Antithrombotic Effect of Captopril and Losartan Is Mediated by Angiotensin-(1-7)

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Abstract—It is well established that renin-angiotensin system blockers exert NO/prostacyclin-dependent antithrombotic effects. Because some beneficial effects of these drugs are mediated by angiotensin (Ang)-(1-7), in the present study we examined if their antithrombotic action could be mediated by Ang-(1-7). Intravenous infusion of Ang-(1-7) (1, 10, or 100 pmol/kg per minute for 2 hours) into rats developing venous thrombosis caused 50% to 70% reduction of the thrombus weight. This effect was dose-dependently reversed by cotreatment with A-779 (selective Ang-[1-7] receptor antagonist) or EXP 3174 (angiotensin type 1 receptor antagonist) but not by PD 123,319 (angiotensin type 2 receptor antagonist). Similarly, the antithrombotic effects of captopril (ACE inhibitor) and losartan (angiotensin type 1 receptor blocker) were attenuated by A-779 in a dose-dependent manner. The effect of Ang-(1-7) was completely abolished by concomitant administration of NO synthase inhibitor (N\textsuperscript{G}-nitro-L-arginine methyl ester) and prostacyclin synthesis inhibitor (indomethacin), as has been shown previously for captopril and losartan. Thus, the antithrombotic effect of renin-angiotensin system blockers involves Ang-(1-7)-evoked release of NO and prostacyclin. (Hypertension. 2002;40:774-779.)

Key Words: angiotensin | venous thrombosis | captopril | losartan | nitric oxide | prostacyclin | rats

The renin-angiotensin system (RAS) serves as a principal regulator of blood pressure and hydroelectrolite balance and, in a paracrine or autocrine manner, modulates numerous cellular functions.\textsuperscript{1–3} Angiotensin (Ang) II is considered the main active peptide of RAS that possesses well-documented prothrombotic activity.\textsuperscript{4–6} It has been demonstrated that Ang II increases the production and secretion of plasminogen activator inhibitor type 1 (PAI-1) from endothelial and smooth muscle cells\textsuperscript{5,7,8} and augments tissue factor (TF) expression,\textsuperscript{8} thus promoting thrombosis.

In addition to Ang II, Ang-(1-7) has been recently identified as a new biologically active component of RAS.\textsuperscript{9–11} Ang-(1-7) is produced either from Ang I or Ang II, reducing their concentration. It has been demonstrated that Ang-(1-7) acts as a counter-regulatory hormone to Ang II, limiting its pressor, proliferative, and angiogenic actions.\textsuperscript{12–14} However, very little is known about the role of Ang-(1-7) in the regulation of hemostasis. In contrast to Ang I, II, and III, Ang-(1-7) does not induce TF and PAI-1 mRNA expression in cultured rat aortic endothelial cells,\textsuperscript{15} indicating that it should not promote thrombosis in vivo. However, the involvement of this peptide in the regulation of coagulation and fibrinolysis in vivo has not been studied so far.

In our previous studies, we have demonstrated the antithrombotic action of captopril (CAP; ACE inhibitor [ACE-I]) and losartan (LOS; angiotensin type 1 [AT\textsubscript{1}] receptor antagonist) in venous thrombosis, which is dependent on the secretion of prostacyclin (PGI\textsubscript{2}) and/or NO.\textsuperscript{16–19} Because it is known that chronic treatment with ACE-Is or AT\textsubscript{1}-receptor antagonists results in a 5- to 50-fold increase in the concentration of Ang-(1-7)\textsuperscript{20–22}—a stimulator of NO and PGI\textsubscript{2}, release\textsuperscript{23,24}—the present study is aimed to determine whether the antithrombotic action of ACE-Is or AT\textsubscript{1} receptor antagonists might be mediated by Ang-(1-7).

Methods

Animals and the Induction of Renovascular Hypertension

Male Wistar rats (160 to 180 g; Brwinow, Poland) were housed in a room with a 12-hour/12-hour light/dark cycle, in group cages as appropriate; given tap water; and fed a standard rat chow. In these animals, 2-kidney, 1-clip (2K-1C) renovascular hypertension was induced by a partial, standardized clipping of the left renal artery\textsuperscript{25} under pentobarbital anesthesia (40 mg/kg IP). After 6 weeks, all the animals developed hypertension (confirmed by tail-cuff blood pressure measurement) and were used for subsequent experiments. Sham-operated rats (SO) served as a control to 2K-1C hypertensive rats. They received the same surgical intervention without the clipping of the renal artery.

Procedures involving the animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research.\textsuperscript{26}

Experimental Protocol

To check if angiotensin fragments exert an antithrombotic effect, the animals received a continuous 2-hour infusion of Ang-(1-7) (1, 10,
100 pmol/kg per minute; Ascor infusion pump), Ang-(3-7) (100 pmol/kg per minute), or 0.9% NaCl (2 mL/kg per hour) into the femoral vein. The infusion started 10 minutes before the induction of venous thrombosis and was continued for the entire period of thrombus development. To assess whether the potential antithrombotic effect of Ang-(1-7) could be receptor specific, the rats were treated with A-779 (selective Ang-[1-7] receptor antagonist\textsuperscript{25}–\textsuperscript{29} D-Ala-Ang-[1-7]; 100 to 10 000 pmol/kg per minute IV), EXP 3174 (AT\textsubscript{1} receptor antagonist; 1.2 \mu mol/kg IV bolus injection), or PD 123,319 (angiotensin type 2 [AT\textsubscript{2}] receptor antagonist; 10 nmol/kg per minute IV) 5 minutes before the induction of Ang-(1-7) (10 pmol/kg per minute) or 0.9% NaCl.

To study the role of Ang-(1-7) in the antithrombotic effect of drugs blocking RAS, a separate set of animals was treated with CAP (25 mg/kg twice daily), LOS (10 mg/kg, once daily), or their solvent (distilled water; 0.5 mL/300g) per os, for 10 days. On the 11th day, when venous thrombosis was performed, the rats received the specific Ang-(1-7) receptor antagonist A-779 (D-Ala-Ang-[1-7]), 10 to 10 000 pmol/kg per minute IV) or 0.9% NaCl (2 mL/kg per hour) infused continuously into the femoral vein. The infusion started 10 minutes before the induction of venous thrombosis and was continued for the entire period of thrombus development.

In experiments concerning the mechanism of antithrombotic action of Ang-(1-7), NO-synthase inhibitor \textsuperscript{N}\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME; 30 mg/kg SC) or PGI\textsubscript{2} synthesis inhibitor indomethacin (INDO; 2.5 mg/kg IV), alone or in combination, was administered additionally to Ang-(1-7) 20 or 10 minutes before venous thrombosis induction, respectively.

**Venous Thrombosis Induction**

Twelve hours before venous thrombosis induction, the rats were deprived of food but had free access to water. The venous thrombosis was performed as previously described by Reyers et al.	extsuperscript{30} On the 11th day of treatment with CAP or LOS, and/or 10 minutes after the IV infusions started, the abdomen was opened under pentobarbital anesthesia (40 mg/kg IP), and the vena cava was carefully separated from the surrounding tissues and then ligated tightly with a cotton thread just below the left renal vein. Subsequently, the abdomen was closed with a double layer of sutures (peritoneum with muscles and skin separately). After 2 hours, the animals were reanesthetized and the abdomen was then reopened, and the vena cava was carefully dissected and inspected for the presence of a thrombus. The thrombus was air-dried at 37°C, and after 24 hours its weight was measured.

**Blood Pressure Measurement**

The systolic blood pressure (SBP) was measured by the tail-cuff method\textsuperscript{31} (Student Oscillograph, Harvard Rat Tail Blood Pressure Monitor) before and after 10 days of CAP or LOS administration. Each value was the average of 3 consecutive readings.

The blood pressure in rats receiving Ang-(1-7) infusion was measured directly through a cannula filled with heparin solution (150 U/mL), placed in the right common carotid artery, and connected to the pressure transducer (Gould P23ID) and a monitor Trendoscope 8031 (SandW Vickers Ltd), as previously described.\textsuperscript{18} In these animals, venous thrombosis was not induced.

**Chemicals and Drugs**

Ang-(1-7) (Sigma Chemical Co or Bachem), L-NAME (RBI), indomethacin (RBI), CAP (RBI), LOS (DuP 753, kindly provided by DuPont Merck Pharmaceutical Co), A-779 (Bachem), EXP 3174 (kindly provided by DuPont Merck Pharmaceutical Co), PD 123,319 (RBI), and pentobarbital (Vetbital) were used in the study.

**Statistical Analyses**

The data are shown as mean±SEM. In calculating the thrombus weight, the lack of the thrombus was regarded as 0 mg.\textsuperscript{18} Therefore, Kruskal-Wallis nonparametric ANOVA, followed by Dunn’s multiple comparison test, was used consistently throughout the study. Values of $P<0.05$ were considered significant.

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**Results**

**Ang-(1-7) Exerts an Antithrombotic Effect in 2K-1C Rats**

To check if Ang-(1-7) produces an antithrombotic effect, we infused this peptide to 2K-1C and SO rats. The 2K-1C hypertensive rats developed thrombi 2.5-times larger than those of SO rats ($P<0.05$) (Figure 1). In 2K-1C rats, Ang-(1-7), but not Ang-(3-7), significantly reduced the thrombus weight (by 65%, 70%, and 50% for 1, 10, 100 pmol/kg per minute; $P<0.01$, $P<0.01$, and $P=NS$, respectively) (Figure 1). In SO rats, Ang-(1-7) (10 pmol/kg per minute) did not cause any change in the thrombus weight (Figure 1).

We chose 10 pmol/kg per minute of Ang-(1-7), which exerted a strong antithrombotic effect, to study the mechanism of its action. The infusion of A-799, an Ang-(1-7) receptor antagonist, dose-dependently reversed the antithrombotic effect of Ang-(1-7) (to 30%, 45%, and 75% of the control value for 100, 1000, 10 000 pmol/kg per minute; $P<0.05$, $P=NS$, and $P=NS$, respectively) (Figure 1). Similarly a bolus injection of EXP 3174, the AT\textsubscript{1} receptor antagonist, before the infusion of Ang-(1-7) reversed its antithrombotic action ($P<0.05$) (Figure 2). EXP 3174 did not change the thrombus weight by itself. In contrast, PD 123,319, an AT\textsubscript{2} receptor antagonist, did not influence the antithrombotic action of Ang-(1-7) (Figure 2).

**Ang-(1-7) Contributes to the Antithrombotic Effect of CAP and LOS**

We then investigated the involvement of Ang-(1-7) in the action of CAP and LOS. Chronic treatment with CAP or LOS
resulted in significant reduction of the thrombus weight (to 7% of the control values; \( P < 0.001 \) for both drugs) (Figure 3).

Ang-(1-7) receptor antagonist A-779 infused to CAP-pretreated rats reversed its antithrombotic action (to 27%, 50%, and 42% of the control values for 100, 1000, and 10 000 pmol/kg per minute; \( P < 0.05, \) \( P = \text{NS}, \) \( P = \text{NS}, \) respectively) (Figure 3), as did infusion to LOS-pretreated animals (an increase to 34%, 53%, and 33% of the control values for 10, 100, and 1000 pmol/kg per minute) (Figure 3). A-779 (1000 pmol/kg per minute), when administered to LOS- or CAP-naive animals, did not change the thrombus weight.

**Antithrombotic Effect of Ang-(1-7) Involves NO and PGI2**

Finally, we evaluated whether the antithrombotic effect of Ang-(1-7) is mediated by NO and/or PGI2. Concurrently, 10 pmol/kg per minute of Ang-(1-7) and either L-NAME (NO synthase inhibitor) or indomethacin (PGI2 synthesis inhibitor) was given. Despite some tendency toward the increase in the thrombus weight, L-NAME or indomethacin failed to fully reverse the antithrombotic action of Ang-(1-7) (Figure 4). However, when L-NAME and indomethacin were given concomitantly, they completely abolished the antithrombotic effect of Ang-(1-7) (to 90% of the control values) (Figure 4). L-NAME and/or indomethacin failed to influence the thrombus weight when administered to Ang-(1-7)-naive animals (data not shown).

**Blood Pressure**

As expected, the SBP in 2K-1C rats compared with SO rats was higher (158 ± 2 mm Hg versus 129.8 mm Hg; \( P < 0.05 \)). Chronic treatment with CAP, and chronic LOS administration, significantly lowered SBP (from 158 ± 2 mm Hg to 131 ± 2 mm Hg or to 126 ± 2 mm Hg, for CAP and LOS, respectively; \( P < 0.05 \)). Two-hour infusion of Ang-(1-7) (1, 10, and 100 pmol/kg per minute) failed to influence SBP (data not shown).

**Discussion**

In the present study, we induced venous thrombosis in renovascular hypertensive rats. Both experimental models are known to be RAS-dependent. In the former, activation of RAS occurs owing to false information about hypovolemia as a result of the vena cava ligation, whereas in the latter, clipping of renal artery leads to the overproduction of renin.

Thus, these models mimic the pathological conditions of RAS overactivation and are therefore particularly useful to study various effects of RAS blockers. They can also be used to unmask the effects of Ang-(1-7), because its actions are much more pronounced when the production or activity of Ang II is increased, and in animal models of hypertension. A further advantage of this model is that it can separate antithrombotic and hypotensive effects of the studied compounds, because only a minor relationship between arterial and venous blood pressure. Thus, the thrombus forming inside the veins is unlikely to be affected by changes in the arterial blood pressure.

Using this experimental model, we have demonstrated previously that the long-term administration of CAP (ACE-I) or LOS (AT1-receptor antagonist) exerts a NO/PGI2-dependent antithrombotic effect. This striking similarity in the mode of action of both RAS blockers suggests a common mechanism leading to the liberation of NO/PGI2 after the inhibition of RAS. Because the concentration of Ang-(1-7)—which liberates NO/PGI2—increases during treatment with RAS blockers, we thought that this peptide might be responsible for their antithrombotic action. To study this, we infused Ang-(1-7) into hypertensive rats developing venous thrombosis and

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*Figure 2.* The effect of angiotensin AT1-receptor antagonist (EXP3174; 1.2 \( \mu \)mol/kg per minute IV) and AT2-receptor antagonist (PD 123,319; 10 nmol/kg per minute IV) on the antithrombotic action of Ang-(1-7) (10 pmol/kg per minute IV). *\( P < 0.05 \) vs control. Each column represents the mean±SEM from 5 to 12 experiments.

*Figure 3.* The effect of A-779 on the antithrombotic action of CAP (25 mg twice daily, 10 days) and LOS (10 mg once daily). *\( P < 0.05, \) **\( P < 0.0001 \) vs control. Each column represents the mean±SEM from 6 to 11 experiments.

*Figure 4.* The effect of pretreatment with NO synthase inhibitor (L-NAME; 30 mg/kg SC) and/or cyclooxygenase inhibitor indomethacin (INDO; 2.5 mg/kg IV) on the antithrombotic action of Ang-(1-7) (10 pmol/kg per minute IV). *\( P < 0.05 \) vs control. Each column represents the mean±SEM from 7 to 11 experiments.
found a reduction of the thrombus weight. To our knowledge, this is the first demonstration of the involvement of this peptide in the thrombotic process. The antithrombotic effect of Ang-(1-7) followed the inverted U-shaped curve. Further experiments excluded a possible involvement of a potentially prothrombotic metabolite of Ang-(1-7), Ang-(3-7),\textsuperscript{5,36,37} in this phenomenon. We did not observe an antithrombotic effect of Ang-(1-7) in SO normotensive animals, consistent with previous findings that the effects of this peptide are most prominent when RAS is activated.\textsuperscript{10,34}

We next attempted to check if the antithrombotic action of Ang-(1-7) was receptor dependent and to characterize this putative receptor pharmacologically. We found that the effect of Ang-(1-7) was dose-dependently reversed by A-779 (a specific antagonist at Ang-[1-7] binding site) and attenuated by EXP3174 (AT\textsubscript{1}-receptor antagonist), but not by PD 123,319 (AT\textsubscript{2}-receptor antagonist). These results indicate that the antithrombotic action of Ang-(1-7) in vivo may be mediated by a binding site distinct from the classical angiotensin receptors. That putative Ang-(1-7) binding site may share homology with the AT\textsubscript{1} receptor because it is sensitive to the AT\textsubscript{1}-receptor antagonist. This possibility seems likely because similar atypical angiotensin receptors have been described previously.\textsuperscript{28,35} Alternatively, an interplay between AT\textsubscript{1} and a putative Ang-(1-7) receptor may be necessary for the antithrombotic action of the peptide to occur. The involvement of AT\textsubscript{1} receptor is in line with studies showing that Ang-(1-7) may either modulate the allosteric binding of Ang II to the AT\textsubscript{1} receptor or compete with Ang II for this binding site.\textsuperscript{38,39} For example, in rabbit\textsuperscript{39} and human arteries,\textsuperscript{40} it has been demonstrated that Ang-(1-7) behaves as a specific noncompetitive antagonist of AT\textsubscript{1}. The involvement of a putative Ang-(1-7) receptor in many actions of this peptide, including the liberation of NO from bovine aortic endothelial cells,\textsuperscript{23} was also demonstrated previously.

In the present study, the antithrombotic effects of CAP or LOS were attenuated by A-779, pointing toward Ang-(1-7) as a specific common mediator of their action. Thus, once we recognized the antithrombotic properties of Ang-(1-7) and found that it mediates the antithrombotic effect of RAS blockers, we tried to clarify if Ang-(1-7) acts in an NO/PGI\textsubscript{2}-dependent manner. We reasoned that Ang-(1-7) is of major importance for the antithrombotic action of RAS blockers, because their effect is NO/PGI\textsubscript{2}-dependent. This is validated by well-established inhibitory effect of these endothelial mediators on platelets and leukocytes—cells involved in thrombus formation.\textsuperscript{41–44} First, we found that NO synthase inhibitor (L-NAME) and PGI\textsubscript{2} synthesis inhibitor (indomethacin) did not alter the thrombus development in hypertensive rats, probably owing to compensatory mechanisms, which might have developed in these animals as a response to the long-term impairment of NO/PGI\textsubscript{2} production by endothelial cells. However, we have demonstrated that L-NAME and indomethacin, when given concomitantly, completely abolished the antithrombotic effect of Ang-(1-7). The involvement of NO and PGI\textsubscript{2} in the observed phenomenon is in accordance with a number of studies pointing to the role of these mediators in the vascular actions of Ang-(1-7). In piglet pial arterioles, vasodepressor action of Ang-(1-7) depends on prostaglandin release,\textsuperscript{24} and in porcine, feline, and canine vessels, this action depends mainly on NO release.\textsuperscript{45–47} Also, in vivo Ang-(1-7) decreases blood pressure in spontaneously hypertensive rats, pithed rats, and hypertensive dogs in an NO/PGI\textsubscript{2}-dependent manner.\textsuperscript{48–50} Therefore, from these results and from our present study, it appears that the hypothetical Ang-(1-7) receptor must be distinct from AT\textsubscript{2}, may share some homology with AT\textsubscript{1}, must be linked to NO/PGI\textsubscript{2} production or release.

Although our present results demonstrated the involvement of Ang-(1-7) in the antithrombotic action of RAS blockers, the actual mechanism may be more complex. Our recent study has shown that during AT\textsubscript{1} receptor blockade, the antithrombotic effect of LOS, at least partly, was related to excessive AT\textsubscript{2} receptor stimulation.\textsuperscript{51} Therefore, AT\textsubscript{2} receptor stimulation may explain its incomplete reversal after the administration of Ang-(1-7) receptor blocker. Additionally, LOS is a weak antagonist of thromboxane A\textsubscript{2}/prostaglandin H\textsubscript{2} (TXA\textsubscript{2}/PGH\textsubscript{2}) receptor on platelets.\textsuperscript{52–54} Because the activation of this receptor is crucial for platelet aggregation, its blockade may also contribute to the antithrombotic effect of LOS. As for captopril, we have shown recently that a thiol group of this drug is important for its antithrombotic action,\textsuperscript{19} most probably by trapping and stabilizing endogenous NO in stable S-nitroso compounds.

Thus, from our current and previous studies a complex but consistent picture emerges for the mode of antithrombotic action of RAS blockers, with Ang-(1-7) being a common mechanism for the effects of both ACE-I\textsubscript{s} and AT\textsubscript{1}-receptor antagonists (Figure 5). The decrease in Ang II level (or AT\textsubscript{1} receptor blockade) prevents the induction of PAI-1 and TF in endothelial cells. High concentration of Ang-(1-7) acting on a putative Ang-(1-7) receptor and/or AT\textsubscript{1} receptors facilitates the production and liberation of antithrombotic endothelial mediators NO/PGI\textsubscript{2}. In the case of LOS, the action of Ang-(1-7) via AT\textsubscript{2}-receptor is likely to be inhibited, but the antithrombotic effect can be augmented by the stimulation of unblocked AT\textsubscript{2} receptor by high amounts of Ang II and by the blockade of platelet TXA\textsubscript{2}/PGH\textsubscript{2} receptor. CAP potentiates
the antithrombotic effect of NO, probably by stabilizing it and prolonging its half-life.

Perspectives
The majority of studies on the role of Ang-(1-7) suggests that it opposes or counter-regulates the cardiovascular effects of Ang II. Our present study confirmed these findings and extended them to the thrombotic process. There is clearly a lot to be done to fully clarify the mechanisms involved in these effect of Ang-(1-7). The identification and cloning of the putative Ang-(1-7) receptor may be an important step toward better understanding of the renin-angiotensin system. This may also lead to the development of new drugs that could target the Ang-(1-7) binding site and, by mimicking Ang-(1-7) effects, would prevent thrombosis, prevent the formation of neointima after coronary angioplasty, or serve as antihypertensive drugs.

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