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# Angiotensin-(1-7) Augments Bradykinin-Induced Vasodilation by Competing With ACE and Releasing Nitric Oxide

Ping Li, Mark C. Chappell, Carlos M. Ferrario, K. Bridget Brosnihan

***Abstract*** Recent studies have shown that angiotensin-(1-7) [Ang-(1-7)] interacts with kinins and augments bradykinin (BK)-induced vasodilator responses by an unknown mechanism. In this study, we evaluated whether the potentiation of the BK-induced vasodilation by Ang-(1-7) may be attributable to inhibition of BK metabolism, release of nitric oxide, or both. Isometric tension was measured in intact canine coronary artery rings suspended in organ chambers. $^{125}$I-[Tyr$^{0}$]-BK metabolism was determined in vascular rings by assessing the degradation of the peptide by high-performance liquid chromatography. Ang-(1-7) augmented the vasodilation induced by BK in a concentration-dependent manner in rings preconstricted with the thromboxane analog U46619. The $EC_{50}$ of BK (2.45±0.51 nmol/L versus 0.37±0.08 nmol/L) was shifted leftward by 6.6-fold in the presence of 2 μmol/L concentration of Ang-(1-7). The response was specific for BK, since Ang-(1-7) did not augment the vasodilation induced by either acetylcholine (0.05 μmol/L) or sodium nitroprusside (0.1 μmol/L). Moreover, neither angiotensin I nor angiotensin II (Ang II) duplicated the augmented BK response of Ang-(1-7). Pretreatment of vascular rings with the nitric oxide synthase inhibitor, $N^{\omega}$-nitro-L-arginine (L-NA, 100 μmol/L) completely abolished the effects of Ang-(1-7) on BK-induced vasodilation whereas pretreatment with indomethacin (10 μmol/L) was without effect. The potent specific BK $B_2$ receptor antagonist, Hoe 140, nearly abolished the BK and the Ang-(1-7) potentiated responses at 2 μmol/L, whereas at a lower concentration (20 nmol/L) Hoe 140 shifted the response curve to the right for both Ang-(1-7) and vehicle; however, the augmented response to Ang-(1-7) persisted. Preincubation of vascular rings with 20 μmol/L of the $AT_1$ (CV11974), $AT_2$ (PD123319), or nonselective (Sar$^{1}$ Thr$^{8}$-Ang II) receptor antagonists had no significant effect on the Ang-(1-7)–enhanced vasodilator response to BK. Lisinopril (2 μmol/L) significantly enhanced the BK-induced vasodilator response while at the same time it abolished the synergistic action of Ang-(1-7) on BK. In addition, pretreatment with 2 μmol/L Ang-(1-7) significantly inhibited the degradation of $^{125}$I-[Tyr$^{0}$]-BK and the appearance of the BK-(1-7) and BK-(1-5) metabolites in coronary vascular rings. Ang-(1-7) inhibited purified canine angiotensin converting enzyme activity with an $IC_{50}$ of 0.65 μmol/L. In conclusion, Ang-(1-7) acts as a local synergistic modulator of kinin-induced vasodilation by inhibiting angiotensin converting enzyme and releasing nitric oxide. **(*Hypertension.* 1997;29[part 2]:394-400.)**

***Key Words*** • angiotensin peptides • coronary artery • angiotensin-converting enzyme • endothelium-derived relaxing factors • nitric oxide • kinins • dog

Angiotensin-(1-7) possesses novel biological functions that are distinct from Ang II.[1,2] In contrast to Ang II, Ang-(1-7) is not a dipsogen or an aldosterone secretagogue,[3,4] but similar to Ang II, it stimulates the release of vasopressin,[5] prostaglandins,[6] and nitric oxide.[7] Ang-(1-7) counteracts several actions of Ang II. In both canine and porcine coronary arteries, Ang-(1-7) causes vasodilation,[8,9] while Ang II divergently constricts the coronary arteries.[8] Freeman et al[10] showed that Ang-(1-7) inhibits cultured vascular smooth muscle cell growth, whereas equal molar concentration of Ang II stimulates cell growth. In transgenic (mRen-2)27 hypertensive rats, central administration of antibodies to Ang-(1-7) or Ang II causes opposite changes in blood pressure.[11] In addition, chronic intravenous infusion of Ang-(1-7) reduces blood pressure in SHR,[12] while acute infusion of Ang-(1-7) causes a long-lasting depressor response in the pithed rat[13] and vasodilation of piglet pial arterioles.[14]

There is evidence that Ang-(1-7) may interact with kinins and augment BK-induced vasodilator responses. Porsti et al[9] and Brosnihan et al[8] reported that the specific BK $B_2$ receptor antagonist Hoe 140 inhibited the Ang-(1-7)–evoked vasodilator response in coronary vessels. In rat kidney, Hoe 140 inhibited Ang-(1-7)–induced natriuresis and diuresis.[15] Paula et al[16] found that Ang-(1-7) infusion potentiated BK-induced hypotensive responses in conscious rats. The ACEI quinaprilat enhanced Ang-(1-7)–induced vasodilation,[9] a finding that may be interpreted as resulting from inhibition of BK degradation.[17-19] Additionally, ACE inhibition is associated with significant elevations of Ang-(1-7) as blockade of ACE activity diverts the pathway of Ang II formation from Ang I into Ang-(1-7).[20-22] Thus, the vasodilator response associated with ACEI therapy may be due to local accumulation of BK and Ang-(1-7) and amplification of the effects of BK in the presence of augmented plasma and tissue concentrations of Ang-(1-7). The present studies were conducted to explore further the mechanisms of action of Ang-(1-7) in the BK-potentiating response in isolated canine coronary arteries.

## Methods

### Vascular Ring Reactivity

Following approval by the Institutional Animal Care and Use Committee, 18 male dogs (body weight: 15 to 25 kg) were anesthetized with a combination of ketamine (15 mg/kg IM) and 2% halothane. Animals were euthanatized with a lethal dose of sodium pentobarbital (50 mg/kg IV). The hearts were removed im-

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**Selected Abbreviations and Acronyms**

ACEI = angiotensin converting enzyme inhibitor
Ach = acetylcholine
Ang = angiotensin
BK = bradykinin
L-NA = $N^{\omega}$-nitro-L-arginine

mediately and kept in ice-cold modified Krebs-Henseleit buffer while the left anterior descending coronary artery was carefully dissected free of fat and adhering connective tissues The artery was cut into 3-mm-long rings and mounted with two stainless steel wire triangles in glass organ chambers containing modified Krebs-Henseleit solution (composition in mmol/L 118 3 NaCl, 4 7 KCl, 2 5 $CaCl_2$, 1 2 $MgSO_4$, 1 2 $KH_2PO_4$, 25 $NaHCO_3$, 0 026 CaNaEDTA, and 11 glucose) The Krebs' solution was aerated with 95% $O_2$ and 5% $CO_2$ at 37°C (pH 7 4) and the rings were allowed to equilibrate for 60 minutes at 1 g initial resting tension Basal tension was increased in a step-by-step fashion until the optimal length-tension relationship was achieved by repeated exposure to 40 mmol/L KCl (the optimal tension was 3 5 to 5 g) Isometric tension of the vascular rings was measured continuously using a polygraph (Grass No 7) The integrity of functional endothelium was confirmed by the presence of Ach-induced relaxation (more than 90% relaxation at 0 1 μmol/L of Ach) in rings preconstricted with 10 nmol/L of the thromboxane $A_2$ analogue U46619 It was demonstrated in a previous study[8] that the stable constriction induced by 10 nmol/L U46619 was 40% to 50% of maximal contraction produced by U46619

## Measurements of ACE Activity

ACE was purified ≈1200-fold from a membrane fraction of canine lung using a lisinopril-coupled affinity column, described elsewhere[23,24] Tissue was homogenized in 25 mmol/L HEPES, 0 2 mol/L NaCl, pH 8 0 (Buffer A) in a blender followed by centrifugation at 10 000*g* for 30 minutes The pellet was washed once with Buffer A and centrifuged, and then the pellet was solubilized in Buffer A with 0 1% Triton X-100 and 0 05 mmol/L $ZnSO_4$ (Buffer B) Following centrifugation at 30 000*g* for 30 minutes, the supernatant was applied (10 mL/h) to a Sepharose 6B affinity column (Sigma Chemical Co) coupled to lisinopril via a 2 8-nm spacer arm The column was washed with 100 mL of Buffer B, 50 mL of Buffer B containing 0 5 mol/L NaCl, and 50 mL of Buffer B at 4°C, and the enzyme was eluted with 10 mL of 50 mmol/L sodium borate, pH 9 5, 0 1% Triton ACE was further purified and concentrated on a DEAE cellulose ion-exchange column (in 10 mmol/L HEPES, pH 7 0, 0 1% Triton) and eluted with Buffer B containing 0 5 mol/L NaCl Protein concentration was determined by a BioRad Bradford assay kit

ACE activity was determined using the synthetic substrate Hip-His-Leu as described previously[25] Purified canine ACE (40 ng) was incubated in 0 48-mL Buffer A with 1 0 mol/L sodium chloride with 1 mmol/L concentration of Hip-His-Leu for 15 minutes at 37°C The reaction was terminated by addition of 1 2 mL 0 34N sodium hydroxide (NaOH) The product (His-Leu) was reacted with 0 1 mL *o*-phthalaldehyde (20 mg/mL methanol) for 10 minutes followed by 0 2 mL 3N hydrochloric acid, and the fluorescence was determined (365-nm excitation and 495-nm emission) The specificity of the assay was assessed by addition of 1 μmol/L lisinopril

## $^{125}$I-[Tyr$^0$]BK Metabolism

We employed a BK analog containing tyrosine ($Tyr^0$) at the amino terminus of the peptide for metabolism studies The $[Tyr]^0$-BK analog (Bachem) was iodinated with $^{125}$I-Na (Amersham) using the chloramine-T method and purified by high-performance liquid chromatography (HPLC, see below) to a specific activity of 2200 Ci/mmol[26] The peak of $^{125}$I-$[Tyr]^0$-BK was diluted in 1.5 mol/L Tris-HCl, pH 8 3, containing 0 05% BSA and stored at 4°C The radiolabeled peptide (final concentration of 1 nmol/L) was added to the tubes containing three rings preincubated for 60 minutes with 1-mL Krebs' buffer gassed with 95% $O_2$ and 5% $CO_2$ at 37°C Aliquots of the incubation medium were obtained at 5, 10, and 20 minutes and the metabolism was terminated by addition of 1% heptafluorobutyric acid

The extent of $^{125}$I-$[Tyr]^0$-BK metabolism was determined by HPLC using the HFBA solvent system[26] This system consisted of 0 1% HFBA (mobile phase A) and 80% acetonitrile/0 1% HFBA (mobile phase B), and the analysis was performed on an Applied Biosystems 400 HPLC equipped with a narrow-bore Waters Nova-Pak $C_{18}$ column (Waters, 2 1×150 mm), an Aquapore $C_8$ guard column (Applied Biosystems, 3 2×15 mm), and a Rheodyne 7125 injector (ABI) Samples (50 μL, 25 to 50 000 cpm) were analyzed under isocratic conditions of 38% mobile phase B at a flow rate of 0 35 mL/min One-minute fractions (0 35 mL) were collected and counted in a Packard gamma counter (Packard Instruments, counting efficiency 75%) $^{125}$I- $[Tyr]^0$-BK-(1-7) and BK-(1-5) standards were prepared enzymatically by incubating $^{125}$I-$[Tyr]^0$-BK with purified ACE, and the peptides were isolated by HPLC

## Experimental Protocol 1

BK-induced relaxation (1 nmol/L) was produced after 1 hour equilibration in intact vascular rings preconstricted with 10 nmol/L U46619 Ang-(1-7) at concentrations of 0.1 to 2 μmol/L was used to pretreat quiescent coronary artery rings for 10 minutes, then 1 nmol/L BK-induced relaxation response was repeated in the preconstricted rings The specificity of the potentiation response for BK was assessed by measurement of the relaxation responses to either Ach (50 nmol/L) or sodium nitroprusside (0 1 μmol/L) before and after pretreatment with 2 μmol/L Ang-(1-7)

## Experimental Protocol 2

For experiments in protocol 2, it was determined that control concentration-dependent, cumulative relaxation response curves to BK ($10^{-10}$ to $10^{-6}$ mol/L) were able to be generated in rings preconstricted from 70% to 80% of maximal contraction with 50 nmol/L of U46619 The contributions of endothelium-derived nitric oxide, vasoactive prostaglandins, ACE, and Ang and kinin receptors to the Ang-(1-7) potentiation response for BK were investigated as follows (1) The contribution of vasodilator prostaglandins was assessed by administration of the cyclooxygenase inhibitor indomethacin (10 μmol/L) applied for 20 minutes before introduction of 2 μmol/L Ang-(1-7) for 10 minutes Then the BK-induced dose-dependent relaxation response curve was repeated (2) In separate experiments, rings were similarly co-pretreated with the nitric oxide synthase inhibitor L-NA (100 μmol/L) for 20 minutes followed by the addition of 2 μmol/L Ang-(1-7) for 10 minutes (3) To evaluate a potential interaction among Ang-(1-7), kinins, and ACE, coronary artery rings were also exposed for 20 minutes to either the specific BK $B_2$ receptor blocker Hoe 140 (2 μmol/L and 20 nmol/L) or the ACEI lisinopril (2 μmol/L), and each was then followed with 2 μmol/L Ang-(1-7) co-pretreatment for 10 minutes (4) The participation of Ang receptor subtypes was determined following administration of selective $AT_1$ (CV11974, 20 μmol/L [the active form of TCV-116][27]), and $AT_2$ (PD123319, 20 μmol/L) receptor antagonists A nonselective competitive peptide Ang II receptor antagonist, $Sar^1$ $Thr^8$-Ang II (20 μmol/L), was also utilized to determine the possible participation of other subtypes of Ang receptors

In addition, to evaluate whether Ang I or Ang II also potentiate the BK-induced relaxation response in isolated coronary rings, the BK-induced relaxation response curves were produced in the presence and absence of rings pretreated for 10 minutes with 2 μmol/L of Ang I or Ang II. All receptor antagonists and enzyme inhibitors (lisinopril and cyclooxygenase) did not change basal tension and the preconstriction induced by U46619 On the other hand, Ang I and Ang II pretreatment caused typical phasic constriction in intact quiescent rings, the nitric oxide synthase inhib-

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itor L-NA resulted in either no effect or a slight rise in basal tension

### Experimental Protocol 3

Competition assays using purified canine ACE were determined using a fixed concentration of the substrate Hip-His-Leu (1 mmol/L) and varying the concentrations of the competing agents [lisinopril ($10^{-10}$ to $10^{-7}$ mol/L), Ang-(1-7) ($10^{-8}$ to $10^{-5}$ mol/L), or Sar[1], Thr[8]-Ang II ($10^{-8}$ to $10^{-5}$ mol/L)] Inhibitory constants ($IC_{50}$) were determined from the respective competition curves

To study the effect of Ang-(1-7) on BK metabolism in intact coronary rings, $^{125}$I-[Tyr$^0$]-BK (final concentration of 1 nmol/L) was added to the tubes containing three rings preincubated with 1 mL Krebs' buffer and aerated with 95% $O_2$ and 5% $CO_2$ at 37°C Lisinopril (2 $\mu$mol/L), Ang-(1-7) (2 $\mu$mol/L), or Krebs' buffer as control were added to the rings 10 minutes before addition of the radiolabeled BK Aliquots of the incubation medium were removed at 5, 10, and 20 minutes and diluted with 1% HFBA to inhibit peptidase activity

### Statistical Analysis

The concentration of BK causing 50% ($EC_{50}$) of the maximal relaxation and the $IC_{50}$ of ACE inhibition were calculated using a nonlinear regression method of a sigmoid curve-fitting program (PRISM, Graphpad Inc) Results are reported as mean±SEM (standard error of mean) One-way ANOVA followed by Newman-Keuls multiple comparisons and Student's $t$ test for paired observations were used for statistical analysis A value of $P<05$ was considered statistically significant

### Drugs and Solutions

Ang peptides were purchased from Bachem Hoe 140 was a gift of Hoechst-Roussell Inc (Frankfurt, Germany) PD123319 was generously supplied by Warner-Lambert Parke-Davis Inc (Ann Arbor, Mich), CV11974 by Takeda Chemical Industries, Ltd (Osaka, Japan), and lisinopril by DuPont Merck Co (Wilmington, Del) Other chemicals were purchased from Sigma Chemical Co Ang peptides were prepared daily in a Krebs-Henseleit buffer solution Indomethacin and CV11974 were dissolved in 0 2N $Na_2CO_3$ in stock solution and diluted with Krebs' buffer upon use U46619 was prepared as stock solution in ethanol and diluted with Krebs' buffer The concentrations of drugs reported are at a final concentration in organ chambers

## Results

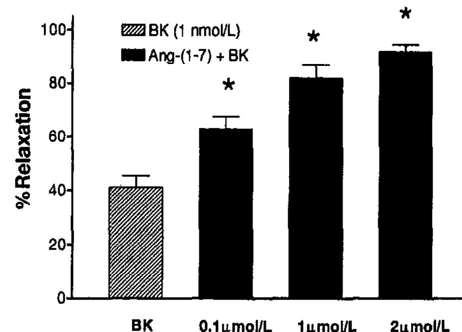
### Synergistic Action of Ang-(1-7) on BK-Induced Vascular Relaxation

BK at a concentration of 1 nmol/L caused endothelium-dependent vasorelaxation of intact coronary artery rings preconstricted with 10 nmol/L of the thromboxane $A_2$ analogue U46619 The BK-induced relaxation response was augmented by Ang-(1-7) (0 1 to 2 $\mu$mol/L) in a dose-dependent manner (Fig 1, top) At a concentration of 2 $\mu$mol/L Ang-(1-7), relaxation to BK was increased 92% compared to BK alone (41±4.4% versus 92±2.5%, $P<01$) The effect of Ang-(1-7) on the BK-induced relaxation response was specific for BK, since Ang-(1-7) did not augment relaxation responses induced by the endothelium-dependent vasodilator Ach (0 05 $\mu$mol/L) (60±5 5% versus 61±6.9%, $P>.05$) or the endothelium-independent vasodilator sodium nitroprusside (0 1 $\mu$mol/L) (70±8 2% versus 68±8.5%, $P>05$) (Fig 1, bottom)

### Contribution of Endothelium-Derived Autacoid Relaxing Factors

BK ($10^{-10}$ to $10^{-6}$ mol/L) elicited concentration-dependent relaxation responses in submaximally preconstricted rings Ang-(1-7) at 2 $\mu$mol/L concentration elicited a significant leftward shift of the BK-induced relaxation response curve. The $EC_{50}$ of BK was reduced 6.6-fold in the presence of 2 $\mu$mol/L Ang-(1-7) ($EC_{50}$, 2 45±0 51 versus 0.37±0 08 nmol/L, $P<.01$) Preincubation with indomethacin (10 $\mu$mol/L) had no significant effect on the BK-induced relaxation response The $EC_{50}$ of BK was not changed in the presence of indomethacin in both control (BK) and Ang-(1-7)–treated groups [control. 2 45±0 51 versus 3.06±0.86, Ang-(1-7) treated 0 37±0 08 versus 0 43±0 11 nmol/L, without versus with indomethacin] The potentiation response to BK produced by 2 $\mu$mol/L Ang-(1-7) was still present in the presence of indomethacin ($EC_{50}$. 3 06±0.86 versus 0.43±0 11 nmol/L, $P<01$) (Fig 2, top) In contrast, pretreatment with the nitric oxide synthase inhibitor L-NA (100 $\mu$mol/L) significantly shifted the BK-induced relaxation response curves to the right of both control and Ang-(1-7)–treated groups [control 2 45±0.51 versus 28 84±9 68 nmol/L, $P<01$, Ang-(1-7)–treated· 0 37±0 08 versus 31.68±9.59 nmol/L, $P<.01$, without versus with L-NA] and abolished the effect of Ang-(1-7) on the BK-induced relaxation response ($EC_{50}$ 28.84±9 68 versus 31.68±9 59 nmol/L, $P>.05$) (Fig 2, bottom)

Pretreatment with the potent specific BK $B_2$ receptor antagonist Hoe 140 at a concentration of 2 $\mu$mol/L nearly abolished the BK-induced relaxation response, resulting in only about 20% relaxation at the highest concentration of BK tested (1 $\mu$mol/L) The Ang-(1-7) potentiating response to



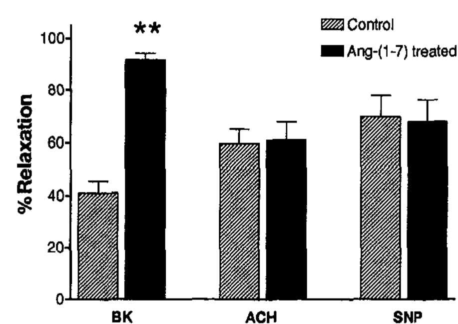




FIG 1 Top, Average dose-response curves to 1 nmol/L BK before (hatched bar) and after (black bars) 10-minute pretreatment with Ang-(1-7) (0 1 to 2 $\mu$mol/L) Bottom, Effect of 2 $\mu$mol/L Ang-(1-7) on BK (1 nmol/L), Ach (ACH, 50 nmol/L), or sodium nitroprusside (SNP, 100 nmol/L) Values are mean±SEM BK responses are the average of 16 rings obtained from 6 dogs All other groups contain 6 to 12 rings from 4 to 6 dogs *$P<05$ vs BK, **$P<01$ vs control

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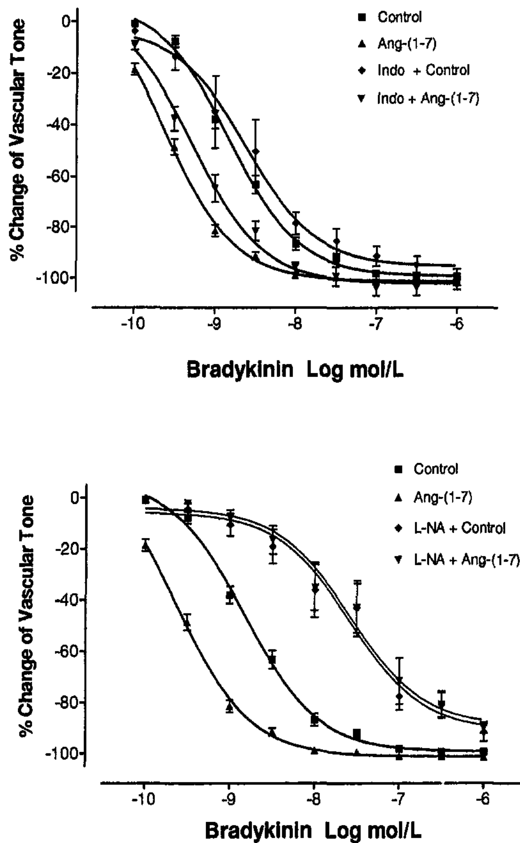


FIG 2 Top, Cumulative dose-response curves to BK ($10^{-10}$ to $10^{-6}$ mol/L) alone (control) and in the presence of 2 $\mu$mol/L Ang-(1-7) Conditions are the same as in Figs 2 to 5 Preincubation with the cyclooxygenase inhibitor indomethacin (Indo, 10 $\mu$mol/L) had no significant effect on the BK-induced relaxation response. Coincubation with indomethacin (10 $\mu$mol/L) and 2 $\mu$mol/L Ang-(1-7) showed no difference from the findings obtained with 2 $\mu$mol/L Ang-(1-7) alone Bottom, Cumulative dose-response curves to BK ($10^{-10}$ to $10^{-6}$ mol/L) alone (control) and in the presence of 2 $\mu$mol/L Ang-(1-7) Preincubation with the nitric oxide synthase inhibitor L-NA (100 $\mu$mol/L) shifted the BK-induced relaxation response to the right Co-pretreatment with L-NA (100 $\mu$mol/L) and 2 $\mu$mol/L Ang-(1-7) showed no difference from the L-NA treated alone L-NA abolished the Ang-(1-7) potentiating effect on the BK-induced relaxation response Values are mean±SEM Control group contains 33 rings from 12 dogs, and Ang-(1-7)–treated group has 44 rings from 10 dogs Other treatments include 6 to 10 rings from 3 to 4 dogs

BK was no longer present (data not shown) Pretreatment with Hoe 140 at a 20 nmol/L concentration shifted the BK-induced relaxation response curves to the right of both control and Ang-(1-7)–treated group [control. 2 45±0 51 versus 547 65±19 63 nmol/L, $P$<.01, Ang-(1-7)–treated 0 37±0 08 versus 115.14±23.96 nmol/L, $P$<.01, without versus with Hoe 140] (Fig 3, top) However, in the presence of 20 nmol/L Hoe 140, Ang-(1-7) still potentiated the BK-induced relaxation [$EC_{50}$: 547 65±19.63 versus 115 14±23 96 nmol/L, $P$<.01, without and with Ang-(1-7)]

Pretreatment with the ACEI lisinopril (2 $\mu$mol/L) markedly shifted the BK-induced relaxation response curves to the left of both control and Ang-(1-7)–treated groups [control: 2450±510 versus 0 25±0.07 pmol/L, $P$<.01, Ang-(1-7)–treated· 370±80 versus 0.24±0 05 pmol/L, $P$<.01, without and with lisinopril]. Pretreatment with lisinopril also abolished the potentiation response to BK produced by Ang-(1-7) [$EC_{50}$· 0.24±0 01 versus 0.25±0.01 pmol/L, $P$>.05, without and with Ang-(1-7)] (Fig 3, bottom)

## Effect of Ang Receptor Antagonists

Preincubation with either the $AT_1$ or $AT_2$ receptor antagonists at a concentration 10 times higher (20 $\mu$mol/L CV11974 and 20 $\mu$mol/L PD123319) than Ang-(1-7) did not significantly inhibit the Ang-(1-7) potentiating response to BK [$EC_{50}$: 0 37±0 08 versus 0.44±0 06 versus 0.52±0.16 nmol/L, Ang-(1-7) versus CV11974 versus PD123319] (Fig 4) Similarly, pretreatment of precontracted rings with the nonselective Ang receptor antagonist $Sar^1$ $Thr^8$-Ang II (20 $\mu$mol/L) had no significant effect on the enhanced response to BK produced by Ang-(1-7) [$Ec_{50}$ 0 37±0.08 versus 0 41±0.11 nmol/L, Ang-(1-7) versus $Sar^1$ $Thr^8$ Ang II] (Fig 4). $Sar^1$ $Thr^8$-Ang II had no effect on the BK-induced relaxation response (data not shown)

## Selectivity of Ang Peptides

In contrast to Ang-(1-7), pretreatment with 2 $\mu$mol/L Ang I or Ang II for 10 minutes did not change the BK-

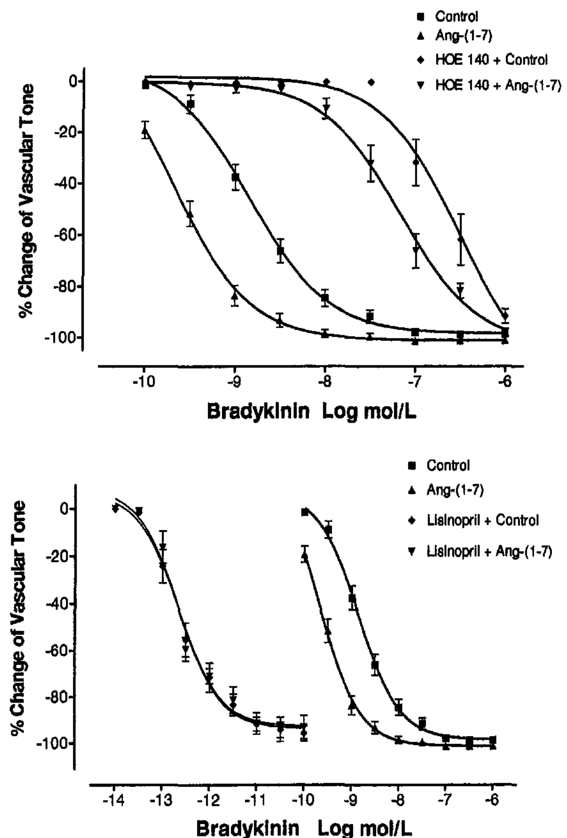


FIG 3 Top, Cumulative dose-response curves to BK ($10^{-10}$ to $10^{-6}$ mol/L) alone (control) and in the presence of 2 $\mu$mol/L Ang-(1-7) Preincubation with the BK $B_2$ receptor antagonist Hoe 140 (20 nmol/L) shifted the BK-induced relaxation response to the right. Preincubation with Hoe 140 (20 nmol/L) and 2 $\mu$mol/L Ang-(1-7) reduced BK-induced response Low concentration of Hoe 140 did not abolish the Ang-(1-7) potentiating effect on the BK-induced relaxation response Bottom, Pretreatment with the ACEI lisinopril (2 $\mu$mol/L) markedly leftward shifted the BK-induced relaxation response curves and that co-pretreated with Ang-(1-7) Pretreatment with lisinopril abolished the potentiation response to BK produced by Ang-(1-7) Values are mean±SEM Each treated group includes 6 to 10 rings from 3 to 4 dogs

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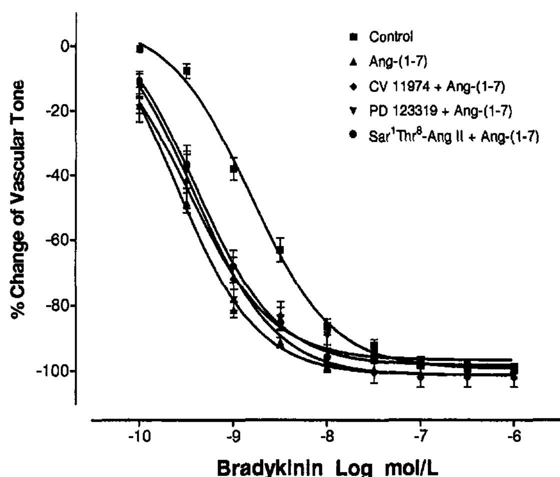


FIG 4 Preincubation with either the $AT_1$ (CV11974, 20 $\mu$mol/L) or $AT_2$ receptor (PD123319, 20 $\mu$mol/L) antagonists at a 10-fold higher concentration than Ang-(1-7) did not significantly block the Ang-(1-7) (2 $\mu$mol/L) potentiating response to BK (control) Similarly, pretreatment with the nonselective Ang receptor antagonist $Sar^1$ $Thr^8$-Ang II (20 $\mu$mol/L) had no significant effect on the potentiating response to BK produced by Ang-(1-7) Values are mean±SEM Each treated group includes 6 to 10 rings from 3 to 4 dogs

induced relaxation response. There was no difference in the $EC_{50}$ of BK in Ang I– and Ang II–treated groups compared with BK alone [$EC_{50}$ 2.45±0 51 versus 2.33±1 24 versus 2 09±0 74 nmol/L, control versus Ang I versus Ang II] (Fig 5)

## Ang-(1-7) Inhibits ACE Activity and Attenuates Metabolism of $^{125}$I-[$Tyr^0$]-BK

Ang-(1-7) inhibited ACE activity purified from canine lungs with an $IC_{50}$ of 0.65 $\mu$mol/L (Fig 6, top). Lisinopril, as expected, was a more potent inhibitor of canine ACE than Ang-(1-7), having an $IC_{50}$ of 1 5 nmol/L. At 10 $\mu$mol/L, $Sar^1$ $Thr^8$-Ang II showed no effect on ACE activity Pretreatment of coronary rings with 2 $\mu$mol/L Ang-(1-7) or 2 $\mu$mol/L lisinopril for 10 minutes significantly attenuated or blocked the rapid degradation of $^{125}$I-[$Tyr^0$]-BK metabolism (Fig 6, bottom). Five minutes after the addition of radiolabeled BK, both Ang-(1-7) and lisinopril were shown to be equally effective in blocking its degradation Thereafter, Ang-(1-7) and lisinopril significantly attenuated the degradation of BK, but at 20 minutes only lisinopril was effective in blocking the degradation of $^{125}$I-[$Tyr^0$]-BK in vascular rings

## Discussion

In isolated canine coronary arteries, Ang-(1-7) has a synergistic, concentration-dependent action on BK-induced vasorelaxation The specificity of the response for Ang-(1-7) was demonstrated by determining the failure of other vasodilator autocoids, namely Ach, sodium nitroprusside, and prostaglandins, to augment the BK-induced relaxation Moreover, neither Ang I nor Ang II given in concentrations equimolar to Ang-(1-7) caused potentiation of the BK response The synergistic effect of Ang-(1-7) on the vasodilator response produced by BK is not mediated by a known Ang receptor since the effect persisted in the presence of $AT_1$, $AT_2$, and $Sar^1$ $Thr^8$-Ang II receptor antagonists. Our studies indicate that the effect of Ang-(1-7) may be produced by release of NO Furthermore, we provide novel evidence that inhibition of endothelial ACE activity may in part also contribute to the synergistic action of Ang-(1-7) on the vasodilator response to BK

Ang-(1-7) acts as a vasodilator opposing the pressor and proliferative actions of Ang II In vivo Ang-(1-7) produces vasodilation in feline mesenteric and hindquarter vascular beds,[28] in piglet pial arterioles,[14] and in pithed rats [13] In porcine and canine coronary arteries,[8,9] Ang-(1-7) dilates vessels by eliciting the release of kinins and endothelial-derived nitric oxide. Our studies provide further evidence that Ang-(1-7) may be acting as a local autocrine or paracrine hormone since renin, angiotensinogen, ACE, and Ang receptors are found in the various cellular elements of the vascular wall [29,30] Local release of both Ang I and Ang II has been demonstrated across the hindlimb and mesenteric beds after bilateral nephrectomy [31] Ang-(1-7) has been shown to be produced locally in endothelial and vascular smooth muscle cells.[21,26]

Kinins are potent endogenous vasodilator peptides causing endothelium-dependent vasodilation by release of endothelium-derived nitric oxide, hyperpolarizing factor, and prostacyclin ($PGI_2$) [17,32,33] The vascular endothelium contains a kinin-generating system,[17,32,34] and kinins are mainly degraded into inactive peptides by local ACE and other endopeptidases.[32,35] Recent studies have shown that ACE inhibition increases local kinin concentration. Kinin levels and the half-life of exogenously added BK in cultured endothelial cells and arterial tissue were increased fourfold and prolonged, respectively, by treatment with ACE inhibitors [36-39] Other studies showed that ACE inhibitors potentiated BK-induced vasodilation mediated by nitric oxide and enhanced cGMP production in arteries [39-41] These studies strongly indicate that ACE inhibition is associated with increases in local kinin concentrations.

In agreement with previous studies,[38,39,42,43] we found that lisinopril inhibited purified canine ACE activity with an $IC_{50}$ of 1 5 nmol/L and also blocked the degradation of BK in vascular rings. Importantly, Ang-(1-7) also inhibited ACE activity with an $IC_{50}$ of 0.65 $\mu$mol/L, possibly by competing with kinins as a substrate for ACE Matsufuji et al[44] showed that in SHR the antihypertensive effects

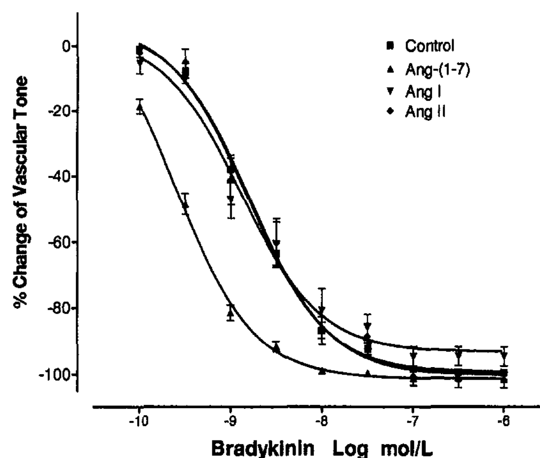


FIG 5 Pretreatment with 2 $\mu$mol/L Ang I or Ang II for 10 minutes had no effect on the BK-induced relaxation (control) response Two micromoles per liter of Ang-(1-7) potentiated the BK-induced relaxation Values are mean±SEM Ang I or Ang II groups include 6 to 8 rings from 3 to 4 dogs

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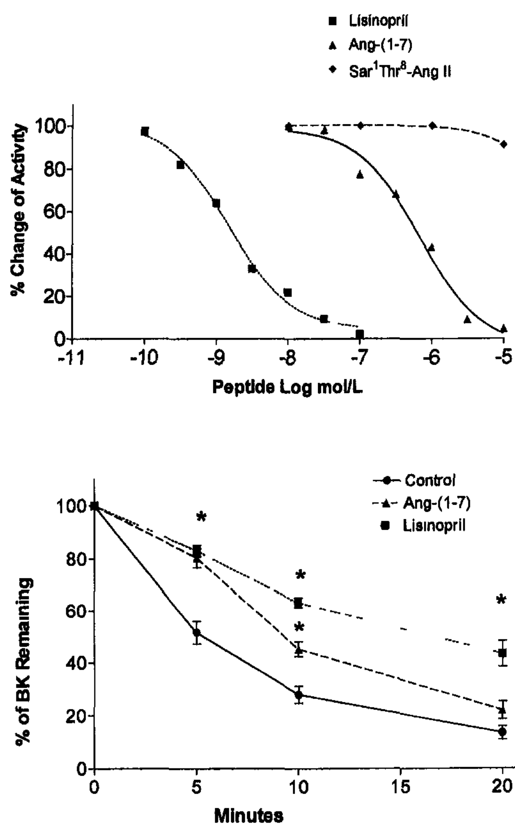


FIG 6 Top, Competition assays using purified canine ACE were determined using a fixed concentration of the substrate Hip-His-Leu (1 mmol/L) and varying the concentrations of the competing agents [lisinopril, $10^{-10}$ to $10^{-7}$ mol/L, Ang-(1-7), $10^{-8}$ to $10^{-5}$ mol/L, and Sar[1] Thr[8]-Ang II, $10^{-8}$ to $10^{-5}$ mol/L] Values are mean±SEM n=3 to 4 dogs Ang-(1-7) had an $IC_{50}$ of 0 65 $\mu$mol/L Lisinopril had an $IC_{50}$ of 1 5 nmol/L At 1 $\mu$mol/L, Sar[1] Thr[8]-Ang II showed no inhibition of ACE. Bottom, $^{125}$I-[Tyr$^0$]BK (final concentration of 1 nmol/L) was added to tubes containing three preincubated coronary rings with 1 mL Krebs' buffer aerated with 95% $O_2$ and 5% $CO_2$ at 37°C Lisinopril (2 $\mu$mol/L), Ang-(1-7) (2 $\mu$mol/L), or Krebs' buffer as control were added to the rings 10 minutes before addition of the radiolabeled BK Aliquots of the incubation medium were removed at 5, 10, and 20 minutes Results are reported as mean±SEM n=4 dogs *$P$< 05 vs control

of small Ang fragments, including Ang-(1-7), were due to ACE inhibition Thus, our findings indicate that Ang-(1-7) may inhibit local endothelial ACE activity, prevent the degradation of kinins in local vessels, and as a consequence augment kinin levels. Furthermore, since it would not be anticipated that ACE inhibition would have an effect on Ach or sodium nitroprusside, our findings demonstrating a specific effect of Ang-(1-7) on BK, but not these other vasodilators, are consistent with Ang-(1-7) competitively binding with ACE and preventing BK degradation The specific interaction of Ang-(1-7) and BK confirms previously reported results in conscious rats [16]

There are some differences between our in vitro studies and those described in whole animal. Paula et al[16] showed that indomethacin but not treatment with enalaprilat blocked the Ang-(1-7) potentiating effect to BK in rats in vivo In contrast, in canine coronary vessels nitric oxide rather than prostaglandins appears to account for the synergistic action of Ang-(1-7) on BK-induced relaxation Similarly, nitric oxide was demonstrated to participate in the potentiation of relaxation to BK by ACE inhibition in canine coronary rings [40] It has previously been shown that Ang-(1-7) stimulated prostaglandin release in pithed rats and cerebral arteriolar vessels in piglets,[13,14] whereas Ang-(1-7) responses were mediated by nitric oxide release in feline hindlimb and mesentery preparations and in vitro preparation of coronary vascular rings.[8,9] The kind of mediator contributing to the modulatory actions of Ang-(1-7) on BK may be tissue or organ specific or less likely reflect differences in species-specific mechanisms

There is another possible explanation for the reported differences between in vivo and in vitro findings ACEIs do not always produce an increase in circulating kinin concentration in vivo.[17] Thus, the lack of an effect of ACE inhibition in the conscious rat may suggest that kinins are either not involved or exist at concentrations below those that will be required to demonstrate a synergistic action between the two peptides In our preparation, lisinopril pretreatment greatly enhanced BK-induced responses, indicating the effectiveness of lisinopril to inhibit local ACE Moreover, lisinopril abolished the Ang-(1-7) potentiating effect to BK Since lisinopril has a much higher affinity to ACE than Ang-(1-7) (≈500-fold), at equal molar concentrations, lisinopril may mask the ACE inhibitory effects of Ang-(1-7) in vascular rings.

Hoe 140 is a potent competitive antagonist of kinin $B_2$ receptors.[17,45,46] Our results showed that high concentrations of Hoe 140 (2 $\mu$mol/L) nearly abolished BK-induced responses and also eliminated the effect of Ang-(1-7) At lower concentrations (20 nmol/L), Hoe 140 caused a rightward shift in the relaxation curve of BK. Under these conditions, however, the potentiating response produced by Ang-(1-7) was still present These findings may be explained by a direct action of Ang-(1-7) on ACE preventing binding of BK to the enzyme Reduced BK metabolism in the presence of Ang-(1-7), as shown in our experiments, would allow increased kinin concentration in the presence of low concentrations of Hoe 140

How then is the synergistic effect of Ang-(1-7) on BK different from the effect of Ang-(1-7) acting as a direct vasodilator of coronary vessels?[8] Both the direct and the synergistic effects of Ang-(1-7) involve a $B_2$ receptor and are mediated by nitric oxide but not prostaglandins Furthermore, in our previous experiment,[8] we found that the direct effect of Ang-(1-7) is mediated by a non-$AT_1$ or -$AT_2$ receptor While Sar[1] Thr[8]-Ang II has been reported by us to block the vasodilator and antiproliferative actions of Ang-(1-7),[8,10] this competitive peptide antagonist did not inhibit the amplification of the BK-induced vasodilator response in the presence of Ang-(1-7). This finding suggests that the interaction between BK and Ang-(1-7) may be mediated by an Ang receptor subtype that is not competed for by Sar[1] Thr[8]-Ang II or the subtype-specific $AT_1$ and $AT_2$ receptor antagonists Alternatively, Ang-(1-7), in these circumstances, may act as an endogenous ligand for $B_2$ receptors or as an ACEI, as discussed above Further studies need to be conducted to evaluate the nature of the receptor and the mechanism accounting for this interaction between Ang-(1-7) and BK receptors.

In summary, we have demonstrated that Ang-(1-7) augments BK-induced vasodilation in coronary arteries by acting as a local modulator of ACE activity and by enhancing the release of nitric oxide. These effects of Ang-(1-7) may contribute to enhanced cardiovascular protection

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