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Angiotensin I-converting enzyme inhibitor peptides derived from the endostatin-containing NC1 fragment of human collagen XVIII

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Abstract

Extracellular matrix and soluble plasma proteins generate peptides that regulate biological activities such as cell growth, differentiation and migration. Bradykinin, a peptide released from kininogen by kallikreins, stimulates vasodilatation and endothelial cell proliferation. Various classes of substances can potentiate these biological actions of bradykinin. Among them, the best studied are bradykinin potentiating peptides (BPPs) derived from snake venom, which can also strongly inhibit angiotensin I-converting enzyme (ACE) activity. We identified and synthesized sequences resembling BPPs in the vicinity of potential proteolytic cleavage sites in the collagen XVIII molecule, close to endostatin. These peptides were screened as inhibitors of human recombinant wild-type ACE containing two intact functional domains; two full-length ACE mutants containing only a functional C- or N-domain catalytic site; and human testicular ACE, a natural form of the enzyme that only contains the C-domain. The BPP-like peptides inhibited ACE in the micromolar range and interacted preferentially with the C-domain. The proteolytic activity involved in the release of BPP-like peptides was studied in human serum and human umbilical-vein endothelial cells. The presence of enzymes able to release these peptides in blood led us to speculate on a physiological mechanism for the control of ACE activities.

Keywords: ACE inhibitors; angiogenesis; bradykinin potentiating peptides; endostatin.

Introduction

Endostatin, an inhibitor of angiogenesis and tumor growth, was first shown to be produced by a cell line derived from a non-metastatic murine hemangioendothelioma (EOMA) (O'Reilly et al., 1997). Endostatin is generated by proteolysis of collagen XVIII, which is the core protein of a heparan sulfate proteoglycan found in vascular and epithelial basement membranes. Collagen XVIII is part of a distinct subgroup within the collagen superfamily characterized by multiple interruptions in the central triple-helical domain and a unique non-triple-helical domain at the C-terminus, non-collagenous-1 (NC1) domain. This NC1 domain of collagen XVIII consists of a 5-kDa N-terminal association domain that is implicated in self-assembly of the homotrimeric collagen XVIII; a central protease-sensitive hinge domain; and a compact 22-kDa C-terminal endostatin domain (Sasaki et al., 1998). The central protease-sensitive hinge domain of the NC1 domain of collagen XVIII is cleaved into smaller fragments ranging from 22 to 32 kDa (Sasaki et al., 1998; Marneros and Olsen, 2001; Iozzo, 2005) by different proteases such as cathepsin L (Felbor et al., 2000), MMPs (Ferreras et al., 2000; Lin et al., 2001; Heljasvaara et al., 2005) or pancreatic elastase (Wen et al., 1999), generating distinct endostatin-containing fragments with varying efficiency. Lysosomal proteases seem to be involved not only in the generation of endostatin, but also in its degradation (Ferreras et al., 2000).

The NC1 domain sequences of human and mouse collagen XVIII are not conserved and the size and sequences are quite different in both organisms. Figure 1 shows the sequence of the human collagen XVIII hinge domain and the cleavage sites for different matrix metalloproteases (MPPs) and cathepsin L as reported previously (Felbor et al., 2000; Heljasvaara et al., 2005). The N-terminal pro-segment of human endostatin contains the sequence LRPARTPSPP (underlined in Figure 1) that is similar to the sequences of the bradykinin potentiating peptides (BPPs) isolated from *Botrops jararaca* snake venom (Ferreira, 1965; Ianzer et al., 2004). These peptides are efficient natural angiotensin I-converting enzyme (ACE) inhibitors and their structure-activity studies were the basis for the development of anti-hypertensive drugs such as captopril and various analogs (Cushman and Ondetti, 1980). However, in the last three decades, different explanations for the BPP potentiating mechanism demonstrated that the enhancement of BK activity cannot be entirely due to ACE inhibition (revised by Erdös and Marcic, 2001). Recently, Mueller et al. (2005), studying synthetic analogs of the bradykinin potentiating peptide BPP9a, provided evidence that ACE inhibition and the bradykinin potentiating activity occur by different mechanisms.

ACE is anchored to the plasma membrane of endothelial cells and is involved in the modulation of angiogenesis (Volpert et al., 1996; Fabre et al., 1999; Silvestre et

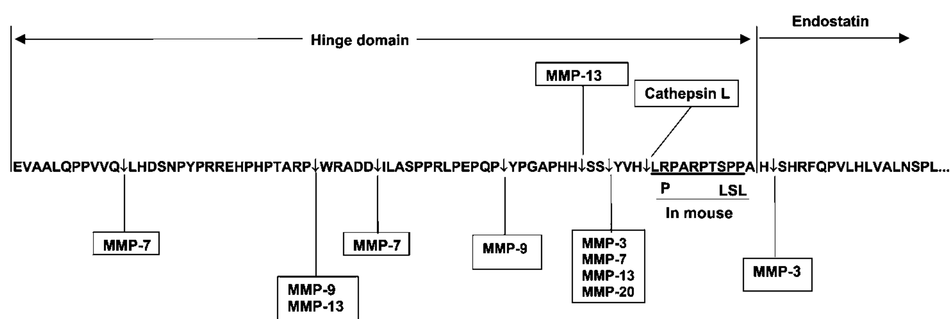

Figure 1 Amino acid sequence of the hinge domain and the N-terminal sequence of endostatin from the NC1 domain of human collagen XVIII.
The cleavage sites for MMPs (Heljasvaara et al., 2005) and cathepsin L (Felbor et al., 2000) are indicated. The beginning of the endostatin sequence was arbitrarily assigned as the same site described for mouse endostatin (Sasaki et al., 1998). The underlined sequence LRPARPTSTSPP corresponds to the BPP-like sequence. Modifications in mouse collagen XVIII in this sequence are indicated below the human sequence.

al., 2001; Yoshiji et al., 2001). In addition, ACE activity was reported to increase angiogenesis and cellular proliferation (Lindberg et al., 2004) through angiotensin-II-induced VEGF expression (Fujita et al., 2002) and AT1 receptors (Lorell, 1999), and both effects were decreased by treatment with ACE inhibitors (Small et al., 1997; Hii et al., 1998). The opposite effect of ACE inhibition has been reported, highlighted in the context of angiogenesis (Small et al., 1997; Hii et al., 1998; Silvestre et al., 2001), through increasing levels of angiotensin II, bradykinin and VEGF signaling (Williams et al., 1995; Pupilli et al., 1999), all of which are involved in endothelial proliferation.

The hydrolysis pattern of the protease-sensitive hinge domain of collagen XVIII (Figure 1) suggests the cleavage and release of peptides containing BPP-like sequences, such as LRPARPTSPPA, as well as longer or shorter regions of this peptide. Owing to the role of ACE in the control of angiogenesis via angiotensin II and bradykinin, we investigated the inhibition of four forms of ACE by synthetic peptides with sequences derived from human collagen XVIII that contain the BPP-like peptides. We report on the inhibition of three human recombinant ACE proteins, namely wild-type protein containing two intact functional domains and two full-length ACE mutants containing only one functional catalytic site (referred to as the C- or N-domain) by these peptides. They were also tested as inhibitors of a purified human testicular ACE, a natural form of the enzyme that only contains the C-domain. We also examined the hydrolysis of synthetic peptides of human collagen XVIII by recombinant human cathepsins B, K and L. These peptides were also assayed with human serum and with an established lineage of human umbilical-vein endothelial cells.

Results

Peptides derived from the C-terminal NC1 domain of human collagen XVIII as ACE inhibitors

The FRET substrate Abz-YRK(Dnp)P-OH was used to evaluate ACE activity (Araujo et al., 2000) and to compare the inhibition profiles of the peptides derived from the C-terminal NC1 domain of human collagen XVIII. The N- and C-termini of these peptides (Table 1) were unprotected and were resistant to hydrolysis by all forms of ACE studied. Moreover, they displayed competitive inhibition, with K_i values in the micromolar range.

Table 1 Inhibition constants (K_i) for angiotensin I-converting enzyme activity with synthetic peptides derived from human collagen XVIII hinge domain with sequences similar to bradykinin potentiating peptides (BPPs) from snake venom.

No.	Sequence	K_i (μM)			
		Testis ACE	Wildtype	C-domain	N-domain
I	SPP	5.0	6.9	19	13
II	PARPTSPP	1.5	5.3	2.1	49
III	RPARPTSPP	1.0	6.8	0.9	23
IV	LRPARPTSPP	0.8	2.6	3.2	17
V	YVHLRPARPTSPP	0.8	5.8	2.0	10
VI	SSYVHLRPARPTSPP	2.4	7.7	3.1	200
VII	LRPARPTSPPA	0.3	17	4.6	12
VIII	(pE)KWAP (BPP5a)	0.8	0.4	0.7	0.6
IX	(pE)WPRPQIPP (BPP9a)	0.01	0.03	0.003	0.1

Kinetic conditions: the enzymes were pre-incubated with increasing concentrations of peptides I–IX for 3 min at 37°C in 0.1 M Tris-HCl buffer, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$. The reaction was initiated by the addition of Abz-YRK(Dnp)P-OH (10 μM) at 37°C in a final volume of 2 ml. Measurements were made in duplicate and differences were less than 5%. pE, pyroglutamic acid.

Testicular ACE, which contains only the C-domain, was more efficiently inhibited by peptides I–VII (Table 1) than the other forms of ACE assayed. The specificity of these peptides for the ACE C-domain was also observed with the mutant enzyme with only the C-domain active. This C-domain selectivity was also observed for BPP9a (peptide IX, Table 1), in accordance with earlier reports regarding the domain selectivity of BPPs (Cotton et al., 2002). Furthermore, this peptide inhibited all forms of ACE in the nanomolar range, which indicates that the amino acids that precede the pair of Pro residues play a significant role in the interaction of BPPs with the non-prime sites of ACE.

Hydrolysis of the sequence connecting the hinge domain and endostatin in human collagen XVIII by human cathepsins B, L and K, human serum and endothelial cell proteases

Cathepsin B, L and K hydrolysis of the peptide Abz-RPTSPPAHSHRDFQPVLHLVALNSPL-NH_2 containing the hinge sequence between the BPP-like peptide (RPTSPP) and the N-terminal region of endostatin, resulted in cleavage of three peptide bonds as determined by HPLC and mass spectrometry (Figure 2). The three cathepsins cleaved at the H-L bond, cathepsins B and L also cleaved at the L-H bond, and only cathepsin L cleaved at the A-L bond. This peptide was more efficiently hydrolyzed by cathepsin L, followed by cathepsin B; however, none of the cathepsins was able to release the RPTSPP fragment that corresponds to the BPP-like peptide.

We also investigated the presence of proteolytic activity in human serum (Figure 3A) and HUVEC cell culture (Figure 3B) that could generate a peptidic ACE inhibitor from Abz-RPTSPPAHSHRDFQPVLHLVALNSPL-NH_2. Incubation with serum or with HUVECs showed that the fragment Abz-RPTSPPA, corresponding to hydrolysis at the A-H bond, was detected after 8 h and remained intact even after 48 h, as demonstrated by HPLC analysis and mass spectrometry. Pre-incubation of human serum with pepstatin A, E-64 and *ortho*-phenanthroline (aspartyl, cysteine and metallopeptidase inhibitors, respectively) did not inhibit peptide cleavage. Only PMSF, a serine peptidase inhibitor, was effective in blocking cleavage.

Discussion

Endothelial ACE exerts its physiological effects through two distinct routes: the renin-angiotensin system by angiotensin II production, a potent vasopressor peptide also involved in cellular proliferation through the AT1 receptor; and the kininogen-kinin system by bradykinin degradation, a vasodilator peptide involved in angiogenesis through the inflammation-inducible B_1 and constitutively expressed B_2 receptors. B_1 signaling plays an important role in reparative angiogenesis in post-ischemic limbs (Emanueli et al., 2002) and rabbit cornea (Parenti et al., 2001), whereas its antagonism results in apoptosis. Using a model of surgically induced hind-limb ischemia, Silvestre et al. (2001) showed that the proangiogenic effect of ACE inhibitors is also mediated by the B_2 receptors. The B_2 signaling pathway is also responsible for nitric oxide and prostaglandin release (Regoli and Barabe, 1980), which contribute to endothelial cell proliferation, a system that enhances VEGF signaling.

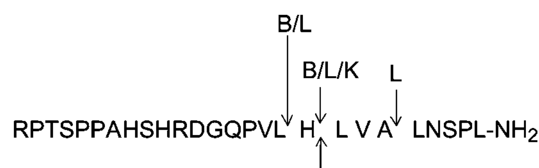

Figure 2 Cleavage sites in the peptide Abz-RPTSPPAHSHRDFQPVLHLVALNSPL-NH_2 of human cathepsins B, K and L. The cleavage sites were determined as described in the materials and methods section.

BPPs are natural ACE inhibitors first reported in *Bothrops jararaca* venom (Ferreira, 1965). Later, other

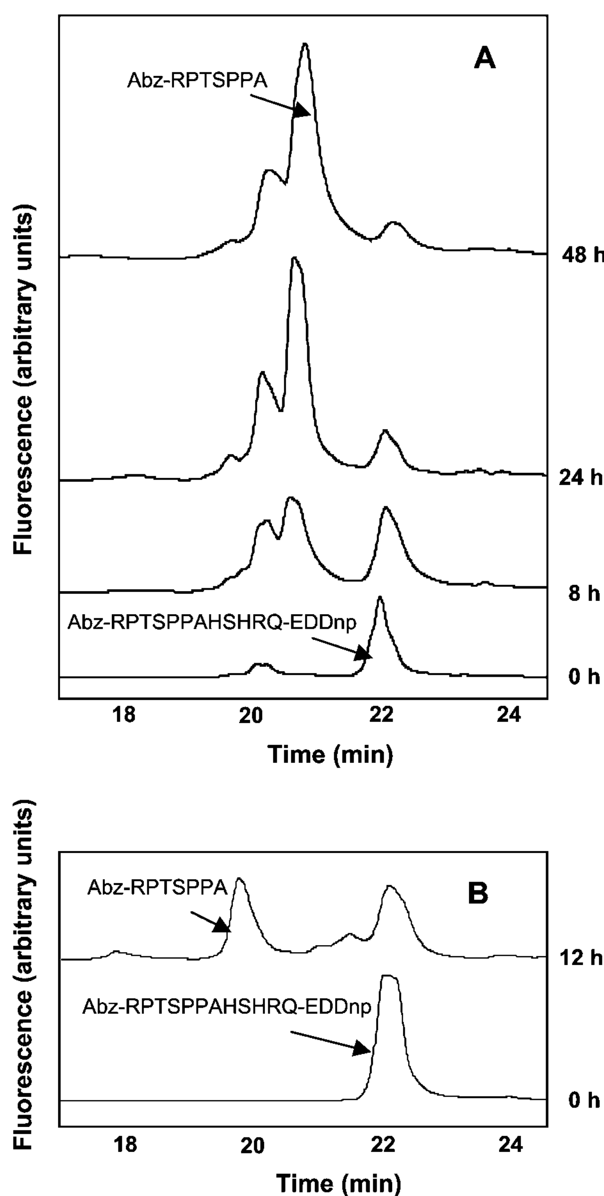

Figure 3 HPLC profile of the peptide Abz-RPTSPPAHSHRDFQPVLHLVALNSPL-NH_2 before and after incubation for 8, 24 and 48 h with human serum (A) and HUVEC supernatant (B). Incubation conditions are described in the materials and methods section. Fractions were monitored by fluorescence at λ_{ex}=320 nm and λ_{em}=420 nm.

BPPs were isolated from the venom of other snakes (Kato and Suzuki, 1971; Cintra et al., 1990; Ferreira et al., 1992), spiders (Akchumov et al., 1992) and scorpions (Ferreira et al., 1993). Peptides with bradykinin potentiating activity can also be formed by hydrolysis of proteins taken from serum (Yamafuji et al., 1996), hemoglobin (Piot et al., 1992; Ivanov et al., 1997), and milk (Henriques et al., 1987; Lebrun et al., 1995). The main effects of natural ACE inhibition are not restricted to the reduction of angiotensin II production, but also include a decrease in bradykinin degradation, synergistically leading to several outcomes in the progression of the cell cycle. Most BPPs are enriched in internal proline residues, which confer resistance to cleavage by aminopeptidades, carboxypeptidases and endopeptidases present in human plasma (Ferreira et al., 1970; Freer and Stewart, 1971). The presence in the hinge domain of a sequence enriched in proline residues and its localization near to endostatin in human collagen XVIII make it tempting to speculate about the existence of combined physiological roles of endostatin and BPPs from human collagen XVIII in the control of angiogenesis. All peptides (I–VII in Table 1) derived from the hinge domain inhibited all forms of ACE in this study, with K_i values two or three orders of magnitude higher than BPP9a, the most efficient natural inhibitor of ACE. However, for physiological control and owing to the possible local release from collagen XVIII, the BPP-like peptides could be effective in human tissues. It is important to note that the peptides assayed in the present study contain peculiar sequences of prolines not found in mice collagen (Figure 1). Endostatin and BPP-like peptides can be released from human collagen XVIII by the proteolytic activity of lysosomal proteases, particularly cathepsin L and MMPs, as illustrated in Figure 1. In addition, another candidate protease in human serum and in HUVECs could be a serine protease that was able to hydrolyze the peptide Abz-RPTSPPAH-SHRDFQPVLHLVALNSPL-NH_2 at the A-H bond, releasing the ACE inhibitory peptide RPTSPPA.

In conclusion, the discrepancy between *in vitro* and *in vivo* endostatin strength of action, which is attenuated *in vivo*, could be related to the absence of other factors present, such as peptides with ACE inhibitory properties. Although the simultaneous activities of endostatin and ACE inhibitors on angiogenesis control are speculative, until recently there has been little molecular information about endogenous ACE fine-tuning of angiogenesis regulation. The effects of natural BPPs on angiogenesis and tumorigenesis require further investigation and we are currently pursuing these areas of interest.

Materials and methods

Materials

The protease inhibitors pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), *ortho*-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), BPP5a and BPP9a were purchased from Sigma (St. Louis, MO, USA). DMEM:F12 was from Gibco (Gaithersburg, USA) and fetal bovine serum was from Cultlab (Campinas, Brazil).

Synthetic peptides

All peptides assayed as inhibitors were synthesized using a solid-phase technique. The fluorescence resonance energy transfer (FRET) peptide Abz-YRK(Dnp)P-OH, (Abz=*ortho*-aminobenzoic acid; Dnp=2,4-dinitrophenyl) was synthesized using Fmoc-Lys(Dnp)-OH and H-Pro-2-chlorotrityl resin (Araujo et al., 2000). The FRET peptide Abz-RPTSPPAHSHRQ-EDDnp (EDDnp=ethylene diamine-2-4-dinitrophenyl) was also synthesized using a solid-phase method (Hirata et al., 1994) with the Fmoc procedure. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system; Shimadzu, Kyoto, Japan) was used for all syntheses. The peptides were purified by semi-preparative HPLC using an Econosil C-18 column. The molecular mass and purity of the synthesized peptides were checked by amino acid analysis and matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, using a TOFSpec E instrument (Micromass, Manchester, UK). Stock solutions of Dnp or EDDnp peptides were prepared in DMSO, and the concentrations were measured spectrophotometrically using a molar absorption coefficient of 17 300 M^{-1} cm^{-1} at 365 nm.

Enzymes

Human testicular ACE was prepared as previously reported (Woodman et al., 2000). The three human recombinant forms of ACE, namely the wild-type form and the two full-length ACE mutants containing only one intact catalytic site, were kindly supplied by Dr. Dulce Elena Casarini (Universidade Federal de São Paulo, Brazil). The enzymes were obtained through stable expression in CHO cells as previously described (Wei et al., 1991). The molar concentrations of the ACE enzymes were determined by active-site titration with the tight-binding inhibitor lisinopril as previously described (Ehlers and Riordan, 1991). Recombinant human cathepsins L and B were expressed as previously described (Carmona et al., 1996; Nagler et al., 1997). Cathepsin K was kindly supplied by Dr. Dieter Brömme from the University of British Columbia (Vancouver, Canada). The molar concentrations of the cathepsins were evaluated by active-site titration with the cysteine peptidase inhibitor E64 as previously reported (Barrett and Kirschke, 1982).

Inhibition assays

ACE inhibition experiments were performed with the three forms of recombinant human wild-type ACE and with human testicular ACE. ACE activity on Abz-YRK(Dnp)P-OH was continuously measured at 37°C in a Shimadzu RF-1501 PC spectrofluorimeter adjusted to λ_{ex}=320 nm and λ_{em}=420 nm. The assays were performed in 0.1 M Tris-HCl buffer, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$. Inhibition assays were carried out under the same conditions after 3-min pre-incubation of the enzymes with increasing concentrations of BPP-like peptides. Fluorescence emission was continuously measured and apparent inhibition constant (K_{iapp}) values were obtained using the equation

$$\frac{V_0}{V_1}=1+\frac{[I]}{K_{iapp}},$$

where V_0 and V_i are the velocity for less than 2% substrate hydrolysis in the absence and presence of different inhibitor concentrations [I], respectively. The assays were performed in duplicate and the K_i parameters were obtained from the equation

$$K_i=\frac{K_{iapp}}{1+\frac{[S]}{K_m}}.$$

Processing of peptides derived from the NC1 fragment of collagen XVIII by human recombinant cathepsins B, K and L

Hydrolysis by cathepsins B, L and K (4 nM) of 20 μM Abz-TSPPAHSHRDFQPVLHLVALNSPL-NH_2 was carried out in 100 mM sodium acetate, 1 mM EDTA, pH 6.0. The incubations were performed at 37°C and aliquots were analyzed after 8, 24 and 48 h. The scissile bonds were identified by isolating the fluorescent peptides on HPLC and determining their sequences using MALDI-TOF mass spectrometry.

Processing of peptides derived from the NC1 region of collagen XVIII by human serum

Human whole blood was collected and a clot was allowed to form and shrink. Unclotted blood was centrifuged at 2000 *g* for 30 min and the serum supernatant was used in experiments. Serum proteolytic activity was determined using substrates Abz-RPTSPPAHSHRQ-EDDnp (20 μM) and Abz-TSPPAHSHRDFQP VLHLVALNSPL-NH_2 (20 μM) in 0.1 M Tris-HCl containing 135 mM NaCl, pH 7.4, at 37°C. The inhibition profile was measured by pre-incubating reaction aliquots with 10 μM pepstatin A, 10 μM PMSF, 5 μM E-64 or 1 mM *ortho*-phenanthroline (aspartyl-, serine-, thiol- and metallo-protease inhibitors, respectively). Aliquots were taken at different times over a period of 48 h and the scissile peptide bonds were identified by isolation of the fluorescent fragments on HPLC, with sequences determined by mass spectrometry, as described above.

Processing of peptides derived from the NC1 domain of collagen XVIII by human endothelial cells

Human umbilical-vein endothelial cells (HUVECs) were cultured in DMEM:F12 medium supplemented with 20% fetal bovine serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were maintained at 37°C in 5% CO_2/95% air and were seeded into six-well plates (Falcon Co.) and grown to confluence. The proteolytic activity of membrane-bound and secreted proteases was assayed using 20 μM Abz-RPTSPPAHSHRQ-EDDnp, washing the monolayer twice with buffered saline solution and incubating peptides in the same buffer at 37°C in 5% CO_2/95% air. Aliquots were taken and the cleavage sites were identified by HPLC/mass spectrometry analysis, as already described.

Acknowledgments

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