Molecular Mechanisms of Inhibition of Vascular Growth by Angiotensin-(1-7)

E. Ann Tallant, Michelle A. Clark

Abstract—Angiotensin (Ang) peptides play a critical role in regulating vascular reactivity and structure. We showed that Ang-(1-7) reduced smooth muscle growth after vascular injury and attenuated the proliferation of vascular smooth muscle cells (VSMCs). This study investigated the molecular mechanisms of the antiproliferative effects of Ang-(1-7) in cultured rat aortic VSMCs. Ang-(1-7) caused a dose-dependent release of prostacyclin from VSMCs, with a maximal release of 277.9±25.2% of basal values (P<0.05) by 100 nmol/L Ang-(1-7). The cyclooxygenase inhibitor indomethacin significantly attenuated growth inhibition by Ang-(1-7). In contrast, neither a lipoxygenase inhibitor nor a cytochrome p450 epoxygenase inhibitor prevented the antiproliferative effects of Ang-(1-7). These results suggest that Ang-(1-7) inhibits vascular growth by releasing prostacyclin. Ang-(1-7) caused a dose-dependent release of cAMP, which might result from prostacyclin-mediated activation of adenylyl cyclase. The cAMP-dependent protein kinase inhibitor Rp-adenosine-3',5'-cyclic monophosphorothioate attenuated the Ang-(1-7)–mediated inhibition of serum-stimulated thymidine incorporation. Finally, Ang-(1-7) inhibited Ang II stimulation of mitogen-activated protein kinase activities (ERK1/2). The antiproliferative effects of Ang-(1-7) were blocked by sarcosine1-threonine8-Ang-(1-7) and by D-alanine7-Ang-(1-7) (D-Ala7-Ang-(1-7)) but were not prevented by subtype-selective AT1 or AT2 receptor antagonists. Furthermore, we recently showed that the heptapeptide fragment of Ang II, Ang-(1-7), also attenuated mitogen-stimulated VSMC growth, Ang-(1-7) caused a dose-dependent inhibition of serum-stimulated VSMC growth. The antiproliferative effects of Ang-(1-7) were blocked by sarcosine1-threonine8-Ang-(1-7) and by D-alanine7-Ang-(1-7) (D-Ala7-Ang-(1-7)), but were not prevented by subtype-selective AT1 or AT2 receptor antagonists. Furthermore, we showed that a 2-fold elevation in circulating Ang-(1-7), produced by an intravenous infusion of the heptapeptide, significantly inhibited neointimal formation after vascular injury but had no effect on blood pressure, heart rate, or medial cross-sectional area. The associated discovery that plasma Ang-(1-7) is increased to a similar level after ACE inhibitor therapy or long-term administration of AT1 receptor antagonists suggests that Ang-(1-7) might either limit the proliferative response of VSMCs in hypertension or contribute to the mechanisms that account for the reversal of vascular hypertrophy or hyperplasia due to inhibition of Ang II formation or activity.
Ang-(1-7) increases prostaglandin formation in various types of cells, and prostaglandins inhibit vascular growth. Infusions of Ang-(1-7) into rats at the concentration that inhibited neointimal formation in balloon-injured rats increased urinary prostaglandin excretion. Thus, Ang-(1-7) might inhibit vascular growth through the production of prostaglandins and the activation of prostaglandin-mediated cellular events. In this study, we investigated the molecular mechanisms that participate in inhibition of vascular growth by Ang-(1-7).

Materials
Ang-(1-7) was obtained from Bachem California. Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL. Indomethacin, cinnamyl-3,4-dihydroxy-α-cyanocinnamate, 17-octadecynoic acid, carbacyclin, PGI₂, and Rp-adenosine-3’,5’-cyclic monophosphorothioate (Rp-cAMPS) were obtained from Biomol. Phospho-specific antibodies against extracellular signal–regulated kinase (ERK) 1/2 and an ERK2 antibody were obtained from Cell Signaling.

VSMC Isolation
VSMCs were isolated by explant culture from the thoracic aortas of 12- to 14-week-old Hannover Sprague-Dawley rats bred and raised at Wake Forest University School of Medicine, as described previously. Cells were used between passages 4 and 10 and were made quiescent by a 48-hour treatment with defined serum-free medium containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, penicillin, streptomycin, 3 μg/mL insulin, 5 μg/mL transferrin, and 0.2 mmol/L ascorbic acid.

Measurement of Thymidine Incorporation
Quiescent cells in 24-well plates were treated for 48 hours in the presence or absence of angiotensin peptides, 1% FBS, and metabolic inhibitors, as indicated. During the last 24 hours of treatment, 0.5 μCi [3H]thymidine per milliliter was added to each well, and [3H]thymidine incorporation was determined as previously described.

Radioimmunoassays
PGI₂ release from cells incubated for 15 minutes with Ang-(1-7) was measured in Krebs-Ringer solution (125 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO₄, 6 mmol/L glucose, 1 mmol/L CaCl₂, and 25 mmol/L HEPES, pH 7.4). PGI₂ was measured by a radioimmunoassay for its stable metabolite, 6-keto-PGF₁α, by using a kit from PerCepptive Diagnostics. The production of cAMP was measured in confluent monolayers of cells preincubated with 1 mmol/L isobutylmethylxanthine and incubated for 15 minutes at 37°C with Ang-(1-7). The medium was extracted twice in ice-cold 95% ethanol and evaporated to dryness under N₂. cAMP was measured by a radioimmunoassay for its stable metabolite, 6-keto-PGF₁α. Results are presented as the percentage of basal release, which averaged 360±120 pg/mL of incubation medium. Data shown are mean±SEM of duplicate samples of VSMCs isolated from 3 different rat aortas.

Statistics
All data are presented as mean±SEM. Statistical differences were evaluated by repeated-measures, 1-way ANOVA followed by the Dunnett post hoc test or by Student t test. The criterion for statistical significance was set at P<0.05.

Results
Role of Prostaglandins in Growth Inhibition by Ang-(1-7)
PGI₂ release from VSMCs stimulated with Ang-(1-7) was measured to determine whether Ang-(1-7) stimulated the production of PGI₂ in VSMCs isolated from Sprague-Dawley rat aortas. VSMCs were incubated with increasing concentrations of Ang-(1-7) for 15 minutes, and PGI₂ release into the medium was measured by a radioimmunoassay specific for the stable metabolite of prostacyclin, 6-keto-PGF₁α. Ang-(1-7) caused a dose-dependent release of PGI₂, with a maximal release of 177.9±25.2% above basal value at 100 nmol/L Ang-(1-7), as shown in Figure 1.

PGI₂ is produced by the cyclooxygenase-mediated conversion of arachidonic acid to PGG₂/prostacyclin H₂, which is subsequently processed by PGI₂ synthase to PGI₂. The cyclooxygenase inhibitor indomethacin was used to determine whether an increase in prostaglandin production contributes to the Ang-(1-7)–mediated inhibition of VSMC growth. Ang-(1-7) caused a significant inhibition of serum-stimulated [3H]thymidine incorporation (79.1±5.1% of total; n=5, P<0.05). Pretreatment with the cyclooxygenase inhibitor indomethacin (10 μmol/L) effectively blocked the growth inhibition mediated by Ang-(1-7), as shown in Figure 2. In contrast, neither the lipooxygenase inhibitor cinnamyl-3,4-dihydroxy-α-cyanocinnamate (1 μmol/L) nor the cytochrome P450 inhibitor 17-octadecenoic acid (10 μmol/L) had any effect on growth inhibition by the heptapeptide.
Role of cAMP in the Antiproliferative Response to Ang-(1-7)

The addition of PGI2 (or stable analogues of PGI2) to VSMCs activates adenylate cyclase, resulting in an elevation in the cellular levels of cAMP. Because Ang-(1-7) stimulates VSMCs to release PGI2, VSMCs were treated with Ang-(1-7), and cellular cAMP production was measured to determine whether the PGI2 released in response to Ang-(1-7) stimulation caused a subsequent release of cAMP. VSMCs were pretreated with 1 mmol/L isobutylmethylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, to prevent breakdown of cellular cAMP. VSMCs were then treated with Ang-(1-7) for 15 minutes. Cellular cyclic nucleotides were extracted with ethanol and measured by radioimmunoassay. Ang-(1-7), at a concentration of 1 μmol/L, caused a significant increase in the cellular levels of cAMP, to 131.9±9.7% of basal value (n=3, P<0.05).

We hypothesize that Ang-(1-7) inhibits vascular growth by the PGI2-mediated increase in cAMP and its activation of the cAMP-dependent protein kinase. To test this hypothesis, quiescent VSMCs were treated with 1% FBS and 1 μmol/L Ang-(1-7) in the presence or absence of the protein kinase A inhibitor Rp-cAMPS. Ang-(1-7) significantly reduced serum-stimulated [3H]thymidine incorporation to 85.9±6.2% of serum-stimulated incorporation; n=3, P<0.05). PGI2 or its stable analogue carbacyclin, at a concentration of 5 μmol/L, also caused a significant inhibition of serum-stimulated thymidine incorporation (82.9±6.0% and 62.6±2.6% of serum-stimulated incorporation, respectively). The PGI2-mediated inhibition of thymidine incorporation was also significantly blocked by pretreatment with the protein kinase A inhibitor.

Figure 2. Effect of inhibitors of arachidonic acid metabolism on Ang-(1-7)-mediated inhibition of serum-stimulated VSMC growth. Confluent monolayers of VSMCs were treated with 1 μmol/L Ang-(1-7)/1% FBS (vehicle, VEH) in the presence or absence of the cyclooxygenase inhibitor indomethacin (IND, 10 μmol/L), the lipoxygenase inhibitor cinnamyl-3,4-dihydroxy-α-cycano-cinnamate (CDC, 1 μmol/L), or the cytochrome P450 inhibitor 17-octadecynoic acid (ODYA, 10 μmol/L) for 48 hours at 37°C. [3H]thymidine was added during the final 24 hours, and thymidine incorporation was determined. Inhibition of [3H]thymidine incorporation by Ang-(1-7) was calculated as the percentage of serum-stimulated VSMCs incubated with the same concentration of each inhibitor. Results are presented as the percentage of inhibition of serum-stimulated growth by Ang-(1-7), which averaged 20.9±5.1%. Data are mean±SEM of triplicate samples from VSMCs isolated from 3 different rat aortas. *P<0.05 vs growth inhibition by 1 μmol/L Ang-(1-7)/1% FBS.

Figure 3. Blockade of the antitrophic effects of Ang-(1-7) by a cAMP-dependent protein kinase A inhibitor. Quiescent VSMCs were pretreated for 48 hours with 1% FBS, 1 μmol/L Ang-(1-7), 5 μmol/L carbacyclin, or 5 μmol/L PGI2 in the presence or absence of the protein kinase A inhibitor Rp-cAMPS (10 μmol/L). [3H]thymidine was added during the last 24 hours, and thymidine incorporation was determined. Inhibition of [3H]thymidine incorporation by Ang-(1-7) was calculated as the percentage of thymidine incorporation into serum-stimulated VSMCs incubated in the presence or absence of Rp-cAMPS. Results are mean±SEM of triplicate samples from VSMCs isolated from 3 different rat aortas. *P<0.05 vs in the presence and absence of Rp-cAMPS.

Reduction in Ang II–Stimulated ERK1/ERK2 Activity by Ang-(1-7)

Ang II stimulates the MAP kinases ERK1 and ERK2 in VSMCs. Ang II caused a dose-dependent increase in both ERK1 and ERK2 activity (37- and 166-fold increase over basal), with maximal stimulation by 100 nmol/L Ang II (data not shown). Incubation of VSMCs with concentrations of Ang-(1-7) up to 1 μmol/L had no effect on ERK1 or ERK2 phosphorylation. However, preincubation with increasing concentrations of Ang-(1-7) caused a dose-dependent reduction in 100 nmol/L Ang II–stimulated ERK1/2 activities, with maximal inhibition at 1 μmol/L Ang-(1-7), as shown in Figure 4. Ang-(1-7) at 1 μmol/L reduced 100 nmol/L Ang II–stimulated ERK1 and ERK2 activation by 42.3±6.2% and 41.2±4.2%, respectively (P<0.01).

Discussion

In previous studies, we showed that Ang-(1-7) inhibited mitogen-stimulated VSMC growth and reduced neointimal formation after vascular injury. Ang-(1-7) stimulated PGI2 release from VSMCs, and the Ang-(1-7)–mediated inhibition of serum-stimulated [3H]thymidine incorporation into VSMCs was blocked by indomethacin, a cyclooxygenase inhibitor that blocks prostaglandin production. Ang-(1-7) also increased the vascular content of cAMP in VSMCs, and Rp-cAMPS, an inhibitor of the cAMP-dependent protein kinase, blocked the Ang-(1-7)–mediated inhibition of serum-stimulated [3H]thymidine incorporation. Finally, Ang-(1-7) reduced the Ang II–stimulated MAP kinase activities of ERK1 and ERK2. These results suggest that Ang-(1-7) inhibits VSMC growth by a cyclooxygenase-mediated increase in PGI2 through an elevation in cellular levels of cAMP and its activation of the cAMP-dependent protein kinase A inhibitor.
Ang-(1-7) inhibited the growth of cultured VSMCs, in agreement with previous studies.\textsuperscript{17–19} Expression of PGI2 synthase in rat VSMCs increased PGI2 release of thromboxane B2 from rat aortic rings, which was stimulated by Ang-(1-7)–mediated reduction in MAP kinase activity. Ang-(1-7) caused a dose-dependent increase in PGI2 production in VSMCs, in agreement with previous studies.\textsuperscript{17–19} PGI2 inhibited the growth of cultured VSMCs,\textsuperscript{8,9} and over-expression of PGI2 synthase, we blocked PGI2 production in response to Ang-(1-7) by using a cyclooxygenase inhibitor. The Ang-(1-7)– or prostacyclin-mediated inhibition of serum-stimulated \textsuperscript{3H}thymidine incorporation was prevented by the cyclooxygenase inhibitor indomethacin, suggesting that a metabolite of the cyclooxygenase pathway mediates the response to Ang-(1-7). In contrast, blockade of the lipoxygenase pathway inhibited the proliferative response to Ang II in Chinese hamster ovary–AT\textsubscript{1A} cells.\textsuperscript{26} Furthermore, a lipoxygenase inhibitor potentiated PGI2 release by Ang-(1-7) in rabbit VSMCs, suggesting that Ang-(1-7) also stimulated the production of lipoxygenase metabolites, which attenuated PGI2 production in rabbit VSMCs.\textsuperscript{19} Indomethacin prevented the vasodepressor response to Ang-(1-7) in the pithed rat\textsuperscript{27} as well as the dilation of piglet pial arteries.\textsuperscript{28} In spontaneously hypertensive rats treated with lisinopril, losartan, or both, an antibody against Ang-(1-7), an inhibitor of Ang-(1-7) formation, or [D-Ala\textsuperscript{7}]Ang-(1-7) caused an increase in blood pressure.\textsuperscript{29} Indomethacin caused a similar increase in blood pressure, and the increase in blood pressure by [D-Ala\textsuperscript{7}]Ang-(1-7) was prevented by pretreatment with the cyclooxygenase inhibitor. These results suggest that the production of prostaglandins represents a common signaling pathway in both the vasodepressor and antiproliferative effects of Ang-(1-7).

PGI2 receptors on VSMCs activate adenylate cyclase to generate cellular levels of cAMP.\textsuperscript{10} Treatment of VSMCs with Ang-(1-7) also resulted in a significant increase in cAMP in conjunction with an increase in PGI2 production. These results suggest that Ang-(1-7) might stimulate the production of PGI2 receptors and produce cAMP. Alternatively, Ang-(1-7) might directly activate adenylate cyclase to generate cAMP. Inhibition of vascular growth by prostaglandins is correlated with an increase in cAMP,\textsuperscript{30} and pharmacologic agents that increase cAMP production in rabbit VSMCs.\textsuperscript{31,32}

Many of the known effects of cAMP are mediated through the activation of the cAMP-dependent protein kinase. The Ang-(1-7)– or prostacyclin-mediated inhibition of serum-stimulated \textsuperscript{3H}thymidine incorporation was blocked by Rp-cAMPS, an inhibitor of the cAMP-dependent protein kinase. The growth-inhibitory response to phosphodiesterase inhibition was also completely reversed by pretreatment with a peptide inhibitor of the cAMP-dependent protein kinase inhibitor.\textsuperscript{32} These results suggest that Ang-(1-7) inhibits vascular growth through activation of the cAMP-dependent protein kinase and subsequent stimulation of downstream signaling pathways by cAMP-mediated protein phosphorylation.

VSMC growth stimulated by PDGF and Ang II was mediated, at least in part, by activation of MAP kinases,\textsuperscript{33} and inhibition of the MAP kinase pathway prevented VSMC proliferation in response to PDGF.\textsuperscript{34} Ang-(1-7) significantly reduced Ang II–mediated ERK1/ERK2 activity in VSMCs, which might attenuate vascular growth. Vascular MAP kinase activation by PDGF was reduced by an increase in the cellular content of cAMP,\textsuperscript{35} which caused cell cycle arrest by stimulating an inhibitor of cyclin-dependent kinase 4, p27\textsuperscript{kip1}. These results suggest that Ang-(1-7) might inhibit vascular growth in thromboxane formation could also contribute to vascular growth inhibition. Inhibition of vascular lipoxygenase activity by cinnamyl-3,4-dihydroxy-\alpha-cyanocinnamate or blockade of cytochrome P450 epoxygenase activity by octadecynoic acid had no effect on growth inhibition by Ang-(1-7). In contrast, blockade of the lipoxygenase pathway inhibited the proliferative response to Ang II in Chinese hamster ovary–AT\textsubscript{1A} cells.\textsuperscript{26} Furthermore, a lipoxygenase inhibitor potentiated PGI2 release by Ang-(1-7) in rabbit VSMCs, suggesting that Ang-(1-7) also stimulated the production of lipoxygenase metabolites, which attenuated PGI2 production in rabbit VSMCs.\textsuperscript{19} Indomethacin prevented the vasodepressor response to Ang-(1-7) in the pithed rat\textsuperscript{27} as well as the dilation of piglet pial arteries.\textsuperscript{28} In spontaneously hypertensive rats treated with lisinopril, losartan, or both, an antibody against Ang-(1-7), an inhibitor of Ang-(1-7) formation, or [D-Ala\textsuperscript{7}]Ang-(1-7) caused an increase in blood pressure.\textsuperscript{29} Indomethacin caused a similar increase in blood pressure, and the increase in blood pressure by [D-Ala\textsuperscript{7}]Ang-(1-7) was prevented by pretreatment with the cyclooxygenase inhibitor. These results suggest that the production of prostaglandins represents a common signaling pathway in both the vasodepressor and antiproliferative effects of Ang-(1-7).

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growth by a cAMP-mediated inhibition of MAP kinase. A reduction in MAP kinase activity by Ang-(1-7) might result from the inhibition or downregulation of the ERK1/2 kinases by the heptapeptide, by a reduction or inhibition of MEK, the enzyme that activates ERK1/2, or by an increase in MAP kinase phosphatase activity. Takeda-Matsubara et al. showed that estrogen activates both the serine/threonine MAP kinase phosphatase (MKP-1) and a tyrosine phosphatase (SHP-1) to inhibit VSMC growth.

In previous studies, we showed that Ang-(1-7) infusion reduced neointimal formation in rat carotid arteries injured with a balloon catheter.13 Neointimal formation in the injured rabbit aorta was reduced by the stable PGI2 analogue TFC-132,38 whereas treatment with ciprofene, a chemically stable PGI2 analogue, reduced restenosis of human coronary arteries after angioplasty.39 Beraprost, another stable PGI2 analogue, had a protective effect on cardiac allograft vasculopathy in rats through an increase in cAMP and downregulation of the cyclin-dependent protein kinase inhibitor p27Kip1.40 Local administration of 8-bromo-cAMP or phosphodiesterase inhibitors reduced neointimal formation in the balloon-injured rat carotid artery,32 and systemic infusion of 8-chloro-cAMP significantly reduced neointimal formation after balloon injury to the rat carotid artery.42 The reduction in intimal hyperplasia by analogues of PGI2 and cAMP is in agreement with our results, showing that Ang-(1-7) stimulates the production of PGI2 and cAMP and blockade of cyclooxygenase or the cAMP-dependent protein kinase attenuates the response to Ang-(1-7). We also demonstrated a reduction in Ang II–stimulated ERK1/ERK2 activity by Ang-(1-7). The MAP kinases ERK1 and ERK2 are rapidly activated in balloon-injured rat carotid arteries.43 Gene transfer of a dominant-negative mutant of ERK2 or the MAP kinase MEK45 or downregulation of ERK1 and ERK2 by antisense oligonucleotides46 prevented neointimal formation in balloon-injured arteries. Because Ang-(1-7) reduced Ang II–stimulated ERK1/ERK2 activity in vitro, a similar reduction in MAP kinase activity by Ang-(1-7) might account for the reduction in neointimal formation after Ang-(1-7) infusion into carotid artery–injured rats.

The results of the present study show that Ang-(1-7) releases PGI2 and stimulates cAMP production in rat VSMCs. Furthermore, inhibition of PGI2 production by indomethacin and blockade of cAMP-dependent protein kinase activity by Rp-cAMPS attenuated the antiproliferative effects of Ang-(1-7). Ang-(1-7) reduced Ang II–stimulated ERK1/ERK2 activation, the signaling pathway that plays a predominant role in the proliferative response to Ang II. These results suggest that Ang-(1-7) releases PGI2, which has autocrine and paracrine effects on vascular PGI2 receptors to increase cAMP, activate the cAMP-dependent protein kinase, and reduce MAP kinase activities to inhibit vascular growth. These mechanisms might account for the Ang-(1-7)–mediated reduction in neointimal formation after vascular injury.

Perspectives

Ang-(1-7) inhibited the growth of cultured VSMCs and reduced neointimal formation after endothelial denudation of the rat carotid artery. We now report that Ang-(1-7) increased PGI2 and cAMP production and attenuated Ang II–stimulated ERK1/ERK2 activities in cultured VSMCs. In addition, blockade of cyclooxygenase or the cAMP-dependent protein kinase attenuated the Ang-(1-7)–mediated inhibition of VSMC growth, suggesting a role for PGI2 and cAMP production and MAP kinase inhibition in the antiproliferative response to Ang-(1-7). Treatment with stable analogues of PGI2 and cAMP or downregulation of ERK1/ERK2 by gene transfer or antisense oligonucleotides reduced neointimal hyperplasia after vascular injury. These data suggest that the beneficial effects of Ang-(1-7) infusion after vascular injury might be mediated through similar mechanisms. Furthermore, because plasma Ang-(1-7) is elevated after treatment with ACE inhibitors or AT1 receptor antagonists, Ang-(1-7) stimulation of PGI2 and cAMP production, as well as inhibition of MAP kinase activity, might account for the reversal of vascular hypertrophy or hyperplasia due to inhibition of Ang II formation or activity. In addition, Ang-(1-7) is the major product of a newly discovered enzyme of the renin-angiotensin system, ACE2.47 Reduced ACE2 was associated with hypertension quantitative trait loci as detected by linkage analysis, and ACE2 ablation results in mice with severe cardiac dysfunction. Reduced ACE2 expression or activity might result in a shift of the balance between Ang II and Ang-(1-7), resulting in loss of the counteracting vasodepressor and antiproliferative effects of Ang-(1-7).

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