Mechanisms of angiotensin-(1–7)-induced inhibition of angiogenesis

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Machado, R. D. P., R. A. S. Santos, and S. P. Andrade. Mechanisms of angiotensin-(1–7)-induced inhibition of angiogenesis. Am J Physiol Regulatory Integrative Comp Physiol 280: R994–R1000, 2001.—Angiotensin-(1–7) [ANG-(1–7)], an endogenous bioactive peptide constituent of the renin-angiotensin system, acts as an inhibitory growth factor in vitro and in vivo. In this study, we evaluated whether the antiangiogenic effect of ANG-(1–7) in the mouse sponge model of angiogenesis might be receptor mediated and involved in the release of nitric oxide (NO). The hemoglobin content (μg/mg wet tissue) of 7-day-old sponge implants was used as an index of the vascularization and showed that daily injections of ANG-(1–7) (20 ng) inhibited significantly the angiogenesis in the implants relative to the saline-treated group. The specific receptor antagonist d-Ala7-ANG-(1–7); A-779 prevented ANG-(1–7)-induced inhibition of angiogenesis. The antiangiogenic effect was also abolished by pretreatment with NO synthase inhibitors aminoguanidine (1 mg/ml) or Nω-nitro-L-arginine methyl ester (0.3 mg/ml). Selective AT1 and AT2 angiotensin-receptor antagonists and an angiotensin-converting enzyme inhibitor, in combination with ANG-(1–7) or alone, did not alter angiogenesis in the implants. These results establish that the regulation of the vascular tissue growth by ANG-(1–7) is associated with NO release by activation of an angiotensin receptor distinct from AT1 and AT2.

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The components of the renin-angiotensin system (RAS) have been shown to function as growth factors, apart from their classical roles in controlling blood volume and homeostasis. Interestingly, two of the components, the octapeptide ANG II and the heptapeptide angiotensin-(1–7) [ANG-(1–7)], act in opposition, both as tissue growth regulators and in the control of some cardiovascular functions. Freeman et al. (11) have shown that ANG II stimulated vascular smooth muscle cells, whereas equimolar concentrations of ANG-(1–7) inhibited cell growth. The vasoconstrictor ANG II also stimulated angiogenesis (2, 8, 15, 19, 42), an essential component of wound healing, tumor growth, and chronic inflammatory diseases. Recently, we have demonstrated that these two angiotensin peptides exert different actions in regulating sponge-induced angiogenesis in mice. Although ANG II exerted positive effects on angiogenesis, ANG-(1–7) was shown to inhibit angiogenesis and fibrovascular tissue infiltration in the sponge. These findings prompted us to suggest that these peptides might participate in the control of vascular tissue growth as endogenous regulators of angiogenesis (23), in a pattern similar to that of other extracellular proteins that are not tissue growth regulators themselves (14, 33).

ANG-(1–7) mediates its biological activities by a variety of actions including the release of vasopressin (37), prostaglandins (17, 36), and nitric oxide (NO) (20). Although there is good evidence that the actions of ANG-(1–7) are mediated through an angiotensin receptor that is distinct from the well-characterized AT1- and AT2-receptor subtypes, there are some conflicting results suggesting the involvement of these angiotensin receptors in mediating several biological actions of ANG-(1–7) in vivo and in vitro. Thus, although the blockade of the antihypertensive effect of ANG-(1–7) as well as the synthesis and release of vasoactive mediators has been achieved by using the selective ANG-(1–7) antagonist d-Ala7-ANG-(1–7), A-779 (35), the release of arachidonic acid and prostanooid synthesis in smooth muscle cells induced by this angiotensin peptide were shown to be partially blocked by the AT2 antagonists PD-123177 or PD-123319 (25). Furthermore, in the feline mesenteric and hindquarter vascular beds, the vasoconstrictor effect of the peptide was mediated by activation of the AT1-receptor subtype (29). Because of the involvement of NO and prostaglandins in modulating angiogenesis and tissue growth (12, 27, 34, 38, 40), we reasoned that the antiangiogenic effect of ANG-(1–7) in the sponge model might be associated with the release of these vasoactive mediators. The fact that the circulating levels of ANG-(1–7) increased after treatment of human and experimental hypertensive animals with angiotensin-converting enzyme (ACE) inhibitors or AT1-receptor antagonist (4, 6, 9, 18, 22) led us to use a similar approach to identify common mechanisms involved in the biological actions of ANG-(1–7) in different systems. Furthermore, the
possibility that the angiotensin peptide could be acting via AT₁ or AT₂ receptors in the modulation of vascular tissue growth was also investigated.

The present study was, therefore, undertaken to examine possible mechanisms involved in the ANG-(1–7)-induced inhibition of angiogenesis in the mouse sponge model. Understanding the mechanisms underlying ANG-(1–7)-induced inhibition of angiogenesis may allow full assessment of the role of the RAS in wound healing, which, in turn, may suggest new strategies in the management of angiogenesis-dependent conditions.

MATERIALS AND METHODS

Animals. Male Swiss mice weighing 25–30 g were used for the experiments.

Preparation and surgical implantation of cannulated sponge discs. Polyether polyurethane sponge discs, 4-mm thick × 8-mm diameter (Vitafoam), were used as the matrix for fibrovascular tissue growth (2, 23, 40). One end of a polythene tubing, 12-mm length × 1.2-mm internal diameter (Portex), was secured with three 5.0 silk sutures (Ethicon) to the center of each disc in such a way that the tube was perpendicular to the disc face. The cannulated sponge discs were soaked overnight in 70% vol/vol ethanol and sterilized by boiling in distilled water for 15 min. The animals were anesthetized (pentobarbital sodium 4 mg/100 g body wt ip), the dorsal hair was shaved, and the skin was wiped with 70% ethanol. The cannulated sponge discs were aseptically implanted into a subcutaneous pouch that had been made with curved artery forceps through a 1-cm long dorsal midline incision. The cannula, exteriorized through a small incision in the pouch, was afterwards sealed with a removable plastic plug. The animals were housed individually and provided with normal diet and water ad libitum. Housing and anesthesia concurred with the guidelines established by our local Institutional Animal Welfare Committee.

Modulation of angiogenesis. ANG-(1–7) (Sigma), 20 ng in 50 μl sterile saline, was administered intraimplant daily for 3 days, from day 3 to day 5 postimplantation. This treatment resulted in the inhibition of sponge-induced angiogenesis. The dose and treatment schedule were based on the results of our previous study (23). To avoid acute vasoactive effects, the last dose of ANG-(1–7) was given 48 h before assay of the implant. The control group (basal angiogenesis) was injected with an equal volume of saline alone, following the same protocol.

Inhibitors or antagonists were given either orally, in drinking water from the day of implantation onwards, or by local intraplantation injection, given 20 min before injection of ANG-(1–7). The latter mode of administration was adopted when the amounts of antagonist available were strictly limited (A-779, PD-123319). The following compounds were given orally and were started on the day of implantation: amino- guanidine (AG; 1 mg/ml in drinking water, which approximates 230 mg·kg⁻¹·day⁻¹; Sigma), N⁶-nitro-L-arginine methyl ester (l-NAME; 0.3 mg/ml or 20 mg·kg⁻¹·day⁻¹; Sigma), the AT₁-receptor antagonist losartan (30 μg/ml or 10 mg·kg⁻¹·day⁻¹; Merck Sharp Dohme), and enalapril (30 μg/ml or 10 mg·kg⁻¹·day⁻¹; Biolab Farmacéutica). The AT₂-receptor antagonist PD-123319 (1 and 10 μg; RBI) and the peptide antagonist A-779 (1 and 10 μg) were administered in 50 μl saline 20 min before the injection of ANG-(1–7). A set of experiments was performed in which the AT₁-receptor antagonist (1 μg losartan) and the AT₂-receptor antagonist (10 μg PD-123319) were given simultaneously intraplant to determine a potential additive effect of the receptors on the antiangiogenic effect of ANG-(1–7).

For all inhibitors and antagonists, a control group of animals was included in which no agonist [ANG-(1–7)] was given to allow for possible effects on the control, basal, and angiogenic responses. The doses of inhibitors and antagonists were derived from previous experience or published experiments and are as follows: AG (40), l-NAME (24), losartan (40), enalapril (6), PD-123319 (40), and A-779 (31, 40).

Vascular density measurement. At the end of the seventh day postimplantation, mice were killed by ether inhalation. The implants were carefully harvested, dissected, weighed, and processed for hemoglobin extraction. Each implant was homogenized (Tekmar TR-10) in 5 ml of Drabkin Reagent (Labtest) and centrifuged at 2,000 g for 20 min. The supernatants were filtered through a 0.22-μm filter. Hemoglobin in the samples was calculated from a known amount of hemoglobin assayed in parallel form. The results are expressed in micrograms of hemoglobin per milligram of wet tissue.

Statistical analysis. Results are reported as means ± SE. One-way ANOVA followed by Newman-Keuls multiple comparison test was used to assign probabilities to individual between-groups comparisons. A value of P < 0.05% was considered to be statistically significant.

RESULTS

In these experiments, we have used the measurement of hemoglobin as the index of angiogenic activity based on our previous results showing good and reliable correlation with other biochemical and functional measures of new vessel formation (23).

The results of the present experiments elucidated possible mechanisms by which ANG-(1–7) caused inhibition of sponge-induced angiogenesis in mice. Daily doses of 20 ng ANG-(1–7) over 3 days reduced neovascularization relative to saline-treated implants in all experimental groups, confirming our earlier results (23). When the specific ANG-(1–7) antagonist A-779 was coadministered with the standard dose of ANG-(1–7) for 3 days, the amount of hemoglobin in the treated implants (1.45 ± 0.19 μg/mg wet tissue) remained within the range of the control untreated group (1.5 ± 0.12 μg/mg wet tissue), suggesting that the antiangiogenic effect of ANG-(1–7) was mediated by the specific ANG-(1–7) receptor. At a lower dose (1 μg), the specific antagonist did not alter the antiangiogenic effect of exogenous ANG-(1–7) (Fig. 1).

Inhibition of angiogenesis induced by ANG-(1–7) was also totally prevented by pretreatment with either NO synthase inhibitors AG or l-NAME given for 6 days, starting on the day of implantation (Fig. 2, A and B). Angiogenesis in the absence of exogenous peptide i.e., basal angiogenesis, was not modified by AG or l-NAME.

To determine whether the antiangiogenic effect of ANG-(1–7) could be mediated via activation of ANG II receptors AT₁ or AT₂, the selective receptor antagonists losartan (AT₁) or PD-123319 (AT₂) were administered. The results (Fig. 3, A and B) showed that continuous treatment with losartan at a dose corresponding to ~10 mg·kg⁻¹·day⁻¹ over 6 days did not prevent the antiangiogenic effect of the standard dose
of ANG-(1–7) given on days 3–5. The amount of hemoglobin in the losartan-ANG-(1–7)-treated group was 1.05 ± 0.11 (n = 10) vs. 1.77 ± 0.11 (n = 11) for the saline-treated group.

The role of the AT$_2$ receptor in mediating the antiangiogenic action of ANG-(1–7) was assessed with the selective AT$_2$ antagonist PD-123319. The antagonist was given into the implant before the daily injection of ANG-(1–7). At a lower dose of 1 µg (n = 6), the antagonist did not alter the action of ANG-(1–7). At a higher dose (10 µg; n = 8), the selective antagonist was still without effect on the antiangiogenic action of ANG-(1–7). The combination of losartan (1 µg) and PD-123319 (10 µg) given simultaneously intratissue 20 min before the agonist ANG-(1–7) was unable to prevent the antiangiogenic effect of the angiotensin peptide (Fig. 4). The hemoglobin value for the losartan-PD-123319-ANG-(1–7)-treated group was 0.68 ± 0.12 (n = 10) vs. 0.66 ± 0.08 (n = 10) for the ANG-(1–7)-treated group. The hemoglobin (µg hemoglobin/mg wet tissue) in the saline-treated, losartan-treated, and PD-123319-treated implants were 1.6 ± 0.13 (n = 10), 1.34 ± 0.2 (n = 8), and 1.5 ± 0.17 (n = 8), respectively.

Because the circulating levels of ANG-(1–7) can rise after ACE inhibitors (4, 6, 22), we assessed the effect of pretreatment with enalapril in drinking water for 6 days. As shown in Fig. 5, this treatment failed to alter the antiangiogenic effect of ANG-(1–7) as detected by the hemoglobin values; ANG-(1–7)-enalapril-treated group (0.82 ± 0.11, n = 8) vs. ANG-(1–7)-treated group (0.69 ± 0.1, n = 7). Furthermore, treatment with enalapril did not change the basal angiogenesis in our model.

It is important to note that none of the treatments with inhibitors or antagonists altered the basal angiogenesis in this sponge model, regardless of their effects on the response to ANG-(1–7).

DISCUSSION

In the present study, using the sponge model of angiogenesis in mice and the hemoglobin content of the implants as an index of vascularization, we have been able to confirm the antiangiogenic effect of ANG-(1–7) and to demonstrate its prevention by the specific antagonist A-779 and by the NO synthase inhibitors AG and L-NAME. We were also able to show that other angiotensin-receptor antagonists, one selective for the AT$_1$ receptor (losartan) and one selective for the AT$_2$ receptor PD-123319 or the ACE inhibitor enalapril, did not reverse the antiangiogenic effect of the exogenous angiotensin peptide.

Because the index of vascularization used here, the hemoglobin content of the implants, was indirect, it is necessary to consider how this variable might be changed by the treatments applied to the model. Direct vasoconstriction or vasodilatation within the implant would alter blood, and thus hemoglobin con-
tent of the neovasculature and ANG-(1–7) is known to be a vasodilator (3, 29, 32). However, because we assayed the implant 2 days after the last application of ANG-(1–7), such an acute vasomotor influence of this peptide on the final hemoglobin is highly unlikely.

Several of the treatments we have used in this series of experiments are likely to change blood pressure. For instance, losartan and enalapril decreased blood pressure (4, 21), whereas L-NAME given orally increased blood pressure in rats (24). AG, the other NO synthase inhibitor used in our experiments, is more selective for the inducible form (13) and has minimal effects on

Fig. 3. Effects of A1–7 and AT₁ angiotensin-receptor antagonist losartan (LO; 10 mg·kg⁻¹·day⁻¹) (A) given orally and AT₂ angiotensin-receptor antagonist PD-123319 (PD; 1 and 10 µg) (B) on the Hb content of cannulated sponge discs 7 days after implantation. A: A1–7 decreased the Hb content (n = 11) compared with the saline-treated group (n = 11). LO (n = 10) coadministered with A1–7 had no effect on the antiangiogenic activity of the exogenous peptide, as demonstrated by the Hb values. Addition of LO alone (n = 11) did not cause any significant change in the Hb values compared with the saline-treated group. B: A1–7 decreased the Hb content (n = 6) compared with the saline-treated group (n = 7). PD (10 µg, n = 8) coadministered with A1–7 had no effect on the antiangiogenic activity of the exogenous peptide, as demonstrated by the Hb values. Addition of the receptor antagonist alone (1 µg PD, n = 6; 10 µg PD, n = 8) did not cause any significant change in the Hb values compared with the saline-treated group. Values represent means ± SE from n animals as specified in each group, *P < 0.05; different from A1–7 treated. Empty bar, saline treated; +, administered; −, not administered.

Fig. 4. Effects of A1–7 and LO (1 µg) and PD (10 µg) applied intratransplant on the Hb content of cannulated sponge discs 7 days after implantation. A1–7 decreased the Hb content (n = 10) compared with the saline-treated group (n = 10). The angiotensin-receptor antagonists (n = 10) coadministered with A1–7 had no effect on the antiangiogenic activity of the exogenous peptide, as demonstrated by the Hb values. Addition of both receptor antagonists together (n = 8) or alone (LO, n = 8; PD, n = 8) did not cause any significant change in the Hb values compared with the saline-treated group. Values represent means ± SE from n animals as specified in each group, *P < 0.05; different from A1–7 treated. Empty bar, saline treated; +, administered; −, not administered.

Fig. 5. Effects of A1–7 and the angiotensin-converting enzyme inhibitor enalapril (Ena; 10 mg·kg⁻¹·day⁻¹) on the Hb content of cannulated sponge discs 7 days after implantation. A1–7 decreased the Hb content compared with the saline-treated group. Ena coadministered with A1–7 had no effect on the antiangiogenic activity of the exogenous peptide, as demonstrated by the Hb values. Addition Ena alone did not cause any significant change in the Hb values compared with the saline-treated group. Values represent means ± SE from 6 to 8 animals in each group, *P < 0.05; different from A1–7 treated. Empty bar, saline treated; +, administered; −, not administered.
systemic blood pressure under normal conditions (13). However, for each systemic treatment, there was no change in the basal control angiogenesis as measured by the hemoglobin content of the implant. From this we would conclude that either there was no change in blood pressure at the doses used here or that any change in blood pressure was without effect on the variable measured. The alternative interpretation that an “artifactual change” due to blood pressure was exactly balanced by a real angiogenic change is much less tenable.

In our experiments, we have shown that ANG-(1–7) exerted its inhibitory angiogenic effect via a selective peptide receptor as the specific antagonist A-779 was able to abolish the antiangiogenic effect of the angiotensin molecule. This is in line with several reports showing that various ANG-(1–7) effects in vivo and in vitro are produced via activation of an ANG-(1–7)-specific receptor (1, 3, 10, 17, 35). Although there was a trend toward inhibition of the response to ANG-(1–7) with the lower amount of A-779, significant reversal was achieved only with the higher dose of the antagonist, in marked excess of the agonist. One explanation for this finding is that in the angiogenic site (the implant), there is also a high level of peptidase/protease activity. Such activity is known to be associated with angiogenesis, for instance, to break down basement membrane proteins. Because A-779 is a peptide, it would be highly susceptible to hydrolysis in such a high protease environment, and much of the A-779 added is probably hydrolyzed before it can act.

Our finding that the antiangiogenic effect of ANG-(1–7) was significantly abolished by the NO synthase inhibitors AG and L-NAME implies that, at least in part, this angiotensin peptide is exerting its inhibitory action in the fibrovascular tissue induced by the sponge implants by releasing NO. This result would be compatible with reports of the antiangiogenic and antiproliferative effects of NO in in vitro and in vivo systems (12, 34, 41) and with reports that some cardiovascular effects of ANG-(1–7) are mediated via release of NO (20, 29). Because AG is considered to be an inhibitor more selective for the inducible NO synthase (13) and L-NAME inhibits the constitutive NO synthase (24), our observations also indicate that ANG-(1–7) stimulates both inducible and constitutive forms of NO synthase in the sponge model.

Our study also investigated the involvement of AT1 and AT2 receptors in mediating the antiangiogenic effect of ANG-(1–7). Treatment with antagonists of AT1 and AT2, angiotensin receptors losartan and PD-123319 applied alone or in combination failed to alter ANG-(1–7)-induced inhibition of angiogenesis in our in vivo system, regardless of the mode of delivery (systemically or intraimplant). Central actions of ANG-(1–7) (10, 35) and the depressor and antiproliferative responses to this peptide were not inhibited by AT1- or AT2-selective antagonists (3, 11). Thus our findings also suggest that in the sponge implant, ANG-(1–7) activates a non-AT1, non-AT2 angiotensin receptor comparable to that already defined (3, 35). However, the effects of ANG-(1–7) in other tissues and under other experimental conditions have suggested activation of both AT-receptor subtypes, because they were blocked by AT1- and AT2-receptor antagonists (25, 29).

Deddish et al. (7) have suggested that some of the ANG-(1–7) actions could be mediated by the binding of the peptide to ACE, facilitating an interaction between ACE and the bradykinin B2 receptor that, in turn, would potentiate local bradykinin producing biological effects. This appears not to be the case in our model because the antiangiogenic effect of ANG-(1–7) was blocked by its selective antagonist A-779. This compound has no detectable ACE inhibitory activity and did not prevent ANG-(1–7) from inhibiting ACE (31).

On the basis of these findings, it has been suggested that A-779 could be used to differentiate ACE-mediated actions of ANG-(1–7) (7, 21, 28). In keeping with this interpretation, in our study, treatment with the ACE inhibitor enalapril did not prevent the antiangiogenic effect of ANG-(1–7).

Our results have also shown that none of the treatments caused further enhancement of angiogenesis inhibition induced by ANG-(1–7), even under specific conditions where the circulating levels of the peptide are increased (ACE or AT1-receptor inhibition) as previously reported (4, 6, 9, 18, 22). One possibility is that the newly formed fibrovascular tissue in the implant compartment has a population of ANG-(1–7) receptors that is still immature on day 7 postimplantation or the availability of functional receptors is limited. Another possibility is that in a multimediated process such as new blood vessel formation, the contribution of one inhibitor for the whole process is also limited and is likely to be counteracted by other regulatory substances.

The lack of effect of any of our treatments on the basal angiogenesis induced in the sponge implant would argue against a physiological role for this peptide. However, the identification and characterization of angiotensin receptors in these models (15, 42) and the opposing actions of exogenous angiotensin peptides on angiogenesis (23) do suggest that components of the RAS may modulate the endogenous healing processes and/or the persistence of chronic inflammatory and proliferative pathologies related to these processes (5, 26, 42, 43).

It has been well established that implantation technique induces an inflammatory angiogenic response that reproduces many of the features of healing after mechanical and naturally occurring injuries such as balloon angioplasty, atherosclerosis, inflamed synovium, and surgical wounds. In our implant model, we have been able to differentiate among several possible mechanisms of antiangiogenic action. These results validate further the hemoglobin assay as a measure of neovascularization (16, 30, 40) and emphasize the value of the sponge implant as an experimental model to assess the roles of angiotensin derivatives or other mediators in angiogenesis and wound healing.
Perspectives

Our study of the mechanisms involved in the ANG-(1–7)-induced inhibition of angiogenesis in the sponge implant in mice shows that the actions of the peptide are receptor mediated by a subtype that is distinct from the pharmacologically characterized AT1 or AT2 receptor as observed in other systems. Moreover, the inhibitory effect is associated with the release of NO in the granulation tissue induced by the implants. Although our results give no evidence for mediation of angiogenesis by endogenous ANG-(1–7), the marked effect of exogenous peptide reveals its ability to modify the angiogenic process. It is possible that augmentation of endogenous production and ANG-(1–7) and/or exogenous administration of this peptide (or its synthetic analogs) hold potential for antiangiogenic therapy in conditions of persistent angiogenesis.

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