Rapid Communication

Ramiprilat Enhances Endothelial Autacoid Formation by Inhibiting Breakdown of Endothelium-Derived Bradykinin

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We studied whether inhibition of angiotensin converting enzyme stimulates the formation of nitric oxide and prostacyclin in cultured human and bovine endothelial cells by an enhanced accumulation of endothelium-derived bradykinin. Nitric oxide formation was assessed in terms of intracellular cyclic GMP accumulation, prostacyclin release by a specific radioimmunoassay. Inhibition of angiotensin converting enzyme by ramiprilat dose- and time-dependently increased the formation of nitric oxide and prostacyclin. These increases, peaking within 10 minutes, were maintained for at least 60 minutes. The ramiprilat-induced cyclic GMP increase was completely abolished by the stereospecific inhibitor of nitric oxide synthase, N\(^\text{G}\)-nitro-L-arginine. The B\(_2\)-kinin receptor antagonist, Hoe 140 (0.1 \(\mu\)M), markedly attenuated the cyclic GMP accumulation and abolished the increase in prostacyclin release. The supernatant of endothelial cells, incubated with ramiprilat (0.3 \(\mu\)M) for 15 minutes, elicited a significant nitric oxide release (as assessed by a guanylyl cyclase assay) in untreated endothelial cells used as detector tissue. Preincubation of the detector cells with Hoe 140 completely abolished this nitric oxide release. These data indicate that cultured endothelial cells from different species are capable of producing and releasing bradykinin into the extracellular space in amounts that lead to a sustained stimulation of nitric oxide and prostacyclin formation. Thus, the protective effect of angiotensin converting enzyme inhibitors observed on endothelial vasomotor function in hypertension may be explained by the local accumulation of endothelium-derived bradykinin that acts in an autocrine and paracrine manner as potent stimulus for endothelial autacoid formation. (Hypertension 1991;18:558-563)

Angiotensin converting enzyme (ACE) inhibitors are widely used in the treatment of hypertension because of their effectiveness in reducing blood pressure.1 Although the primary action of these agents is the inhibition of systemic and local formation of angiotensin II, a number of experimental and clinical data suggest that other dilator mechanisms may be involved in the hypotensive effect of ACE inhibitors.2,3 Since ACE is identical to the kininase II of the kallikrein-kinin system that inactivates bradykinin by liberating the C-terminal dipeptide phenylalanyl-arginine,4 it has been suspected that a significant part of the blood pressure-lowering effect of ACE inhibitors in vivo is mediated by the accumulation of kinins.2,3 Kinins are vasodilators through the release of different autacoids, mainly generated by the endothelium. Activation of B\(_2\)-kinin receptors on endothelial cells leads by stimulating phospholipases A\(_2\) and C to the formation of the potent dilator nitric oxide (NO), of prostacyclin, and of platelet activating factor (PAF).5-7 However, the few studies on plasma kinin levels in hypertensive patients after ACE inhibitor treatment present conflicting data on the contribution of kinin-mediated vasodilations in the hypotensive effects of ACE inhibitors.8,9 Thus, it has been proposed that locally generated kinins in the vascular wall are responsible for dilator action of ACE inhibitors rather than circulating kinins.10 The potential sources of intravascular kinins have not yet been identified.

In recent years it has been shown that endothelial cells are able to synthesize and release potent vasoconstrictor peptides such as angiotensins11 and endothelin.12 Furthermore, there is some evidence that the potent stimulators of endothelial NO formation

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acetylcholine, ATP, and substance P, are released from endothelial cells themselves,\textsuperscript{13,14} in this way establishing an effective paracrine dilator system. The present study was designed to examine whether cultured endothelial cells from different species synthesize and release bradykinin and related peptides that may accumulate in the presence of ACE inhibitors with a subsequent stimulation of NO and prostacyclin formation.

**Methods**

**Endothelial Cell Culture**

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by a modification of the method originally described by Jaffe et al.\textsuperscript{15} Under sterile conditions, the cords were cleaned and the umbilical veins were cannulated and rinsed free of blood with 50 ml physiological salt solution (PSS). Enzymatic digestion was performed in the veins filled with PSS containing dispase II (2.4 IU/ml, Boehringer, Mannheim, FRG) for 30 minutes at 37°C. Thereafter, the veins were gently squeezed out and rinsed with 30 ml culture medium (M-199) containing 25% heat-inactivated fetal calf serum. The cells were washed by centrifugation (120g, 5 minutes) and then were resuspended in culture medium. Endothelial cells from bovine (BAEC) and porcine aorta (PAEC) were isolated by digestion with dispase and were cultured as previously described.\textsuperscript{16} All cell types were seeded on either 6- or 24-well plates (Falcon, Heidelberg, FRG, and Nunc Intermed, Wiesbaden, FRG) (for HUVEC and PAEC precoated with fibronectin, 25 μg/ml) and grown to confluence. The medium used for BAEC and PAEC was Dulbecco's modified Eagle's/Ham's F-12 medium (1:1) containing 20% fetal calf serum. All culture media were supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml), L-glutamine (1 mmol/l), glutathion, and L(+)-ascorbic acid (each 5 mg/ml; Biotect protection medium).

**Measurement of Cyclic GMP and 6-ketoprostaglandin F\textsubscript{1α}**

Primary cultures of endothelial cells were used. After removal of the culture medium by aspiration, the monolayer was washed twice with 2 ml HEPES-Tyrode's solution (37°C). Thereafter the cells were preincubated for 15 minutes at 37°C with 3-isobutyl-1-methyl-xanthine (IBMX), (10⁻⁴ mol/l). After this time, drugs or solvents were added to the cells at the concentrations and times indicated in the results. At the appropriate time, the incubation medium was quickly removed from the monolayers and frozen at −20°C until it was assayed for its content of 6-ketoprostaglandin F\textsubscript{1α} (6-keto PGF\textsubscript{1α}), the stable hydrolysis product of prostaglandin I\textsubscript{2} (PGI\textsubscript{2}) by a specific radioimmunoassay as described previously.\textsuperscript{7} 6-keto PGF\textsubscript{1α} content was expressed as nanograms 6-keto PGF\textsubscript{1α} per milligram protein. The cells were then immediately extracted with 0.6 ml ice-cold trichloroacetic acid (TCA), (6%) and scraped off with a rubber scraper. The cell suspension was then sonicated for 10 seconds before being centrifuged for 5 minutes at 4,000g. Supernatants were extracted with four volumes of water-saturated diethylether, and the samples were kept frozen (−20°C) until analysis. The protein contents of the samples were measured according to Lowry et al.\textsuperscript{17} and cyclic GMP was determined in the acetylated samples using a commercially available radioimmunoassay (New England Nuclear, Dreieich, FRG). Cyclic GMP (cGMP) content was expressed as picomoles cGMP per milligram protein.

**Measurement of Nitric Oxide Release**

Release of NO from endothelial cells was assayed on the basis of the stimulatory effect of NO on the activity of soluble guanylyl cyclase (GC). Soluble GC was purified from bovine lung. The activity of the enzyme was determined in test tubes in terms of the formation of cyclic [³²P]GMP from α-[³²P]GTP. Reactions were carried out in a reaction mixture containing 30 mM triethanolamine-HCl (pH 7.4), 1 mM reduced glutathione, 4 mM MgCl\textsubscript{2}, 0.1 mM cGMP, and 0.1 mg/ml bovine γ-globulin (total volume of 0.18 ml) at 37°C in the presence of α-[³²P]GTP (0.03 mM; 0.2 μCi) and soluble GC (4 μg). For the determination of NO in the supernatants of endothelial monolayers (24-well plates) 10-μl samples were quickly transferred to the reaction mixture. Enzymatic formation of cGMP was allowed to proceed for 60 seconds and then stopped by the addition of 450 μl zinc acetate (120 mM) and 500 μl sodium carbonate (120 mM). Details of the method have been described elsewhere.\textsuperscript{7}

**Materials**

3-Isobutyl-1-methyl-xanthine (IBMX), N⁵-nitro-L-arginine, and superoxide dismutase (bovine erythrocytes, specific activity 3,300 IU/mg) were purchased from Serva, Heidelberg, FRG. The B2-kinin receptor antagonist Hoe 140 (D-Arg, [Hyp³, Thr², D-Tic⁵, Oic⁶]-bradykinin)\textsuperscript{18} and ramiprilat were obtained from the Department of Pharma Synthesis, Hoechst AG, Frankfurt a.M., FRG. All other substances were purchased from Sigma, Deisenhofen, FRG.

**Statistical Analysis**

Unless indicated otherwise the data are reported as mean±SEM. Statistical evaluation was performed with Student's t test for paired or unpaired data and with Dunnett's test when more than two groups were compared. For data not normally distributed, Wilcoxon signed rank test for paired data was applied. A value of p<0.05 was considered statistically significant.

**Results**

**Effects of Bradykinin and Ramiprilat on Cyclic GMP Formation in Endothelial Cells**

Bradykinin induced time- and concentration-dependent increases in the cGMP content of all endothelial cell types investigated. The maximum in-
creases of cGMP were reached by 1 minute and returned after a short plateau to baseline by 15 minutes (Figure 1A). The concentration–response relation of bradykinin on cGMP content in bovine endothelial cells is shown in Figure 1B. Similar results were obtained in porcine and human endothelial cells with threshold concentrations of about 0.3 nM bradykinin and maximal increases (about 18-fold the basal level in PAEC and 20-fold in HUVEC) between 10 and 30 nM bradykinin. Preincubation of endothelial cells (5 minutes) with 0.1 μM of the B2 receptor antagonist Hoe 140, a concentration that totally suppressed bradykinin-induced responses in endothelial cells, did not significantly affect the basal cGMP content, but completely abolished the bradykinin-induced increases (Figure 1B). A complete inhibition of bradykinin-induced cGMP formation was also observed in monolayers that were preincubated for 30 minutes with the stereospecific inhibitor of NO synthase, Nω-nitro-L-arginine (30 μM), while basal cGMP levels were not significantly affected (Figure 1B). Time- and concentration-dependent increases in cGMP production were also induced after incubation of endothelial monolayers with the ACE inhibitor ramiprilat (Figures 2A and 2B). In contrast to the transient cGMP kinetic after stimulation with bradykinin, the ramiprilat-induced increases in cGMP content developed slowly, reached a plateau level after 10 minutes, and remained stable for at least 30 minutes (Figure 2A). Maximal increases were obtained between 0.1 and 1 μM ramiprilat at threshold concentrations of about 1 nM (Figure 2B). Preincubation of the cells with either Hoe 140 (0.1 μM) or Nω-nitro-L-arginine (30 μM) abolished the increases in cGMP content (Figure 2B). Ramiprilat (0.01 to 1 μM) caused also a more than sixfold increase in cGMP accumulation in HUVEC, that was significantly suppressed with either Hoe 140 or Nω-nitro-L-arginine (Figure 3). It should be noted that the basal content of cGMP in HUVEC, obviously due to a high basal NO formation, was remarkably higher than in BAEC. Similar findings to those with ramiprilat were obtained with captopril (1 μM) in HUVEC and in PAEC, although the increases in cGMP content were smaller (2.3±0.4-fold of the basal level in PAEC and 3.1±0.9-fold in HUVEC) than those observed after incubation with ramiprilat (data not shown).

**Release of Nitric Oxide by Bradykinin and Supernatants From Ramiprilat-Incubated Cells**

Release of NO by bradykinin and supernatants from ramiprilat-incubated cells was assessed in HUVEC in
terms of increases in the activity of soluble GC (Figure 4). Bradykinin at a concentration of 3 nM elicited a transient increase in GC activity, which was completely abolished by preincubation of the cells with either Hoe 140 (0.1 μM) (Figure 4) or Nω-nitro-L-arginine (30 μM) (data not shown). Aliquots of the supernatants taken from endothelial monolayers after 15 minutes incubation with ramiprilat (0.1 μM) induced a small but significant NO release as detected by the increase in GC activity, whereas the supernatant from untreated cells and from cells incubated with ramiprilat only for 1 minute had no stimulatory effect on NO release. Preincubation of the detector cells with Hoe 140 (0.1 μM) abolished the NO release, elicited by the supernatants from ramiprilat-incubated cells (Figure 4). Hoe 140 (0.1 μM) had no effect on NO release induced by other receptor-dependent and -independent agonists. The increase in GC activity elicited by histamine (10 μM) amounted to 9.3±0.8 nmol×mg protein⁻¹×min⁻¹ in the absence and to 9.7±0.9 nmol×mg protein⁻¹×min⁻¹ in the presence of Hoe 140 (n=5). Likewise, there was no change in NO release induced by the calcium ionophore A23187 (0.1 μM) (5.2±0.4 in the absence and 5.4±0.5 nmol×mg protein⁻¹×min⁻¹ in the presence of Hoe 140, n=4).

Effects of Bradykinin and Ramiprilat on Prostaglandin I₂ Biosynthesis in Endothelial Cells

Bradykinin stimulated the release of PGI₂ in the three endothelial cell types studied in a time- and concentration-dependent manner. Near maximal increases were reached after 60 seconds of stimulation and thereafter declined despite continuous stimulation. Thus, the time course of PGI₂ release was quite parallel to that obtained for cGMP accumulation.

Threshold concentrations were in the range of about 0.1 nM and maximal increases (about 12- to 15-fold the basal level) were observed between 0.1 and 1 μM bradykinin. Similar findings have already been reported in a number of previous studies. Incubation of human and bovine endothelial cells with ramiprilat (15 minutes) elicited in the range between 10 nM and 1 μM an increase in PGI₂ release that was significantly inhibited after preincubation with Hoe 140 (0.1 μM) (Figure 5A). Compared with the rapid increase in PGI₂ release within 60 seconds after direct bradykinin stimulation, there was a remarkable delay in the ramiprilat-mediated PGI₂ release; Statistically significant increases were only observed after 10 minutes incubation (Figure 5B).

Discussion

It has recently been documented that ACE inhibitors prevent and reverse functional or morphological alterations of the endothelium in spontaneously hypertensive rats and in hypercholesterolemic rabbits. The mechanism by which ACE inhibitors enhance agonist-induced endothelium-dependent relaxation in normotensive, hypertensive, and hypercholesterolemic animals is unknown, but seems to be distinct from the blood pressure-lowering action of ACE inhibitors. It has been speculated that ACE inhibitors promote vasodilation by increasing the level of bradykinin generated in subthreshold concentration in the vascular wall by a local kininogen-kinin system.

The present study provides circumstantial evidence of the formation of bradykinin by cultured endothelial cells of different species. It was shown that ACE inhibitors stimulate the formation of NO and PGI₂ in endothelial cells, most likely by inhibiting the break-
down of endothelium-derived kinins. This was supported by using the selective B₂-receptor antagonist Hoe 140, that abolished the enhanced NO and PGI₂ formation observed after ACE inhibition. In the meantime, determinations of bradykinin were performed by a specific radioimmunoassay on HUVEC in the absence and presence of ramiprilat. These preliminary experiments revealed at least a 10-fold increase in bradykinin concentration in the supernatants of the cells within 15 minutes after ACE inhibition; the amount of bradykinin rose from a level below detection limit of the assay to values between 0.8 and 1.8 ng bradykinin/ml supernatant (C. Bossaller and R. Busse, unpublished data).

The present study does not establish the origin and the identity of the bradykinin precursors. The presence of high molecular weight kininogen (H-kininogen) has recently been demonstrated in cultured human endothelial cells. Although a small de novo biosynthesis of kininogens (less than 1%) could not be unequivocally excluded in this study, it was shown that endothelial cells bind and subsequently internalize H-kininogen in a specific and time-dependent way from serum-supplemented culture medium. Since in our study formation of cGMP by ramiprilat still occurs in serum-free medium after removal of cell-bound kininogen by dextran sulfate, it seems likely that internalized kininogen rather than kininogens bound on specific binding sites of the cell surface is cleaved by kallikrein.

The enhanced production of cGMP in endothelial cells in response to NO-releasing agonists such as bradykinin, ATP, ADP, and calcium ionophore has been documented in several studies. The complete inhibition of this increase by the stereospecific inhibitor of NO synthesis, N²-nitro-L-arginine, indicates that an increased formation of NO, which in turn stimulates endothelial soluble GC is responsible for the observed cGMP increases. In contrast to the fast transient increase in cGMP content elicited by exogenously added bradykinin, the increase in response to ramiprilat developed slowly, peaking only after 10–15 minutes and was maintained for several hours (data not shown). The rapid development of homologous desensitization of B₂-receptors in endothelial cells after exposure to high concentration of bradykinin has been described. The question why the endogenously formed kinins do not lead to receptor desensitization cannot be satisfactorily answered by the present study. It is likely, however, that desensitization is a process occurring only at higher concentrations of bradykinin (more than 3 nM), which were not reached after incubation of the cells with ramiprilat.

The accumulation of kinins in the culture medium of endothelial cells grown in dishes under no-flow conditions is not comparable with the physiological situation in vivo, where a considerable dilution of the kinins released from the endothelium may occur by the streaming blood. However, it has to be considered that the blood flow itself, by generating shear stress (viscous drag) on the surface of the endothelium, may enhance the release of kinins. Evidence has been presented recently that ATP, acetylcholine, and substance P are released from cultured human endothelial cells in vitro by increased flow. Furthermore, substance P has been shown to be released by high flow rates from the rat hind limb vasculature in vivo, but only when endothelial cells were present. Thus, it is conceivable that flow facilitates also the release of bradykinin from the endothelium. In agreement with this hypothesis, an endothelium-dependent relaxation by captoril has been documented in perfused canine carotid arteries.

The sustained elevation of endothelial NO production after ACE inhibition as assessed by intracellular cGMP levels might have important physiological implications. In addition to its vasodilator and antithrombotic effects, NO may function as an inhibitor of smooth muscle cell mitogenesis and proliferation. Inhibition of DNA synthesis and cell growth by NO-generating vasodilators and 8-bromo-cGMP in cultured rat vascular smooth muscle cells has been reported. The concept that the endothelium exerts a crucial role in maintaining the medial vascular smooth muscle cells in a quiescent state is supported by several experimental studies.

Intimal injury or the absence of endothelium is an integral part in the development of atherosclerosis, which is characterized by excessive smooth muscle proliferation in the intima and the media. Therefore, improvement and restoration of endothelial NO synthesis by ACE inhibitors may prevent the development of proliferative atherosclerotic lesions and may have an important beneficial effect in the treatment of hypertension.

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References


21. Key Words • endothelial cells • angiotensin converting enzyme inhibitors • nitric oxide • bradykinin-B1-receptor antagonist
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