Complete Structure of an Increasing Capillary Permeability Protein (ICPP) Purified from Vipera lebetina Venom

ICPP IS ANGIgenic VIA VASCular ENDOTHELIAL GROWTH FACTOR RECEPTOR SIGNALING*

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The partial sequence of the increasing capillary permeability protein (ICPP) purified from Vipera lebetina venom revealed a strong homology to vascular endothelial growth factor (VEGF)-A. We now report its complete amino acid sequence determined by Edman degredation and its biological effects on mouse and human vascular endothelial cells. ICPP is a homodimeric protein linked by cysteine disulfide bonds of 25115 Da revealed by mass spectrometry. Each monomer is composed of 110 amino acids including eight cysteine residues and a pyroglutamic acid at the N-terminal extremity. ICPP shares 52% sequence identity with human VEGF but lacks the heparin binding domain and Asn glycosylation site. Besides its strong capillary permeability activity, ICPP was found to be a potent in vitro angiogenic factor when added to mouse embryonic stem cells or human umbilical vein endothelial cells. ICPP was found to be as potent as human VEGF165 in activating p42/p44 MAPK, in reinitiation of DNA synthesis in human umbilical vein endothelial cells, and in promoting in vitro angiogenesis of mouse embryonic stem cells. All these biological actions, including capillary permeability in mice, were fully inhibited by 1 μM of a new specific VEGF receptor tyrosine kinase inhibitor (ZM317450) from AstraZeneca that belongs to the anilinocinnoline family of compounds. Indeed, up to a 30 times higher concentration of inhibitor did not affect platelet-derived growth factor, epidermal growth factor, FGF-2, insulin, α-thrombin, or fetal calf serum-induced p42/p44 MAPK and reinitiation of DNA synthesis. Therefore, we conclude that this venom-derived ICPP exerts its biological action (permeability and angiogenesis) through activation of VEGF receptor signaling (VEGF-R2 and possibly VEGF-R1).

Angiogenesis is a tightly regulated process occurring physiologically during embryonic development, during the menstrual cycle, and in wound healing. It is also associated with a number of pathological situations including diabetic retinopathy, inflammation, brain edema following ischemic stroke, solid tumor growth, and metastasis. A number of polypeptide growth factors have been demonstrated to induce and regulate angiogenesis in vivo, among them fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF)-A (1, 2). Although the mode of activation at the receptor level differs, all these mitogens activate the ubiquitously expressed isoforms of mitogen-activated protein kinases referred to as p42/p44 MAPK or Erk, two essential transducers of growth, survival, and differentiation signals.

VEGF was the first mitogenic growth factor proven to have endothelial cell specificity and to be critical for blood vessel formation. The vascular endothelium-specific growth factors are now known to include five members of the VEGF family, four members of the angiopoietin family, and at least one member of the large ephrin family (3). VEGF is a multifunctional cytokine that is produced by virtually every tissue and overexpressed upon hypoxic stress and oncogenic transformation (4). It is a homodimeric glycoprotein, expressed as several spliced variants; the major forms contain 121, 165, 189, and 206 amino acids. VEGF121 differs from the larger VEGF isoforms in that it is the only VEGF type that does not possess heparin binding ability (5). These isoforms act in a coordinate fashion to recruit and expand the tumor vasculature (6). The main receptors that seem to be involved in initiating signal transduction cascades in response to VEGFs comprise a family of closely related receptor tyrosine kinases that are expressed almost exclusively on neovascularature and on the tumor endothelium. They consist of three members now termed VEGFR-1 (Flt-like tyrosine kinase, Flt-1), VEGFR-2 (kinase insert domain-containing receptor (KDR)), and the VEGF-C and -D receptor, VEGFR-3 (known previously as Flt-3). Some other accessory receptors (neuropilin) that seem to be involved primarily in modulating binding to the main receptors have also been reported (7). Their roles in signaling have not yet been fully elucidated (3). VEGFR-2, however, via activation of intrinsic tyrosine kinase activity, appears to mediate all the transduction cascades and is essential for the biological activity of VEGF.

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** The abbreviations used are: FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; svVEGF, snake venom VEGF; VEGFR, VEGF receptor; VTKI, VEGF receptor tyrosine kinase inhibitor; PDGF, platelet-derived growth factor; ICPP, increasing capillary permeability protein; KDR, kinase insert domain-containing receptor; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; ES, embryonic stem; HF, hypotensive factor; SFM, serum-free media; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.
major actions of VEGF: capillary permeability, chemotaxis, cell survival, and cell division (8).

The possibility that vascular growth factors may help prevent or repair damaged and leaky vessels offers therapeutic hope for ischemic diseases, diabetic retinopathy, or inflammatory setting (9, 10). In opposition, new antitumoral approaches targeting the tumor vasculature via inhibition of VEGF signaling are actively being developed; they include neutralizing anti-VEGF antibodies, anti-VEGF receptor antibodies, soluble VEGF receptors, antisense VEGF techniques, and VEGF receptor tyrosine kinase inhibitors (11).

We have previously isolated a protein from Vipera lebetina venom based on its potent ability to increase capillary permeability. The partial sequence of this protein, referred to as increasing capillary permeability protein (ICPP), revealed a VEGF-like structure (12). In this report, we present the complete amino acid sequence of ICPP and demonstrate that this venom-related-VEGF is capable of inducing p42/p44 MAPK activity and DNA synthesis in human umbilical vein endothelial cells (HUVEC) and of promoting in vitro angiogenesis. Interestingly, all these ICPP-induced biological actions are fully inhibited by a new VEGF receptor tyrosine kinase inhibitor, as presented here.

**EXPERIMENTAL PROCEDURES**

**Materials**—ICPP was purified from V. lebetina venom (12). Reverse phase analytical columns C8 (5 μm, 4.6 × 250 mm) were purchased from Beckman Instruments. Endoproteinases Asp-N, Arg-C, and Lys-C were of sequencing grade and were obtained from Roche Molecular Biochemicals. Recombinant human FGF-2 and VEGF165 were produced in our laboratory from Escherichia coli and Pichia pastoris, respectively, after purification on heparin binding affinity columns, whereas human recombinant PDGFβ and EGF were from Sigma. All other reagents used were of analytical grade from commercial sources.

The AstraZeneca compound 4-floro-5-[(6-methoxy-7-(2-methoxy-thoxy)cinnolin-4-yl]amino]-2-methylphenol (ZM317450), referred to here as VEGF receptor tyrosine kinase inhibitor (VTKI), belongs to the anilinocinnoline family of compounds. It was prepared according to the protocol presented in the patent WO 9734976 by A. P. Thomas and L. F. Hennequin.

**Reduction and Alkylation of ICPP**—Reduction was performed by incubating ICPP for 1 h at 37 °C in 6 mM guanidine-HCl, 0.5 mM Tris-HCl, 2 mM EDTA, 1.4 mM DTT (dithiothreitol), pH 7.5. Then, alklylation occurred following addition of 4-vinylpyridine (9 μmol final concentration) and terminated after 5 min by addition of DTT to a final concentration of 14 μM. The mixture was desalted on a reverse phase HPLC on a C8 column. Solvents A and B were 0.1% trifluoroacetic acid (v/v) and 80% of solvent B in 60 min. Effluent was continuously monitored at 214 nm and peaks were collected manually and subjected to sequence analysis.

**Enzymatic Digestions of ICPP**—Digestions of reduced and alkylated ICPP were carried out by the endoproteinases Arg-C, Lys-C, or Asp-N. The denatured protein was incubated in appropriate buffer medium. The suitable time, temperature, enzyme/substrate ratio, and termination of the reactions were performed according to the manufacturer’s instructions. Urea (2 M) was added to the reaction mixture to ensure solubility. The resulting peptides were subjected to a reverse phase chromatography on C8 column and eluted by an increasing gradient of 10–60% of solvent B in 60 min at a flow rate of 1 ml/min and monitored at 214 nm, and peaks were collected manually and subjected to sequence analysis.

**Amino Acid Analysis and Sequence Comparison**—The sequences of N-terminal subunits were determined after chemically unblocking with HCl in anhydrous methanol (13) by Edman degradation with an Applied Biosystems 470A liquid-phase sequencer equipped with on-line p-p-nitroanilide and reverse HPLC using an RP18 column. The sequences of peptides obtained from enzymatic digests of reduced and alkylated ICPP were performed as described previously. A search for similar proteins was performed following computer analysis with the BLAST data base search program.

**Mass Spectral Analysis**—Determination of the molecular mass of native ICPP was carried out on a Voyager DE-REP matrix-assisted laser desorption ionization time-of-flight mass spectrometer (PerSeptive BioSystems, Inc., Framingham, MA). A sinapinic acid matrix at 10 mg/ml in 50% acetonitrile/50% H2O/0.1% trifluoroacetic acid was used.

**Culture, MAPK Activity, and DNA Replication of HUVECs**—HUVECs were isolated from umbilical cord veins by collagenase perfusion as described previously (14) and cultivated in SFM (Invitrogen) supplemented with 20% fetal bovine serum. Cells were grown in plastic flasks coated with 10 μg/ml collagen (Sigma). For reinitiation of DNA synthesis, HUVEC were serum-starved for 24 h, trypsinized, and replated in 24-well plates coated with gelatin at 50,000 cells/well in 0.5 ml of SFM. Six h later, cells were incubated in 0.5 ml of SFM containing 1 μg/ml of tritiated thymidine and stimulated with growth factors or ICPP in the presence or absence of various concentrations of VTKI. After 24 h, the tritiated sample was analyzed as described previously (16). The blots were incubated with 1/5000 dilution of the anti-phospho p42/p44 MAPK monoclonal antibody (Sigma).

**Receptor Tyrosine Kinase Assays**—The ability of VTKI to inhibit the kinase activity associated with the VEGF receptors R1 (Flt-1) and R2 (Flk) was determined using a previously described enzyme-linked immunosorbent assay (17). Receptor tyrosine kinases in solutions isolated in enzyme assay es were generated as insect cell lysates following cell infection with recombinant baculoviruses containing kinase domains.

Briefly, compounds were incubated with enzyme, 10 μM MnCl2, and 2 μM ATP in 96-well plates coated with a poly(Glu)4Ala,Tyr 6:3:1 random copolymer substrate (Sigma). The ATP concentration used was adjusted to achieve 80% of enzyme activity, the respective Km value. Phosphorylated tyrosine was detected by sequential incubation with mouse IκB anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY), a horse radish peroxidase-linked sheep anti-mouse Ig antibody (Amersham Biosciences), and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Roche Molecular Biochemicals). Microcal Origin software (Version 3.78, Microcal Software Inc., Northampton, MA) was used to interpolate values by non-linear regression (four-parameter logistic equation).

**Protein Determination**—Protein concentration was determined by the procedure of Lowry et al. (18) with the folin phenol reagent and with bovine serum albumin as a standard.

**In Vitro Angiogenesis**—Culture and differentiation of embryonic stem cells was used as an angiogenesis test in vitro. Mouse ES cells (E14Tg2A.IV clone, initially provided by Dr. M. Hooper, University of Edinburgh, UK and subcloned by Dr. A. Smith, Edinburgh, UK) were grown in Dulbecco’s modified Eagle’s medium with Glutamax-1 and sodium pyruvate (Invitrogen) containing 10% fetal calf serum (Duchater, Brunath, France), 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM β-mercaptoethanol, and non-essential amino acids (all reagents from Invitrogen). They were kept undifferentiated by the addition of either 10% fetal calf serum or recombinant leukemia inhibitory factor purchased from Sigma or 100 units/ml leukemia inhibitory factor produced in COS cells as described previously (19).

For differentiation, ES cells were cultured in hanging drops as described previously (20) with some modifications. Briefly, ES cells were detached by trypsin/EDTA and aggregated into embryoid bodies in the above described Dulbecco’s modified Eagle’s medium lacking supplementing leukemia inhibitory factor. Aggregation was performed in 20-μl drops hanging from the lids of bacteriological Petri dishes and containing 800 cells. The lids were then placed over PBS-filled dishes and incubated at 37 °C. This was designated as day 0. At day 3, the resulting embryoid bodies were then transferred to gelatin-coated 24-well tissue culture plates. When indicated, the medium was supplemented with human 10 ng/ml VEGF165 (Sigma) or various concentrations of murine 12/15 β2 integrin-specific antibody (clone MEC13.3; BD PharMingen) for 1 h at room temperature. Staining was revealed by incubating first with a biotin-conjugated donkey anti-rat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and second with Alexa Fluor-conjugated...
ICPP Stimulates \( p_{42/44} \) MAP Kinase Activity in HUVEC, Implication of VEGF Receptors—Since ICPP and human VEGF have a high sequence similarity, it was appropriate to compare the effect of these proteins on signaling and angiogenic potential using HUVEC. The Raf-1 \( \rightarrow \) MEK \( \rightarrow \) p42/p44 MAPK has been demonstrated to rapidly convey growth and survival signals from a variety of receptor tyrosine kinases. We therefore tested the biological action of ICPP on HUVEC that are responsive to VEGF and FGF for growth and differentiation. Fig. 4A shows that like human VEGF165 and FGF-2, \( V. \) lebetina ICPP stimulates p42/p44 MAP kinases in HUVEC. This action is rapid, detected within 2 min, peaks around 10–15 min, and decreases to basal level after 4 h of stimulation (data not shown). This temporal action as well as the maximal intensity of MAPK activation parallel that observed with human VEGF165. In the same cells, however, p42/p44 MAPK activation could reach 3–5 times higher levels in response to FGF-2. We then compared the potency of ICPP and human VEGF165 using MAP kinase activation as a reporter system. On a molar basis, ICPP purified to homogeneity is at least 1.5 times more potent than human recombinant VEGF165 obtained from Sigma or freshly prepared from \( P. \) pastoris and purified on heparin affinity columns (data not shown).

We then sought to determine whether ICPP can signal through endogenous VEGF receptors. To answer this point, we exploited the specificity of tyrosine kinase inhibitors developed by AstraZeneca. These compounds attain selectivity by competing with the non-conserved hydrophobic pocket of the ATP binding site in kinases. The inhibitor used in this study (ZM317450) is a new molecule of the anilinocinnoline family of compounds (Fig. 3), referred to here as VTKI. In isolated enzyme assay, VTKI is a potent inhibitor of VEGFR-2 tyrosine kinase (IC \(_{50} \) = 50 nM) with submicromolar activity versus the kinase activity of VEGFR-1 (Table I). In comparison with its inhibitory activity versus VEGFR-2 tyrosine kinase, VTKI demonstrated >2000-fold selectivity versus that associated with FGFRI and EGFR (Table I). Selectivity is also conserved in endothelial cells (HUVEC); 10 \( \mu \)M VTKI ablates fully ICPP- and VEGF-induced MAPK activation but does not affect that stimulated by FGF-2 (Fig. 4A). Inhibition of ICPP-stimulated MAPK activation was even found to be complete at 1 \( \mu \)M. The dose response of VTKI was explored on HUVEC stimulated with equally potent growth factors: VEGF (30 ng/ml) or ICPP (10 ng/ml). The VTKI concentration inhibiting half of the response (EC \(_{50} \)) is 100 nM for both VEGF (Fig. 4B) and ICPP (data not shown). Next we explored the specificity of VTKI by evaluating its action on the ER22, a derivative of the fibroblastic cell line CCL39 that express several receptor tyrosine kinases—PDGF-R, FGF-R, and EGF-R (28). Fig. 5 shows that MAPK stimulation by all agonists remains unaffected by VTKI, indicating that associated with FGF-R1 and EGFR (Table I). Selectivity is also conserved in endothelial cells (HUVEC); 10 \( \mu \)M VTKI ablates fully ICPP- and VEGF-induced MAPK activation but does not affect that stimulated by FGF-2 (Fig. 4A). Inhibition of ICPP-stimulated MAPK activation was even found to be complete at 1 \( \mu \)M. The dose response of VTKI was explored on HUVEC stimulated with equally potent growth factors: VEGF (30 ng/ml) or ICPP (10 ng/ml). The VTKI concentration inhibiting half of the response (EC \(_{50} \)) is 100 nM for both VEGF (Fig. 4B) and ICPP (data not shown). Next we explored the specificity of VTKI by evaluating its action on the ER22, a derivative of the fibroblastic cell line CCL39 that express several receptor tyrosine kinases—PDGF-R, FGF-R, and EGF-R (28). Fig. 5 shows that MAPK stimulation by all agonists remains unaffected by VTKI.
Function of B. insularis Vipera lebetina Venom VEGF

Inhibition of isolated receptor tyrosine kinase activity (IC_{50} \text{ (M) by VTKI})

| VEGF-R1  | VEGF-R2  | EGFR | FGFR1 |
|----------|----------|------|-------|
| 0.50 ± 0.04 | 0.05 ± 0.01 | >100 | >100 |

The ability of VTKI to inhibit recombinant kinase activity was examined using a 96-well ELISA assay with 2 \muM ATP. Data represent the mean ± S.E. of at least three separate determinations.

The second in vitro system exploited to assess ICPP-induced angiogenesis is more demanding since it records the capacity of a cytokine to promote the proliferation of embryonic vascular endothelial cell precursors and their capacity to differentiate into a vascular network. In comparison with FGF-2, VEGF has been reported to be an extremely potent cytokine in this biological assay (30). Interestingly, as shown in Fig. 6C, ICPP is a potent molecule that, like VEGF, can induce a vascular-like network positive for the vascular specific marker CD31. Although this assay does not allow an accurate quantitation, ICPP has always been more potent than VEGF165 in establishing a vascular differentiated network. This ICPP-induced growth and differentiation is again fully prevented by VTKI (data not shown).

The general blocker of MAPK signaling and highlight the specificity toward VEGF receptor kinase. Collectively, these data suggest that ICPP mediates its effect on MAPK signaling via VEGF receptor signaling.

**ICPP Is a Potent Angiogenic Factor through VEGF Receptor Signaling**

Since we demonstrated in the previous section that ICPP can signal through VEGF receptors, the next question was to investigate whether, besides MAPK signaling, ICPP is capable of mimicking all the biological actions of VEGF. We first tested its capacity to induce angiogenesis in two in vitro systems, DNA proliferation in HUVEC and growth and differentiation of mouse vascular endothelial cells. Fig. 6A shows that serum-starved HUVEC are able to reinitiate DNA synthesis in response to VEGF165 or ICPP. FGF-2, a more potent agonist for stimulation of MAPK signaling, is also a much more potent mitogen for HUVEC (Fig. 6A). In our assays, DNA synthesis was stimulated 3-fold above basal with ICPP and 5–10-fold above basal with FGF-2. Interestingly the mitogenic action of ICPP or VEGF is fully suppressed by VTKI, whereas the activity of FGF is practically unaffected. We regularly observed a 10–15% inhibition of FGF-induced mitogenic action that we attributed to the autocrine production of VEGF in response to FGF, a proposal consistent with a previous observation (29). This biological assay was then used to compare the sensitivity of ICPP and VEGF toward the VTKI. As shown in Fig. 6B, reinitiation of DNA synthesis was fully suppressed at 100 nM of VTKI, indicating that reinitiation of DNA synthesis appears slightly more sensitive than the MAPK inhibition. The VTKI dose-response curves for ICPP and VEGF are identical, suggesting that ICPP and VEGF are using the same receptor system for VEGF-R2 signaling. Indeed, expression of VEGF-R2 alone in the fibroblastic cell line CCL39, devoid of VEGF-R, was sufficient to elicit MAPK activation and DNA synthesis in response to ICPP. This action was fully abolished by VTKI (data not shown).

The action of ICPP (Vleb ICPP) with the hypotensive factor (Vasp HF) purified from V. aspis aspis venom (23), the deduced svVEGF amino acid sequences characterized from B. insularis (Vleb svVEGF) and B. jararaca (Bjar svVEGF) venoms (24), respectively, and human VEGF (22). Human VEGF residues identified by site-directed mutagenesis as being important for receptor binding (25) are in bold. Residues implicated in VEGF R2 (Flk-1 fetal liver kinase-1/KDR) binding along with the VEGF crystal structure are underlined (26). The heparin binding domain (55 residues) is indicated by italics. The box indicates the N-glycosylation site, and the asterisks indicate identical residues.

![Structure of VTKI](image)

**Structure of VTKI**

**4-fluoro-5-[(6-methoxy-7-(2-methoxyethoxy)cinolin-4-yl]amino]-2-methylphenol**

**Table I**

### Inhibition of isolated receptor tyrosine kinase activity (IC_{50} \text{ (M) by VTKI})

| VEGF-R1 | VEGF-R2 | EGFR | FGFR1 |
|---------|---------|------|-------|
| 0.50 ± 0.04 | 0.05 ± 0.01 | >100 | >100 |

The ability of VTKI to inhibit recombinant kinase activity was examined using a 96-well ELISA assay with 2 \muM ATP. Data represent the mean ± S.E. of at least three separate determinations.

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**ICPP-stimulated Increased Capillary Permeability Is Mediated through VEGF Receptor Signaling**

Since we demonstrated in the previous section that ICPP can signal through VEGF receptors, the next question was to investigate whether, besides MAPK signaling, ICPP is capable of mimicking all the biological actions of VEGF. We first tested its capacity to induce angiogenesis in two in vitro systems, DNA proliferation in HUVEC and growth and differentiation of mouse vascular endothelial cells. Fig. 6A shows that serum-starved HUVEC are able to reinitiate DNA synthesis in response to VEGF165 or ICPP. FGF-2, a more potent agonist for stimulation of MAPK signaling, is also a much more potent mitogen for HUVEC (Fig. 6A). In our assays, DNA synthesis was stimulated 3-fold above basal with ICPP and 5–10-fold above basal with FGF-2. Interestingly the mitogenic action of ICPP or VEGF is fully suppressed by VTKI, whereas the activity of FGF is practically unaffected. We regularly observed a 10–15% inhibition of FGF-induced mitogenic action that we attributed to the autocrine production of VEGF in response to FGF, a proposal consistent with a previous observation (29). This biological assay was then used to compare the sensitivity of ICPP and VEGF toward the VTKI. As shown in Fig. 6B, reinitiation of DNA synthesis was fully suppressed at 100 nM of VTKI, indicating that reinitiation of DNA synthesis appears slightly more sensitive than the MAPK inhibition. The VTKI dose-response curves for ICPP and VEGF are identical, suggesting that ICPP and VEGF are using the same receptor system for VEGF-R2 signaling. Indeed, expression of VEGF-R2 alone in the fibroblastic cell line CCL39, devoid of VEGF-R, was sufficient to elicit MAPK activation and DNA synthesis in response to ICPP. This action was fully abolished by VTKI (data not shown).

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**ICPP-stimulated Increased Capillary Permeability Is Mediated through VEGF Receptor Signaling**

ICPP was isolated by its capacity to stimulate capillary permeability on mice (12), and indeed, when compared with VEGF, the best permeability factor known to date, ICPP was found to be an extremely potent permeability factor (Fig. 7A). This biological activity was examined using the Miles assay (21), which involved intradermal injection of purified ICPP and VEGF165 in mice and measurement of the leakage of Evan’s Blue dye into the extravascular space (Fig. 7B). Intradermal injection of PBS was used as a control. For this biological action, ICPP was found to be as potent if not more potent than VEGF165. The ICPP- and VEGF-induced permeability was inhibited by VTKI when the inhibitor was preinjected 60 min before the peptide growth factor (Fig. 7). This inhibition reflects VEGF receptor targeting since VTKI is unable to prevent increased permeability trig-
FIG. 4. ICPP, like VEGF, activates p42/p44 MAP kinases in HUVEC; the dose response of the VTKI on VEGF-induced MAP kinase activation is shown. Serum-starved HUVEC were stimulated for 15 min with 10 ng/ml VEGF165, ICPP or FGF-2 in the absence (0) or presence (10 μM) of VTKI (A). The kinase inhibitor (dissolved in Me2SO) was added 15 min prior to stimulation, and Me2SO alone was added to the media of cells that did not receive VTKI (1% final concentration). Total extracts (50 μg of proteins) were separated on SDS-polyacrylamide gel electrophoresis and immunoblotted with a specific anti-phospho p42/p44 MAP kinase monoclonal antibody as described under “Experimental Procedures.” The doublet represents both ERK isoforms. C, control. HUVEC were stimulated for 15 min with 10 ng/ml VEGF165 (+) and preincubated with various concentrations of VTKI (B). Me2SO was kept constant at 1%. Activation of p42/p44 MAPK was monitored as indicated in “Experimental Procedures.”

FIG. 5. VTKI is a specific VEGF receptor antagonist. ER22 fibroblastic cells, a derivative of CCL39 expressing 300,000 EGF receptors (28), were serum-starved for 24 h and stimulated with five different agonists: human PDGFb (10 and 30 ng/ml), human FGF-2 (10 and 30 ng/ml), human EGF (10 and 30 ng/ml), 5% fetal calf serum (FCS), or human α-thrombin (THR) at 1 unit/ml. Where indicated, cells were preincubated with either 1 or 10 μM of VTKI. Activation of p42/p44 MAPK was monitored as described under “Experimental Procedures.”

gered via the α-thrombin receptor peptide (31) activating PAR-1.

DISCUSSION

Snake venoms represent an extraordinary source of biological molecules that have been invaluable to our knowledge and understanding of basic biological processes. Besides the toxins acting directly in the nervous system, many venom proteins target blood capillaries, preventing blood coagulation via disintegrins and enzymes degrading fibrinogens, whereas others increase the permeability of blood capillaries. This is the case for bradykinin potentiating factors purified from B. jararaca that convert angiotensin I into angiotensin II and phospholipase A2 purified from Trimeresurus mucrosquamatus, which induces release of histamine from mast cell degranulation (32). In this context, it is remarkable to see the emergence of a new set of selected molecules with permeability capacity in snake venoms. ICPP, an increasing capillary permeability protein of V. lebetina venom, is indeed a VEGF-related molecule highly homologous to the HF with vascular permeability activity (23). During the preparation of this manuscript, another report demonstrated the expression of related VEGF molecules in the venom of Bothrops jararaca (pit viper) (24), a result extending the notion that many snakes have evolved to specifically express in their venoms VEGF-like molecules, the most potent permeability factors described so far in vertebrates. During evolution, the co-selection in snake venoms of potent toxins together with the most effective permeability factors was certainly crucial for rapid dissemination of the toxins in the general circulation of the prey.

In the present study, we determined the complete amino acid sequence of ICPP, confirming that it is structurally related to the PDGF family. This protein consists of two homodimers of 110 amino acids having a molecular mass of 25,115 Da. ICPP shares the highest sequence identity with HF, the hypotensive factor purified from V. aspis aspis (23), and the recently identified svVEGF from B. insularis pit viper venom (24). ICPP, together with the other snake svVEGFs, shares about 50% identity with human VEGF-A (22). ICPP, like HF and svVEGF, differs in length from VEGF165, lacking a heparin binding domain and a potential N-linked glycosylation site. The recombinant protein svVEGF from the B. insularis pit viper was biologically characterized only by its ability to increase vascular permeability (24). However, in addition to increasing permeability, HF has been shown to also exert a strong hypertensive effect and have a mitogenic effect on endothelial cells. The mitogenic activity of HF was 5–10 times lower than that of VEGF and was inhibited by cycloheximide. The authors speculated that this protein may induce a signaling response and increase protein synthesis in endothelial cells, but studies examining the mechanism of action have not been reported (23).

VEGF functions by interacting with two well characterized high affinity tyrosine kinase receptors, VEGF-R1 and VEGF-R2, that are selectively expressed on endothelium. VEGF-R2

2 V. Vouret-Craviéri, unpublished results.
appears to be the dominant signaling receptor in VEGF-induced mitogenesis, and permeability increases (33, 34), whereas the role of VEGF-R1 in endothelial cell function is much less clear. Recent findings suggest that VEGF-R1 may have a negative role, either by acting as a decoy receptor or by suppressing signaling through VEGF-R2 (35).

Plasmin cleavage of VEGF165 generates a 110-residue long N-terminal fragment (VEGF110) that lacks the heparin-binding domains. VEGF110 is thought to maintain the ability to bind to the VEGF receptors, but its endothelial cell mitogenic potency is decreased substantially (100-fold) relative to VEGF165, indicating that the heparin binding domains are critical for stimulating endothelial cell proliferation (36). This finding also concurs with experiments using VEGF121, which does not bind to either heparan sulfates or to the extracellular matrix. VEGF121 has been described as being 10–100-fold less potent than VEGF165 at inducing biological responses in endothelial cells (37). The effect of glycosylation of VEGF165 on receptor binding has also been studied by Keyt et al. (25) using a constructed unglycosylated form of VEGF. They showed that N-linked carbohydrate at Asn-75 does not appear to have an effect in mediating VEGF receptor binding and the precise role of glycosylation remains to be elucidated.

ICPP and VEGF165 were tested in parallel in various bioassays including MAP kinase activation, in vitro angiogenesis, and capillary permeability. In this regard, our studies have shown that ICPP, like VEGF165 and FGF-2, stimulates p42/p44 MAP kinases in HUVEC. The activity of ICPP was found to
be at least 1.5 times more potent than human recombinant VEGF165 and mediated via signaling through endogenous VEGF receptors. The requirement for VEGF receptor signaling in transduction of a biological response to ICPP was demonstrated using VTKI, an antagonist that can selectively inhibit the kinase activity associated with VEGF-R2 and VEGF-R1. This inhibitor ablates fully ICPP- and VEGF-induced MAPK activation (IC_{50} of ~100 nM versus both) but does not affect that induced by FGF-2. The specificity of VTKI has been further demonstrated in ER22 cells, where it was found to not affect PDGF-, EGF-, or FGF-2-mediated MAPK activation at a concentration of 30 μM. Furthermore, VTKI did not inhibit the MAP kinase activation in response to the G protein-coupled receptor agonist, α-thrombin, or fetal calf serum.

When the mitogenic action of ICPP was studied, it was found to be as potent if not slightly more potent than VEGF165 at reintroducing DNA synthesis in HUVEC. This ICPP-induced response was fully suppressed by VTKI. In addition, we have observed that ICPP promotes DNA proliferation in HUVEC and induces growth and differentiation of mouse embryonic endothelial cells. VTKI can also inhibit these ICPP-induced angiogenic and permeability responses, indicating an involvement of VEGF receptor signaling. In each biological response examined, ICPP was found to possess remarkably similar and at least three times more potent bioactivity than VEGF165 despite the absence of a heparin binding domain. The structural differences between ICPP and VEGF, in particular the absence in ICPP of heparin binding domains and consensus N-glycosylation sites, suggest that some of their biological activities might be different.

In conclusion, we have shown that the amino acid sequence of ICPP displays a high similarity to that of VEGF and that capillary permeability, angiogenesis, endothelial cell mitogenicity, and MAP kinase activation induced by ICPP were mediated through VEGF receptor signaling. These findings provide the first evidence that ICPP is a novel member of the family of VEGF-like growth factors. Moreover, considering the therapeutic impact of VEGF-A in the treatment of coronary heart disease or critical limb ischemia (38, 39), ICPP, which appears more potent than VEGF, may represent a novel candidate for therapeutic angiogenic approaches aimed at growing new vasculature.

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