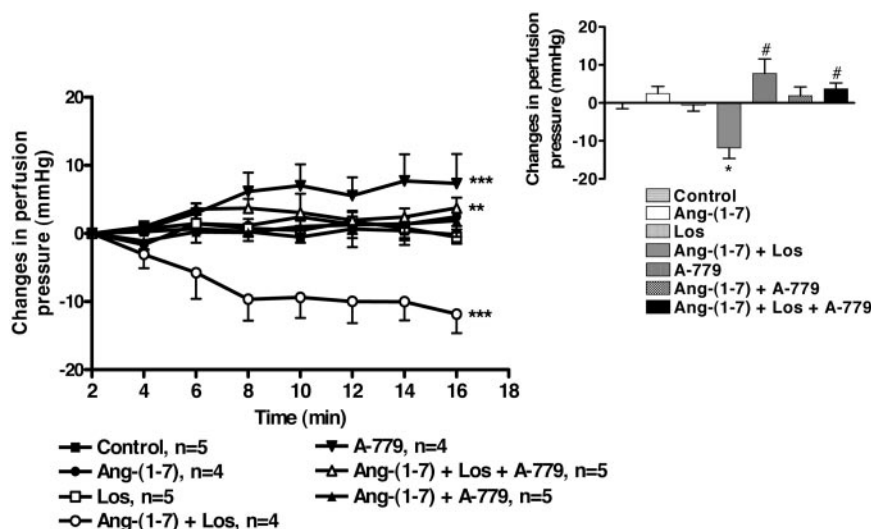


Figure 2. Time course of the changes in perfusion pressure of isolated mouse hearts. The hearts were perfused with KRS (control) or KRS containing Ang-(1-7) (0.22 pmol/L), losartan (2.2 μmol/L), Ang-(1-7) + losartan, A-779 (115 nmol/L), Ang-(1-7) + A-779, or Ang-(1-7) + losartan + A-779. ****P*<0.0001, ***P*<0.001 compared with the control group (2-way ANOVA followed by the Bonferroni test). The insert represents the maximal changes in perfusion pressure observed during the experimental period. **P*<0.01, #*P*<0.05 vs control group (Student's *t* test). Los indicates losartan. Other abbreviations are as defined in text.

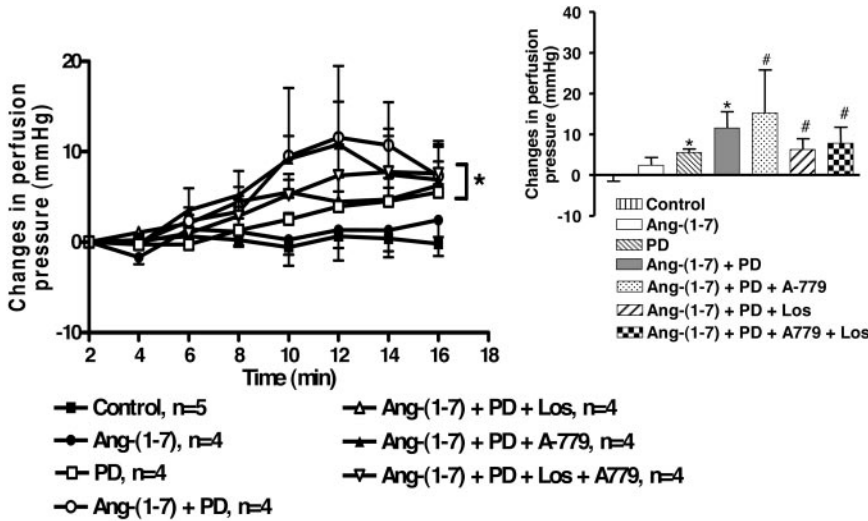


Figure 3. Time course of the changes in perfusion pressure of isolated mouse hearts. The hearts were perfused with KRS (control) or KRS containing Ang-(1-7) (0.22 pmol/L), PD 123319 (130 nmol/L), Ang-(1-7) + PD 123319, Ang-(1-7) + PD 123319 + losartan (2.2 μmol/L), Ang-(1-7) + PD 123319 + A-779 (115 nmol/L), or Ang-(1-7) combined with Ang receptor antagonists (PD123319, A-779, and losartan). **P*<0.01 compared with the control group (2-way ANOVA followed by the Bonferroni test). The insert represents the maximal changes in perfusion pressure observed during the experimental period. **P*<0.01, #*P*<0.05 vs control group (Student's *t* test). Los indicates losartan and PD, PD123319. Other abbreviations are as defined in text.

AT₁, AT₂, and Mas receptor antagonists, alone or in combination, with Ang-(1-7). Strikingly, in the presence of losartan, Ang-(1-7) produced coronary vasodilation, indicated by a significant drop in perfusion pressure (Figure 2). The Ang-(1-7) receptor antagonist A-779 in combination with Ang-(1-7) did not change perfusion pressure. However, it completely blocked the vasodilatory effect of Ang-(1-7) observed in the presence of AT₁ receptor blockade (Figure 2). A more complex response was obtained in the presence of the AT₂ receptor antagonist PD123319. The blockade of AT₂ receptors by itself produced an increase in perfusion pressure (Figure 3). Addition of Ang-(1-7) induced a further increase in perfusion pressure, which was not affected by A-779 cotreatment. On the other hand, the vasoconstriction observed in the presence of PD123319 combined with Ang-(1-7) was decreased but not abolished by losartan or by A-779 combined with losartan (Figure 3).

Further confirmation of the involvement of the Ang-(1-7) receptor Mas in the decrease of perfusion pressure in response to Ang-(1-7) combined with losartan was obtained in isolated hearts from Mas-knockout mice. As shown in Figure 4, the

Ang-(1-7)-induced vasodilator effect was absent in Mas-knockout mice.

We next evaluated the participation of prostaglandins and NO in the Ang-(1-7) effects. Indomethacin completely blocked the vasodilation produced by Ang-(1-7) combined with losartan (Figure 5A). Indeed, in the presence of indomethacin, an increase in perfusion pressure was observed in response to Ang-(1-7) combined with losartan. In addition, the vasodilation produced by Ang-(1-7) in the presence of losartan was also abolished by pretreatment with the NO synthase inhibitor L-NAME (Figure 5B).

Discussion

The major finding of this study was the observation that the Mas-mediated vascular actions of Ang-(1-7) in the mouse heart are importantly influenced by AT₁- and AT₂-related mechanisms. The blockade of AT₁ receptors unmasked a Mas-mediated vasodilator effect of Ang-(1-7) at a very low concentration (0.22 pmol/L). The importance of AT₂ receptors in the vascular actions of Ang-(1-7) in the mouse heart was demonstrated by a significant increase in perfusion

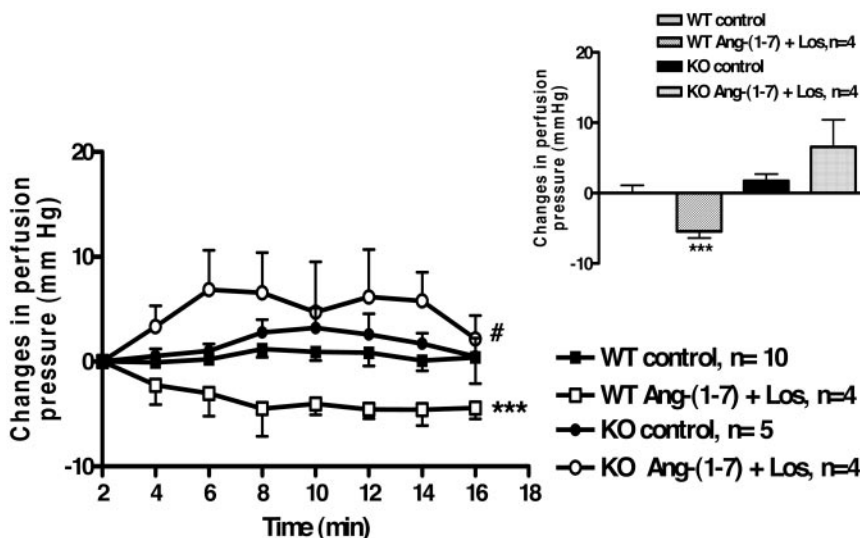


Figure 4. Time course of the changes in perfusion pressure of isolated hearts from Mas-knockout (KO) and wild-type (WT) mice. The hearts were perfused with KRS (control) or KRS containing Ang-(1-7) (0.22 pmol/L) + losartan (2.2 μmol/L). #*P*<0.05, ****P*<0.0001 compared with the control group (2-way ANOVA followed by the Bonferroni test). The insert represents the maximal changes in perfusion pressure observed during the experimental period. ****P*<0.0001 vs control group (Student's *t* test). Los indicates losartan. Other abbreviations are as defined in text.

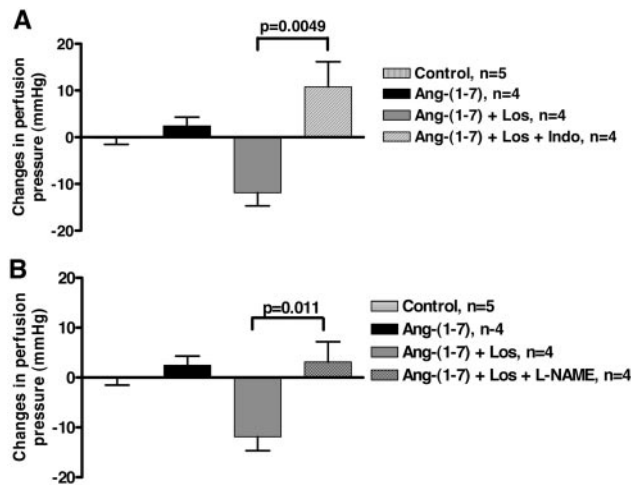


Figure 5. Effects of indomethacin (A) and L-NAME (B) on the maximal changes in perfusion pressure induced by Ang-(1-7) (0.22 pmol/L) during the experimental period. Data were analyzed by Student's *t* test. Indo indicates indomethacin and Los, losartan. Other abbreviations are as defined in text.

pressure produced by Ang-(1-7) in the presence of PD123319. Moreover, AT₂ and the Ang-(1-7) receptor Mas appear to be involved in the maintenance of basal murine coronary vascular tone, as suggested by the increase in perfusion pressure produced by blockade of these receptors.

Strikingly, when Ang-(1-7) was administered with losartan, a significant decrease in perfusion pressure was observed. This effect was completely blocked by A-779, indicating that the receptor Mas mediates the vasodilator effect of Ang-(1-7) in this condition. In keeping with this finding, Ang-(1-7) administered with losartan did not induce a vasodilator effect in Mas-knockout mice.

Contrasting with what was observed with losartan, Ang-(1-7) administered with the AT₂ receptor antagonist PD123319 produced a significant increase in perfusion pressure. This increase was not changed by cotreatment with A-779 and was reduced but not abolished by losartan. Combination of losartan with A-779 did not further reduce the slight increase in perfusion pressure induced by Ang-(1-7) in the presence of PD123319. These data suggest the involvement of a vasoconstrictor mechanism, not yet identified, in this condition.

Taken together, our results suggest that a complex interaction between these receptors leads to the final Ang-(1-7) effect in the isolated, perfused mouse heart. Because no measurements of Ang II in the heart perfusate were made in the present study, it is not clear whether the effects observed were also dependent or not on endogenous Ang II release. Many putative mechanisms could be involved in this interaction, including functional antagonism, cross-talk, or oligomerization. Ang-(1-7) has been shown to antagonize the vasoconstrictor effect of Ang II in many vascular beds²⁹⁻³¹ and cultured cells.^{30,32,33} Likewise, the AT₁ receptor appears to modulate the Ang-(1-7) effects, as suggested before⁸ and illustrated by our results. Several studies have demonstrated the formation of heterodimers between different receptors.³⁴⁻³⁸ AbdAlla et al³⁵ found that the AT₁ and bradykinin

B₂ receptor form stable heterodimers, leading to an increased activation of G proteins. In addition, the signaling transduction of both receptors changed with heterodimerization. The AT₁ receptor and Ang-(1-7) receptor Mas can also interact directly with each other, leading to an altered response to Ang II in cultured mammalian cells³⁶ and in the amygdala of the mouse.³⁷ Moreover, the Mas agonist, nonpeptide AVE 0991, induced an antidiuretic effect in water-loaded mice that was totally blocked by the Ang-(1-7) antagonist A-779 and AT₂ antagonists and partially blocked (≈60%) by AT₁ antagonists.³⁹ Whether this is true for the Ang-(1-7)-induced vasodilation in the isolated, perfused mouse heart remains to be elucidated.

It should be mentioned that subtypes of Ang-(1-7) receptors could be present in some situations. For instance, although Ang-(1-7) has a poor affinity for the AT₁ receptor,⁴⁰ some effects of Ang-(1-7) occurred through an A-779 site that is also recognized by losartan and CV-11974,⁴¹ suggesting the existence of Ang-(1-7) receptor subtypes beyond the Ang-(1-7) receptor Mas or nonreceptor mechanisms, such as binding to Ang-converting enzyme. In keeping with this hypothesis, Vianna et al⁴² demonstrated that Ang-(1-7)-induced vasodilation in isolated aortic rings of Sprague-Dawley rats was abolished by the recently described Ang-(1-7) antagonist D-Pro⁷-Ang-(1-7),⁴³ but not by A-779. However, this hypothesis remains to be confirmed.

The participation of AT₂ receptors in the Ang-(1-7) effects is suggested by the observation that in the presence of PD123319, the vasodilation produced by Ang-(1-7) in mouse hearts pretreated with losartan was turned into a vasoconstrictive effect. Moreover, addition of Ang-(1-7) in a heart preparation pretreated with PD123319 produced an increase in perfusion pressure. Considering the very low affinity of Ang-(1-7) for AT₂ receptors^{40,44} and the very low concentration of Ang-(1-7) used, a direct interaction of Ang-(1-7) with AT₂ receptors to explain our results is unlikely. Blockade of the Ang-(1-7) receptor Mas with PD123319 is also unlikely, because this compound was unable to displace the binding or functional responses to Ang-(1-7) in Mas-transfected cells.^{15,39} In addition, in Mas-deficient mice, the specific binding of Ang-(1-7) to kidney slices was abolished, whereas the binding of Ang II to AT₂ receptors was fully preserved.¹⁵ Thus, a functional interaction such as a cross-talk mechanism or a permissive role for the AT₂ receptor for some Mas-mediated effects, as recently suggested for B₂ receptor-mediated bradykinin effects,⁴⁵ should be considered. It should be pointed out that, despite its putative interaction with the Ang-(1-7) receptor Mas, the role of AT₂ receptors within the RAS is still unclear. Many puzzling aspects of its functional interaction with the AT₁ receptor remain to be elucidated. For example, contradictory results have been obtained, even by the same group, concerning its modulatory role on the pressor effect of Ang II mediated through AT₁ receptors.^{46,47} Of note is the possibility that PD123319 could interfere with nonreceptor-mediated effects or with other receptors, homodimers, or heterodimers, such as AT₁/Mas.³⁶

In the presence of indomethacin and L-NAME treatment, the decrease in perfusion pressure produced by Ang-(1-7) in the presence of the AT₁ receptor blocker losartan was blunted.

These observations indicate that the vasodilator effect of Ang-(1-7) in the isolated mouse heart is dependent on vasodilator prostaglandins and NO release. These findings are in agreement with previous reports in coronary and other blood vessels.⁶

Perspectives

Our results unmasked an important functional interaction between Mas and AT₁ and AT₂ receptors in the mouse heart. According to our data, when AT₁ receptors are blocked, Ang-(1-7) produces a Mas-mediated vasodilation at a subpicomolar concentration, which is influenced by a PD123319-sensitive mechanism. These findings indicate a complex interaction between Mas-mediated actions of Ang-(1-7) and AT₁- and AT₂-related mechanisms. It remains to be established whether a similar interaction exists in other tissues and species, which may have important physiopathologic and therapeutic implications.

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