Potentialiation by ACE inhibitors of the dilator response to bradykinin in the coronary microcirculation: interaction at the receptor level

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1 To examine the possibility that angiotensin-converting enzyme (ACE) inhibitors modulate the action of bradykinin at the receptor level, their effect on the dilator response to bradykinin was studied in the isolated saline-perfused heart of the rabbit.

2 Continuous infusion of bradykinin (10 nM) elicited a transient decrease in coronary perfusion pressure (CPP) and increased prostacyclin (PGI₂) release which returned to baseline values within 30 min.

3 Subsequent co-infusion of ramiprilat (≥ 10 nM) or moexiprilat, but not of the less potent ACE inhibitor n-octyl-ramipril (RA-octyl), caused another fall in CPP and an increase in PGI₂ release, the magnitude and time course of which were almost identical to the first response to bradykinin. No change in CPP or PGI₂ release was observed when the ACE inhibitors were administered in the absence of exogenous bradykinin.

4 Infusion of D-Arg[Hyp³]-bradykinin (10 nM), a specific B₂-receptor agonist which was significantly more resistant to degradation by ACE than bradykinin, produced virtually identical changes in CPP and PGI₂ release when compared to bradykinin. Subsequent co-infusion of ramiprilat was similarly effective in restoring the fall in CPP and increase in PGI₂ release elicited by D-Arg[Hyp³]-bradykinin as in the presence of bradykinin.

5 In concentrations which should block the degradation of bradykinin by ACE in the coronary vascular bed, two ACE substrates, hippuryl-L-histidyl-L-leucine (0.2 mM) and angiotensin I (0.3 μ M), were unable to elicit a significant change in CPP or PGI₂ release while ramiprilat and another ACE inhibitor, quinaprilat, were still active in the presence of these substrates.

6 To reveal the potential B₂-receptor action of ramiprilat, its effect on the constrictor response to bradykinin was studied in the rabbit isolated jugular vein. Ramiprilat (0.1 μ M), but not RA-octyl (1 μ M), potentiated the endothelium-independent, B₂-receptor-mediated constrictor response to bradykinin, but not that to the thromboxane-mimetic U46619 (9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α}). Moreover, ramiprilat but not RA-octyl caused a concentration-dependent, B₂-receptor antagonist-sensitive increase in tone when administered alone.

7 These findings suggest that an interaction of ACE inhibitors with the B₂-receptor or its signal transduction pathway rather than an accumulation of bradykinin within the vascular wall is responsible for the restoration of the endothelial response to bradykinin (dilatation, PGI₂ release) in the coronary vascular bed of the rabbit.

Keywords: Angiotensin-converting enzyme; bradykinin; endothelium; heart; rabbit

Introduction

Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of essential hypertension and congestive heart failure due to their unique mechanism of action and favourable safety profile. Their acute blood pressure-lowering effect in patients with an activated renin-angiotensin system, but not in those with normal or low levels of renin, can be explained by an inhibition of systemic and local angiotensin II formation. Since ACE or kininase II (EC 3.4.15.1) also catalyzes the degradation of the potent vasodilator, bradykinin (Regoli & Barabé, 1980), it has long been suspected, but not yet proven, that an increase in the concentration of endogenous kinins also plays an important role in the antihypertensive effect of this class of compounds (Kiowski *et al.*, 1992). Studies on plasma kinin levels in hypertensive patients receiving ACE inhibitors do not support a major role for circulating kinins in the dilator res-

ponse to these drugs (Iimura & Shimamoto, 1989), so that an interference with the degradation of bradykinin within the vascular wall may be assumed. Indeed, vascular endothelial cells, like smooth muscle cells, contain considerable amounts of high-molecular weight kininogen (Schmaier *et al.*, 1988) which upon activation of kallikrein-like serine proteases can be cleaved to yield bradykinin (Bhoola *et al.*, 1992).

The potent vasodilator action of bradykinin is mainly brought about by the subsequent release of nitric oxide (NO) and prostacyclin (PGI₂) from the vascular endothelium through activation of B₂-receptors (Schini *et al.*, 1990). Although B₁ and B₂-receptors may coexist on the same cell, B₁ receptors are normally absent, but their expression in vascular cells may be enhanced after exposure to pro-inflammatory or noxious stimuli (Bhoola *et al.*, 1992). Both ACE and the B₂-receptor are located in the luminal plasma membrane of endothelial cells (Erdős, 1990). It is conceivable, therefore, that the proteolytic activity of this enzyme influences the local concentration of bradykinin at the receptor and hence the amounts of NO and PGI₂ being formed in response to the kinin. By promoting the accumulation of endogenous kinins, presumably bradykinin, we have recently shown that ACE inhibitors can enhance the basal

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release of both PGI₂ and NO from cultured endothelial cells, an effect which is mediated by a B₂-receptor-dependent increase in intracellular Ca²⁺ (Wiemer *et al.*, 1991; Busse & Lamontagne, 1991). In the absence of exogenous bradykinin, ACE inhibitors can also elicit endothelium-dependent, largely NO-mediated relaxations of bovine and porcine coronary arteries which are antagonized by the specific B₂-receptor antagonist, Hoe 140 (Hecker *et al.*, 1993a). Thus, in certain vascular beds there may be a continuous synthesis of vasoactive kinins from an endogenous source, the release of which can stimulate endothelial autacoid formation in an auto- or paracrine manner when their local concentration exceeds a critical threshold level.

However, besides promoting the accumulation of endothelium-derived kinins, ACE inhibitors may also amplify the action of bradykinin at the receptor level (Auch-Schwelk *et al.*, 1993; Hecker *et al.*, 1993a). To address this possibility further, we have (i) investigated the effects of an active and a very weakly active, structurally related ACE inhibitor on the vasodilator response to bradykinin in the isolated saline-perfused rabbit heart, (ii) compared the magnitude and time course of these responses with those elicited by the putatively ACE-resistant B₂-receptor agonist, D-Arg[Hyp³]-bradykinin (Rhaleb *et al.*, 1990), and (iii) examined the effects of these ACE inhibitors on the constrictor action of bradykinin in ring segments of the rabbit jugular vein. Our findings demonstrate that ACE inhibitors enhance the efficacy of bradykinin by modulating its interaction with the B₂-receptor rather than by promoting its accumulation within the vascular wall.

Methods

Isolated rabbit heart (Langendorff preparation)

Hearts from anaesthetized mongrel rabbits (1.0–1.5 kg body weight) of either sex were excised and perfused with warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs-Henseleit solution, pH 7.4 (composition in mM: Na⁺ 142.0, K⁺ 5.2, Cl⁻ 127.0, Ca²⁺ 2.5, Mg²⁺ 1.0, KPO₄²⁻ 1.2, HCO₃⁻ 24.0, D-glucose 5.0, pyruvate 2.0) as described previously (Lamontagne *et al.*, 1992). Coronary perfusion pressure (CPP) and isovolumetric left ventricular pressure (LVP) were measured by means of pressure transducers connected to a sidearm of the aortic perfusion cannula and to a fluid-filled latex balloon inserted into the left ventricle, respectively. Heart rate was derived from the LVP signal by a cardiometer, and the flow rate (20–30 ml min⁻¹) was adjusted to obtain a coronary perfusion pressure of approximately 60 mmHg.

Determination of PGI₂ release

The concentration of 6-keto-PGF_{1α}, the stable hydrolysis product of PGI₂, in the coronary effluent (collected for 20 s before and during the infusion of each substance at various intervals, double determination) was measured by a specific radioimmunoassay (Lamontagne *et al.*, 1992), and PGI₂ release was expressed as ng 6-keto-PGF_{1α} released per g heart weight and minute.

Bioassay

Rabbit jugular veins were cleaned of adventitial adipose and connective tissue and cut into rings of 5 mm width which were mounted between K30 force transducers and a rigid support for measurement of isometric force (Schuler-Organbad, kindly made available to us by Hugo Sachs Elektronik, March, Germany). Four rings were simultaneously incubated in 10 ml of warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs-Henseleit solution (pH 7.4), and basal (passive) tension was adjusted over a 30 min equilibration period to 1 g. In some experiments, the endothelium of the ring segments was removed mechanically and its absence con-

firmed by the lack of a relaxant response to the endothelium-dependent vasodilator acetylcholine (0.1–1 μM) after preconstriction with U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin F_{2α}, 10–30 nM) to 3 g of tension. Relaxations to the endothelium-independent vasodilator, glyceryl trinitrate (0.01–0.1 μM) were not different between endothelium-intact and -denuded ring segments, demonstrating that smooth muscle function was not affected by abrasion of the endothelium.

Preparation of rabbit lung microsomes

Rabbit lungs were rinsed with saline (0.9% NaCl, w/v), minced into small pieces with scissors and homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.8, ~10 ml per lung) containing 30 mM KCl, 5 mM MgSO₄ and 0.25 M sucrose (Das & Soffer, 1975) in a Potter-Elvehjem-type homogenizer fitted with a low-clearance Teflon pestle. Subcellular fractions were prepared by differential ultracentrifugation at 2,000 g (10 min), 10,000 g (20 min) and 100,000 g (60 min). The 100,000 g-sediment (microsomes) was resuspended in the same buffer containing 10% (v/v) glycerol. Protein concentrations were measured according to the method of Bradford (1976) by using the Bio-Rad protein assay and bovine serum albumin as a standard.

Determination of ACE activity

Aliquots of the microsomal fraction (33–100 μg of protein) were incubated with 1 mM [1-¹⁴C]-hippuryl-L-histidyl-L-leucine (specific activity 0.33 Ci mol⁻¹) in 50 mM HEPES buffer, pH 8.0 (total volume of 100 μl) containing 100 mM NaCl and 600 mM Na₂SO₄ at 37°C. After 10, 30 and 60 min, samples were acidified to pH 3.0 with 80 μl 1 M HCl, and extracted with 200 μl ethyl acetate. A 100 μl-aliquot of the organic layer was mixed with 5 ml scintillation fluid and counted for radioactivity (d.p.m.) in a β-liquid scintillation counter. The calculation of ACE activity was based on the recovery of labelled material in the organic layer, the dilution factor (2) and an extraction coefficient of 0.693 (Ryan, 1984).

The ACE activity in rabbit isolated perfused (20 ml min⁻¹) hearts was determined by infusing [1-¹⁴C]-hippuryl-L-histidyl-L-leucine (specific activity 0.66 Ci mol⁻¹) at a rate of 200 nmol min⁻¹ over 25 min. A 1 ml-sample of the effluent was collected at 30 s intervals, acidified to pH 3.0 with 5 μl 5 M HCl, and stored at 0–4°C. Thereafter the samples were extracted with 1 ml ethyl acetate, and an 800 μl-aliquot of the organic layer was mixed with 10 ml scintillation fluid and counted for radioactivity as above.

Comparison of the degradation of bradykinin and D-Arg[Hyp³]-bradykinin by ACE

The two kinins (final concentration 10 μM) were incubated with the microsomal ACE preparation (2.5 mu ACE activity) in 500 μl HEPES buffer (50 mM, pH 8.0) containing 100 mM NaCl and 600 mM Na₂SO₄ for 0–120 min at 37°C. In some experiments either hippuryl-L-histidyl-L-leucine (2 mM) or ramiprilat (1 μM) were incubated together with bradykinin for 0–90 min and 120 min respectively. At regular intervals (10 min), an aliquot of the incubation mixture was removed (10 μl) and added to endothelium-intact ring segments of rabbit jugular vein suspended in 10 ml organ chambers. The resulting constrictions were compared graphimetrically to the control responses of the bioassay tissues in the presence of bradykinin or D-Arg[Hyp³]-bradykinin (10 nM).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as mean ± s.e.mean. Statistical evaluation was performed by unpaired two-tailed Student's *t* test or one-way

analysis of variance (ANOVA), where appropriate, with a *P*-value <0.05 considered statistically significant.

Materials

Hoe 140 (D-Arg[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]-bradykinin), RA-octyl (n-octyl ester of ramipril, RA-octyl) and ramiprilat were kindly provided by Hoechst (Frankfurt/Main, Germany), moexiprilat by Schwarz Pharma (Monheim, Germany), and quinaprilat by Goedecke (Freiburg, Germany). D-Arg[Hyp³]-bradykinin was generously supplied by Prof. D. Regoli (University of Sherbrooke, Canada); ICI-D8731 (2-ethyl-4-[(2'-(1*H*-1,2,3,4-tetrazol-5-yl)biphenyl-4-yl)-methoxy]quinoline hydrochloride) was from ICI Pharmaceuticals (Macclesfield, U.K.). [¹⁴C]-hippuryl-L-histidyl-L-leucine and the [³H]-6-keto-prostaglandin F_{1α} radioimmunoassay kit were from DuPont (Dreieich, Germany), and hippuryl-L-histidyl-L-leucine and angiotensin I from Bachem (Heidelberg, Germany). All other substances and chemicals were either from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany) or Boehringer Mannheim (Germany).

Figure 1 Change in coronary perfusion pressure (CPP) during the continuous infusion of bradykinin (BK) into the rabbit isolated heart and effect of the subsequent co-infusion of ramiprilat (RA; *n* = 18).

Results

The average weight, flow rate and coronary perfusion pressure (CPP) of the rabbit hearts were 5.1 ± 0.2 g, 26.7 ± 0.9 ml min⁻¹ and 63.8 ± 0.5 mmHg (*n* = 22), respectively. Neither ramiprilat nor moexiprilat had any significant effect on CPP (101 ± 8 and $100 \pm 1\%$ of control respectively, *n* = 8–14) or basal PGI₂ release (99 ± 5 and $115 \pm 16\%$ of control respectively, *n* = 7–14) when administered alone at a concentration of 0.3 μM. In separate experiments bradykinin was found to elicit a half-maximal decrease in CPP when infused at a concentration of 10 nM ($21 \pm 2\%$, *n* = 22; maximum fall in CPP of $36 \pm 3\%$ at 1 μM bradykinin, *n* = 5). Despite the continuous infusion of the kinin, this dilator response was rapid and transient with CPP returning to baseline values within 30 min (Figure 1). Subsequent co-infusion of ramiprilat (0.3 μM), after a lag phase of 0.7 ± 0.1 min (*n* = 15), elicited another fall in CPP, the magnitude and time course of which was very similar to the initial response caused by bradykinin (Figure 1). Both bradykinin alone and the subsequent co-infusion of ramiprilat induced a substantial release of PGI₂ (Table 1). These effects of the ACE inhibitor were comparable at concentrations of 0.01 and 0.3 μM (25 ± 3 and $21 \pm 2\%$ decrease in CPP with bradykinin alone and 30 ± 2 and $28 \pm 3\%$ following the subsequent co-infusion of 0.01 and 0.3 μM ramiprilat respectively, *n* = 4–18; for PGI₂ release see Table 1). Moexiprilat produced very similar effects at a concentration of 0.3 μM ($16 \pm 3\%$ decrease in CPP with bradykinin alone and $17 \pm 3\%$ following the subsequent co-infusion of the ACE inhibitor, *n* = 3; for PGI₂ release see Table 1). The B₂-receptor antagonist, Hoe 140 (0.1 μM) completely suppressed both the fall in CPP and the increase in PGI₂ release caused by both ACE inhibitors (*n* = 3, data not shown).

Increasing the concentration of bradykinin in the perfusate from 10 nM to 1 μM after 30 min also elicited another fall in CPP, the time course of which was virtually identical to the initial response elicited by 10 nM bradykinin. Its amplitude, however, was significantly greater (23 ± 3 vs. $40 \pm 1\%$ decrease in CPP, *n* = 3, *P* < 0.01). The whole protocol with ramiprilat (0.3 μM) and bradykinin (10 nM) could be repeated at least once in the same heart after a washout period of 30 min (*n* = 3, data not shown). However, ramiprilat failed to elicit a fall in CPP when administered 10 min after stopping the infusion of bradykinin (21 ± 3 and $0 \pm 0\%$ decrease in CPP respectively, *n* = 4).

To verify that ramiprilat blocks the ACE activity present in the coronary vascular bed, we compared its effect on ACE

Table 1 Effects of different ACE inhibitors and substrates on the release of prostacyclin (PGI₂) from the rabbit isolated perfused heart (expressed as net increase in 6-keto-PGF_{1α} release (ng g⁻¹ min⁻¹) over basal levels) elicited by infusions of bradykinin (BK) or D-Arg[Hyp³]-bradykinin (D-Arg, 10 nM each)

ACE inhibitor (μM)	BK alone	Δ 6-keto-PGF _{1α} release (ng g ⁻¹ min ⁻¹) + ACE inhibitor	n
RA (0.01)	3.6 ± 1.4	3.0 ± 1.0	4
RA (0.3)	4.5 ± 1.6	9.5 ± 3.7	18
RA-octyl (0.01)	2.6 ± 0.9	0.0 ± 0.1*	3
RA-octyl (1.0)	2.6 ± 0.9	0.3 ± 0.1	3
Mxp (0.3)	7.8 ± 0.6	9.4 ± 2.9	6
	D-Arg alone	+ ACE inhibitor	
RA (0.3)	2.2 ± 0.3	0.6 ± 0.1**	5
	BK alone	+ Hip-His-Leu	
RA (0.3)	1.8 ± 0.5	0.2 ± 0.2*	4
		+ ACE inhibitor	
		1.0 ± 0.3	
		+ AI/ICI-D8731	
Qlp (0.3)	8.9 ± 5.7	0.1 ± 0.1	5
		+ ACE inhibitor	
		17.3 ± 12.5	

The asterisks (**P* < 0.05, ***P* < 0.01) denote significant differences from the corresponding control group. Additional abbreviations and concentrations: RA, ramiprilat; RA-octyl, n-octyl ramipril; Mxp, moexiprilat; Qlp, quinaprilat; Hip-His-Leu, 0.2 mM; angiotensin I/ICI-D8731, 0.3/1.0 μM.

activity both *in vitro* (rabbit lung microsomes) and *in situ* (rabbit heart). Ramiprilat inhibited the microsomal ACE activity with an IC₅₀ value of 10 ± 1 nM ($n = 4$). At this concentration, the ACE inhibitor almost completely inhibited the coronary ACE activity (Figure 2) which was approximately ten fold higher (50.4 ± 4.5 mu, $n = 3$) than in the microsomal preparation (5.2 ± 0.1 mu, $n = 4$). RA-octyl was less effective in inhibiting the microsomal ACE activity by two orders of magnitude (IC₅₀-value 1.22 ± 0.28 μ M, $n = 3$, $P < 0.01$) as compared to ramiprilat. Moreover, RA-octyl was virtually inactive in the rabbit heart at a concentration of 10 nM, and affected ACE activity only when its concentration was raised to 1 μ M (Figure 2). Unlike ramiprilat, RA-octyl did not restore the dilator response (25 ± 3 and $0 \pm 0\%$ decrease in CPP in the presence of bradykinin and bradykinin plus RA-octyl respectively, $n = 3$) or augment the release of PGI₂ (Table 1) during the continuous infusion of 10 nM bradykinin. Raising the concentration of RA-octyl in the perfusate from 10 nM to 1 μ M after 10 min caused a fall in CPP ($38 \pm 11\%$, $n = 3$) which, however, was not associated with a concomitant increase in the release of PGI₂ (Table 1).

To examine the possibility that ACE inhibitors modulate the action of bradykinin at the receptor level, we studied the effects of ramiprilat on the responses to D-Arg[Hyp³]-bradykinin, a more specific, and due to its N-terminal elongation, potentially ACE-resistant B₂-receptor agonist (Rhaleb *et al.*, 1990). To verify that D-Arg[Hyp³]-bradykinin is indeed more resistant to inactivation by ACE than bradykinin, we compared the constrictor effect of both kinins after incubation with the rabbit lung ACE preparation on ring segments of endothelium-intact rabbit jugular veins. In control experiments, bradykinin and D-Arg[Hyp³]-bradykinin elicited marked equipotent constrictions of the venous segments with ED₅₀-values of 6 ± 3 and 6 ± 2 nM respectively ($n = 3-5$). Hoe 140 (0.1 μ M) completely prevented the constrictor responses to both kinins, while the B₁-receptor agonist des-Arg⁹-bradykinin was completely inactive in concentrations ≤ 10 μ M ($n = 3$, data not shown). Bradykinin and D-Arg[Hyp³]-bradykinin (10 μ M) were incubated with the microsomal ACE preparation for different periods of time and aliquots of the supernatant (equivalent to a final concentra-

Figure 2 Inhibition of the ACE activity in the rabbit isolated heart by ramiprilat and RA-octyl. The figure shows two individual experiments representative of three experiments with each ACE inhibitor. Ramiprilat (○) or RA-octyl (△) were infused at a concentration of 10 nM or 1 μ M (RA-octyl, ▲) respectively. Due to the difference in ACE activity between individual hearts, ACE activity was expressed as percentage of the mean activity measured prior to the infusion of the ACE inhibitors.

tion of 10 nM in the organ bath) were assayed for their constrictor activity. To allow a more precise determination of the relative stability of the two kinins, the ACE activity in the incubation medium was reduced to 2.5 mu which, on the basis of the estimate that the molecular activity of purified rabbit lung ACE for hippuryl-L-histidyl-L-leucine is 30 times higher than that for bradykinin (Das & Soffer, 1975), should slowly degrade bradykinin over a period of 60–90 min. Under these conditions, bradykinin was significantly less stable ($t_1 = 51 \pm 4$ min, $n = 5$) than D-Arg[Hyp³]-bradykinin ($t_1 = 123 \pm 15$ min, $n = 3$), while the addition of ramiprilat (1 μ M) largely prevented the degradation of bradykinin (Figure 3).

In the rabbit isolated perfused heart, D-Arg[Hyp³]-bradykinin (10 nM) was as potent as bradykinin in decreasing CPP (maximum fall in CPP of 16 ± 2 as compared to 14 ± 1 mmHg, $n = 5-18$) and also elicited a comparable increase in PGI₂ release (Table 1). However, during the infusion of the B₂-receptor agonist, co-infusion of ramiprilat was less effective in causing another fall in CPP as compared to bradykinin, while the time course of these dilator responses was virtually identical (lag phase in the presence of the ACE inhibitor: 0.7 ± 0.2 min, $n = 5$, Figure 4). There was a more marked difference between the two kinins in terms of the ramiprilat-induced PGI₂ release which was identical to the initial response in the presence of bradykinin, but reduced by approximately 75% in the presence of D-Arg[Hyp³]-bradykinin (Table 1). It should be noted, however, that the release of PGI₂ elicited by the co-infusion of ramiprilat in the presence of D-Arg[Hyp³]-bradykinin still represented a sizeable increase over basal levels (8.9 ± 1.9 fold, $n = 5$).

To exclude further an involvement of ACE in the restoration of the bradykinin-induced coronary dilatation by the ACE inhibitors, we tested the effects of two ACE substrates, hippuryl-L-histidyl-L-leucine and angiotensin I. At a concentration 20,000 times higher than that of bradykinin, the synthetic peptide was unable to elicit a significant increase in

Figure 3 Comparison of the rate of inactivation by ACE of bradykinin (○) and D-Arg[Hyp³]-bradykinin (□). The two kinins were incubated with the microsomal ACE preparation for 0–120 min. At regular intervals (10 min), aliquots of the incubation mixture (corresponding to a kinin concentration of 10 nM in the organ bath) were assayed for their constrictor effect on endothelium-intact rings of rabbit jugular vein. Constrictions were expressed relative to the control responses of the bioassay tissues to 10 nM bradykinin or D-Arg[Hyp³]-bradykinin before and after the incubations. Note that the degradation of bradykinin was largely prevented by hippuryl-L-histidyl-L-leucine (2 mM, ●) and ramiprilat (1 μ M, ▲) respectively. The asterisks denote significant differences ($P < 0.01$ by ANOVA) between the indicated curves, ($n = 3-5$).

Figure 4 Comparison of the magnitude and duration of the dilator response (expressed as a percentage decrease in coronary perfusion pressure, CPP) to ramiprilat ($0.3 \mu\text{M}$) in the presence of bradykinin (○) or D-Arg[Hyp³]-bradykinin (●). The asterisk denotes a significant difference ($P < 0.05$ by ANOVA) between the two curves ($n = 5-18$).

Figure 5 Co-infusions of ramiprilat (RA) and quinaprilat (Qpl, $0.3 \mu\text{M}$ each), but not hippuryl-L-histidyl-L-leucine (0.2 mM) or angiotensin I (Ang, $0.3 \mu\text{M}$ in the presence of $1 \mu\text{M}$ ICI-D8731), restore the decrease in coronary perfusion pressure (CPP, expressed as a percentage of basal pressure) elicited by bradykinin (BK, 10 nM) in the rabbit isolated perfused heart ($n = 4-5$; $**P < 0.01$ vs. bradykinin alone).

CPP or PGI₂ release while ramiprilat was still active in the presence of the ACE substrate (Figure 5, Table 1). A 200 fold excess of hippuryl-L-histidyl-L-leucine, on the other hand, almost completely prevented the breakdown of bradykinin by the microsomal ACE preparation (Figure 3). To prevent the vasoconstrictor action of angiotensin I, its effect on the endothelial response to bradykinin was evaluated in the presence of the AT₁ receptor antagonist ICI-D8731. In control experiments, angiotensin I ($0.3 \mu\text{M}$) caused a $4 \pm 1 \text{ mmHg}$ increase in CPP in the presence of ICI-D8731 ($1 \mu\text{M}$, $n = 5$). Although the affinity of ACE for bradykinin and angiotensin I is virtually identical (Das & Soffer, 1975), a 30 fold higher concentration of angiotensin I failed to induce a significant change in CPP or PGI₂ release. However, like ramiprilat or moexiprilat, another ACE

Figure 6 Comparison of the effects of ramiprilat ($0.1 \mu\text{M}$, ●) and RA-octyl ($1 \mu\text{M}$, ▲) on the constrictor responses to cumulative doses of bradykinin (○) in endothelium-denuded ring segments of rabbit jugular vein ($n = 3-12$). The asterisks denote a significant difference ($P < 0.01$ by ANOVA) between the indicated curves.

Figure 7 Effects of increasing concentrations of ramiprilat in the absence (●) or presence of Hoe 140 ($0.1 \mu\text{M}$, ○), on the tone (g) of endothelium-denuded ring segments of rabbit jugular vein ($n = 6-10$; $*P < 0.05$; $**P < 0.01$). Note that the small increase in tension caused by the ACE inhibitor ($0.09 \pm 0.03 \text{ g}$ at $0.1 \mu\text{M}$, $n = 10$) was subtracted when comparing the concentration-response curve of bradykinin in the absence and presence of ramiprilat. The figure also depicts the lack of effect of RA-octyl (■) at a concentration of 1 and $10 \mu\text{M}$ respectively.

inhibitor, quinaprilat, completely restored the sensitivity of the rabbit isolated perfused heart to bradykinin despite the continued presence of angiotensin I (Figure 5, Table 1).

In a separate series of experiments, the effects of ramiprilat on the interaction of bradykinin with the B₂-receptor were studied in ring segments of the rabbit jugular vein. To minimize the possible contribution of endogenous ACE activity, the endothelium was mechanically removed from the veins, a treatment which did not enhance the potency of bradykinin (ED_{50} -value $11 \pm 3 \text{ nM}$, $n = 9$). Co-incubation with RA-octyl ($1 \mu\text{M}$) slightly shifted the concentration-response curve of bradykinin to the right (ED_{50} -value

24 ± 15 nM, *n* = 3, Figure 6). This effect contrasted with the marked shift of the concentration-response curve to the left (ED₅₀-value 0.6 ± 0.1 nM, *n* = 8) in the presence of ramiprilat (0.1 μM, Figure 6). At this concentration, ramiprilat had no effect on the constrictor responses to U46619 (3–300 nM), another receptor-dependent agonist (*n* = 7, data not shown). Moreover ramiprilat, but not RA-octyl (in concentrations of up to 10 μM, Figure 7), elicited a distinct increase in tone even in the absence of exogenous bradykinin. This constrictor response was concentration-dependent and completely antagonized by Hoe 140 (0.1 μM, Figure 7).

Discussion

The present study shows that ACE inhibitors restore the endothelium-dependent dilator response and release of PGI₂ elicited by bradykinin in the coronary vascular bed of the rabbit. Moreover, these agents not only potentiate the contractile responses elicited by bradykinin in the rabbit isolated jugular vein, but also induce a concentration-dependent constriction on their own.

The rabbit isolated jugular vein is a suitable and sensitive bioassay tissue for studying the effects of B₂-receptor stimulation and/or blockade, even though it may contain some residual ACE activity (Regoli & Barabé, 1980). Judged by the complete lack of effect of des-Arg⁹-bradykinin, the rabbit jugular vein is also devoid of any significant B₁-receptor activity (Regoli & Barabé, 1980). According to our data, however, there is very little, if any, ACE activity present in this vascular preparation, since removal of the endothelium, the primary source of ACE (Erdős, 1990), had no effect on the potency of bradykinin. Moreover, at a concentration which should block any remaining ACE activity in the smooth muscle, RA-octyl decreased rather than enhanced the constrictor response to bradykinin.

The amplification by ramiprilat of the constrictor response to bradykinin in this bioassay system, therefore, suggests that the ACE inhibitor potentiates the action of bradykinin independently of an inhibition of ACE. Since ramiprilat did not affect the constrictor response to another receptor-dependent agonist, U46619, the potentiation of the action of bradykinin is likely to be mediated by an effect of the ACE inhibitor at the level of the agonist-B₂-receptor complex. This notion is substantiated by the finding that ramiprilat, but not RA-octyl, also elicited a distinct Hoe 140-sensitive constrictor response in the absence of exogenous bradykinin. This rise in vascular tone was concentration-dependent and even increased at concentrations of the ACE inhibitor which should have completely suppressed any residual ACE activity in the endothelium-free bioassay tissue. Considering that an involvement of endogenous kinins is unlikely, these results point to a direct interaction of the ACE inhibitor with the B₂-receptor.

A direct effect of ACE inhibitors on the B₂-receptor also seems to be responsible for the instantaneous increase in the intracellular concentration of free Ca²⁺ elicited by moexiprilat or ramiprilat in cultured endothelial cells in the absence of exogenous bradykinin. This effect is completely antagonized by Hoe 140 (Hecker *et al.*, 1992). Moreover, ACE inhibitors can also relax bovine, canine, human and porcine coronary arteries in an endothelium-dependent manner when administered in the presence of subthreshold concentrations of bradykinin, and potentiate the dilator response to bradykinin (Mombouli *et al.*, 1992; Auch-Schwelk *et al.*, 1993; Hecker *et al.*, 1993a). Since the ACE activity present in these coronary artery preparations is apparently too low to influence the dilator response to bradykinin (Auch-Schwelk *et al.*, 1993), these findings support the concept that ACE inhibitors modulate the action of bradykinin at the receptor level or interfere with the subsequent signal transduction pathway.

It is important to note that the present investigation of the

ACE inhibitor action in the rabbit isolated perfused heart differs from the aforementioned studies, because the restoration of the sensitivity to bradykinin and not the potentiation of its vascular action(s) was examined. Even though ACE inhibitors can promote an accumulation of endothelium-derived kinins in superfused bovine and porcine coronary arteries (Hecker *et al.*, 1993a), they do not have such an effect in the coronary vascular bed of the rabbit (Busse *et al.*, 1993; Hecker *et al.*, 1993b). Moreover, none of the ACE inhibitors tested in the present study had any effect on coronary perfusion pressure or PGI₂ release on their own. Thus, endogenously produced kinins do not seem to contribute to the restoration by these agents of the sensitivity to bradykinin in the rabbit coronary microcirculation. In addition, an accumulation of the exogenously administered kinin in or at the vascular wall is also unlikely to play a role, since the dilator response to D-Arg[Hyp³]-bradykinin was affected by ramiprilat virtually in the same way as that to bradykinin, although the B₂-receptor agonist was clearly more resistant to inactivation by ACE than bradykinin. The rapid and identical fading of the dilator response and PGI₂ release elicited by both kinins, on the other hand, suggests that the duration of their action is governed by the desensitization of the B₂-receptor. On the contrary, if the coronary ACE activity would determine the duration of these responses, one would expect D-Arg[Hyp³]-bradykinin to cause a more prolonged effect than bradykinin.

The finding that ramiprilat was somewhat less effective in restoring the sensitivity to D-Arg[Hyp³]-bradykinin as compared to bradykinin may be explained by the fact that, due to its greater stability, the B₂-receptor agonist may have caused a greater degree of regulated receptor loss (down-regulation) than bradykinin (Bathon & Proud, 1991; Munoz & Leeb-Lundberg, 1992). As a consequence, the total number of available B₂-receptors would decrease, hence attenuating the subsequent response to the ACE inhibitor. Enhanced desensitization would also explain the more pronounced difference in PGI₂ release which requires a more pronounced increase in intracellular Ca²⁺, i.e. a greater degree of cell activation than, e.g. NO release, to be initiated (Newby & Henderson, 1990).

Another argument against an accumulation of bradykinin in the presence of ramiprilat is the virtually identical time course after which the dilator response to bradykinin or the ACE inhibitor occurred. This brief lag phase contrasts with the significantly longer period of time that was necessary to attain a substantial blockade of the coronary ACE activity. Moreover, the dilator response to ramiprilat was comparable to that elicited by a 100 fold increase in the concentration of bradykinin in the perfusate. However, it is difficult to envision how bradykinin can accumulate in the vicinity of the B₂-receptor to such an extent and in such a short period of time.

It may be argued that the lack of effect of RA-octyl in the rabbit heart indicates that effective ACE blockade is a prerequisite for the dilator response to ramiprilat or moexiprilat. However, two ACE substrates, hippuryl-L-histidyl-L-leucine and angiotensin I, in concentrations which should competitively block the metabolism of bradykinin by ACE or displace the kinin from the active site of the enzyme, were unable to restore the endothelial response to bradykinin in the coronary vascular bed (dilatation and PGI₂ release). In contrast, ramiprilat and quinaprilat were still active despite the presence of these peptides. Thus, it is more likely that, similar to its lack of effect in the jugular vein, RA-octyl is unable to interact with the B₂-receptor in the same way as the other three ACE inhibitors.

At present, we can only speculate about the possible mechanism involved in the restoration of the sensitivity to bradykinin by ACE inhibitors. Although a decrease in receptor affinity cannot be ruled out (Etscheid *et al.*, 1989), the majority of studies with cultured endothelial cells, fibroblasts and smooth muscle cells suggest that the desensitization of

the B₂-receptor is mainly due to an internalization of the ligand-receptor complex following bradykinin occupancy (Roscher *et al.*, 1984; Munoz & Leeb-Lundberg, 1992; Weintraub *et al.*, 1992). These sequestered receptors, however, can be recycled to the cell surface and due to the limited number of B₂-receptors on the surface, this process must be continuous to ensure an adequate responsiveness of the cells to bradykinin (Munoz & Leeb-Lundberg, 1992). It may be that ACE inhibitors such as ramiprilat, moexiprilat or quinaprilat accelerate the recycling of these receptors, e.g. by a flip-flop type of mechanism.

In summary, the pharmacological evidence obtained in this study supports the conclusion that ACE inhibitors restore the sensitivity of the B₂-receptor system to bradykinin in the rabbit isolated perfused heart independently of an inhibition of ACE. Presumably via a similar effect on the desensitiza-

tion mechanism of the B₂-receptor, ramiprilat also potentiates the constrictor response to bradykinin in the rabbit jugular vein. Apart from blocking vascular angiotensin II formation and protecting endogenously produced kinins from inactivation by ACE, this additional mode of action of ACE inhibitors may play an important role in the vascular effects of this class of compounds.

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