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Vascular endothelial growth factor and the *in vivo* increase in plasma extravasation in the hamster cheek pouch

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1 The purpose of this study in the hamster cheek pouch was to determine whether or not vascular endothelial growth factor (VEGF) induced changes in plasma extravasation and if so, the mechanism(s) involved.

2 The cheek pouch microcirculatory bed of the anaesthetized hamster was directly observed under microscope and the number of vascular leakage sites, as shown by fluorescein isothiocyanate (FITC-dextran, 150 kD) extravasation, was counted. Drugs and VEGF were applied topically. VEGF from 0.05 to 0.5 μg ml^{-1} (1.2 to 12 nM) produced a dose-dependent increase in the number of microvascular leakage sites from virtually none in basal conditions to up to 250 in some pouches. The effects of VEGF (0.1 μg ml^{-1} or 2.4 nM) were blocked in a concentration-dependent manner by the non-specific heparin growth factor antagonist TBC-1635 (0.1, 1 and 3μM). The placenta growth factor (PlGF-1: 0.1 and 0.5 μg ml^{-1} or 3.4 and 17 nM) did not increase plasma extravasation, *per se*, but abolished the effects of VEGF (2.4 nM).

3 The increases in microvascular leakage produced by VEGF (2.4 nM) were partially but significantly ($P<0.05$) inhibited by genistein (5 and 10 μM, up to 33% inhibition), LY 294002 (30 μM, 41%), bisindolylmaleimide (1 μM, 65%) and virtually abolished by indomethacin (3 μM, 88%) and L-nitro-arginine (10 μM, 95%), these drugs being inhibitors of tyrosine kinase, phosphatidylinositol-3-kinase, protein kinase C, cyclo-oxygenase and nitric oxide synthase respectively. None of these inhibitors, at the concentration tested, induced alone an increase in plasma extravasation.

4 These results indicate that the VEGF-induced plasma extravasation may involve the stimulation of VEGF-R2 (Flk-1/KDR) and the activation of phosphatidylinositol-3-kinase and protein kinase C. The production of both nitric oxide and prostaglandin is required to observe an increase in vascular leakage.
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Abbreviations: PlGF, placenta growth factor; VEGF, vascular endothelial growth factor

Introduction

Vascular endothelial growth factor (VEGF) has been independently isolated as both an endothelial growth factor (Connolly *et al*., 1989; Leung *et al*., 1989) and as a vasopermeability factor (VPF, Senger *et al*., 1983; Keck *et al*., 1989). The designation VEGF currently includes a family of six known members: VEGF, placenta growth factor (PlGF-1 and PlGF-2), VEGF-B, VEGF-C, VEGF-D and the VEGF of viral origin or VEGF-E. These peptides can interact with at least six different membrane 'receptors'. These include the signalling receptors VEGFR-1 (flt-1), VEGFR-2 (Flk-1/KDR), VEGFR-3 (flt4), members of the tyrosine kinase receptor family, the accessory isoform specific receptors neuropilin-1 and 2, and the VEGF binding heparan-sulphate proteoglycans (for review: Stacker & Achen, 1999; Veikkola & Alitalo, 1999, Neufeld *et al*., 1999). VEGF itself consists of five polypeptides of different sizes that derive from the same gene by alternative splicing (121, 145, 165, 189 or 206 amino acid residues) but $VEGF_{165}$ is likely to be the predominant molecular species (Veikkola & Alitalo, 1999; Neufeld *et al*., 1999).

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VEGF induces an increase in vascular permeability in various vascular beds from different species *in vivo* (Senger *et al*., 1983, Keck *et al*., 1989; Collins *et al*., 1993; Murohara *et al*., 1998) and *in vitro* either in isolated perfused blood vessels (Wu *et al*., 1996, Bates & Curry 1996) or in cultured endothelial cell monolayers (Wang *et al*., 1996; Kevil *et al*., 1998; Feng *et al*., 1999a). However, the effects of VEGF in the hamster cheek pouch, a model that has been extensively used to study microcirculation and the changes in microvascular permeability (Duling 1973, Svenjö et al 1978), are unknown. The purpose of this work was to determine whether or not topical administration of VEGF could alter plasma extravasation in the hamster cheek pouch and if so to characterize the mechanism involved.

Methods

Hamster cheek pouch

The method has been described in detail elsewhere (Svenjö *et al*., 1978). Male Harlan golden hamsters (110–120 g; Charles River, France) were anaesthetized with sodium pentobarbi-

tone (80 mg kg^{-1}; i.p.) and the body temperature controlled at 37°C with a rectal temperature probe and a homeothermic blanket control unit (Harvard, Ealing, Les Ullys, France). The trachea was cannulated, in order to facilitate the spontaneous breathing of the animal, and the femoral vein catheterized. The cheek pouch was exteriorized, dissected with care under microscope, fixed upon a Plexiglass perfusion chamber and superfused (6 ml min^{-1}, Ismatec, Zurich, Switzerland) with a modified Ringer's solution of the following composition (mM): NaCl 124, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 2.0, $NaHCO_3$ 25, HEPES 30 (pH 7.4; 36°C, bubbled with 5% CO_2-95% N_2 gas mixture). The cheek pouch could be observed under the microscope (modified Leitz Ergolux) either under visible light or under UV illumination (magnification: 50). After a 30 min stabilization period, fluorescein isothiocyanate dextran (FITC dextran, 150 kD) was injected through the femoral vein (250 mg kg^{-1}, 0.5 ml 100 g^{-1}). Anaesthesia was maintained with chloralose (25 mg kg^{-1}, 0.1 ml; i.v.) when needed as previously described (Bouskela *et al*., 1990; Félétou *et al*., 1996).

VEGF-induced increase in vascular leakage

Bradykinin (300 nM), topically applied for 5 min on the dissected hamster cheek pouch which was continuously perfused with non-recirculating modified Ringer's solution at a rate of 6 ml min^{-1}, produced a reversible increase in plasma extravasation. Changes in plasma extravasation could be directly observed by the formation, under UV illumination of the preparation, of leakage sites. Quantification was performed either by counting the number of leakage sites or by measuring the accumulation of FITC dextran in the superfusion fluid with a spectrofluorimeter (495/520 nm, Shimadzu). Both methods of measuring the changes in plasma extravasation produced data that were qualitatively similar and significantly correlated (Félétou *et al*., 1996). Under the same experimental conditions, application of VEGF, up to 1.8 nM, did not produce a consistent increase in plasma extravasation. Increasing either the duration of the VEGF perfusion or the concentration of VEGF in the perfusate could not be envisaged (due to the cost of VEGF). Furthermore, reducing the perfusion rate altered the response of the pouch to bradykinin (data not shown). The responses to VEGF (0.05 to 0.5 μg ml^{-1} or 1.2 to 12 nM) were therefore studied by still perfusing the pouch at a rate of 6 ml min^{-1}, but with a recirculating solution. The recirculation of modified Ringer's solution did not *per se* produce any changes in plasma extravasation and under these conditions, bradykinin (300 nM, for 5 min) induced the formation of leakage sites. The number of these leaks was not significantly different to the number observed with non-recirculating saline solution (data not shown). However, the measurement of the accumulation of FITC-dextran in the recirculating solution became meaningless.

The following protocol was subsequently applied. At the beginning of the experiment the pouch was superfused with non-recirculating solution. Preparations with more than 10 spontaneous leakage sites (before the bradykinin superfusion), an objective sign of damage during the surgical procedure, were rejected. Similarly, preparations developing petechia during the course of the experiments, a gross sign of circulatory and vascular dysfunction, were excluded. Forty minutes after the FITC-dextran administration, bradykinin (300 nM) was superfused for 5 min. In order to homogenize the various groups, preparations with less than 80 or more than 250 leakage sites in response to the bradykinin test were also discarded (surface area of the microscope field: 0.2 cm^2). The preparation was then washed for 40 min by which time the number of leakage sites had returned to zero and the extravasation of FITC dextran to its control value. Groups were established in order to present a homogeneous response toward bradykinin (average number of leakage sites per group before any treatment: 130–170). The pouch was then superfused at the same rate (6 ml min^{-1}) with recirculating control solution (total volume: 20 ml) for 20 min. Drugs or solvent and subsequently VEGF were directly added to the recirculating solution (maximum volume added: 500 μl). The incubation time with the various inhibitors was 30 min. Each animal was exposed only once to a single concentration of VEGF and only one pouch was studied for a given hamster.

Substances used

Bisindolylmaleimide, borax, bradykinin, chloralose, L-nitroarginine, indomethacin, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one: LY 294002 (Sigma/RBI, La Verpillère, France), sodium pentobarbitone (Sanofi, Libourne, France), human recombinant vascular endothelial growth factor ($VEGF_{165}$), human recombinant placenta growth factor ($PlGF_{129}$ or PlGF-1, R&D systems, Abington, U.K.), Sodium salt of 2,6-bis-[4-(10,13-dimethyl-3-sulphooxy-hexadecahydro-cyclopenta[α] phenanthren-17-yl)-pentanoylamino]-hexanoic acid (2-{2,6-bis-[4-(10,13-Dimethyl-3-sulphooxy-hexadecahydro- cyclopenta [α] phenanthren-17-yl) -pentanoylamino]-hexanoylamino}-pentyl)-amide: TBC 1635 (a generous gift from Dr R. Tilton: Texas Biotechnology Corporation, Houston, TX, U.S.A.). Drugs were prepared daily as stock solution in saline or dimethyl sulphoxide (DMSO). Chloralose (25 g l^{-1}) was dissolved in borax solution (23 g l^{-1}).

Statistical evaluation

Data are shown as means ± s.e.mean; *n* represents the number of animals studied. Statistical evaluation was performed using an unpaired *t*-test and a one or two-way analysis of variance. When a significant interaction was observed ($P<0.05$), a complementary analysis was undertaken (Newman-Keul's test) to identify differences among groups.

Results

VEGF-induced changes in plasma extravasation

In the hamster cheek pouch, the non-cumulative, topical application of VEGF (0.05–0.5 μg ml^{-1} or 1.2–12 nM) induced a concentration-dependent formation of leakage sites situated at the level of post-capillary venules (Figure 1), as observed with other inducers of changes in plasma extravasation such as bradykinin or histamine. The maximum increase in the number of leakage sites was reached 10–15 min after the application of VEGF and then progressively declined (Figure 2). However, diffuse extravasation could

thereafter be observed along venules and arterioles but could not be properly quantified as the superfusing fluid was recirculating. Even after a prolonged wash-out (up to 1 h), the number of leakage sites in response to a second VEGF administration was significantly decreased ($\approx$ 25% of the first response). However, the increase in plasma extravasation produced by bradykinin (300 nM) was not significantly influenced by previous exposure to VEGF and was similarly observed in the presence of the growth factor or after wash-out (data not shown). In the subsequent experiments each hamster cheek pouch was exposed to a single 30 min application of VEGF (0.1 μg ml^{-1} or 2.4 nM) and only the leakage sites situated on the post-capillary venules were quantified.

PlGF-1 (0.1 and 0.5 μg ml^{-1} or 3.4 and 17 nM) produced no changes or a very minimal increase in vascular leakage. However, in the presence of PlGF (3.4 nM), the effects of VEGF (2.4 nM) were abolished while the changes in plasma extravasation produced by bradykinin (300 nM) were only partially inhibited (Figure 2). In the presence of the highest concentration of PlGF (17 nM), the effects of bradykinin were also abolished. After the wash-out of PlGF (for both concentrations), the increase in plasma extravasation produced by bradykinin was fully restored.

Mechanism of the changes in plasma extravasation induced by VEGF (2.4 nM)

TBC 1635 (0.3, 1.0 and 3.0 μM), a non-specific heparin-binding growth factor antagonist, did not produce any significant vascular leakage when compared to the solvent (DMSO, maximum number of leakage sites <6). TBC 1635 produced a statistically significant concentration-dependent inhibition of the increase in plasma extravasation produced by VEGF (Figure 3). In contrast, TBC 1635 (1.0 μM) did not

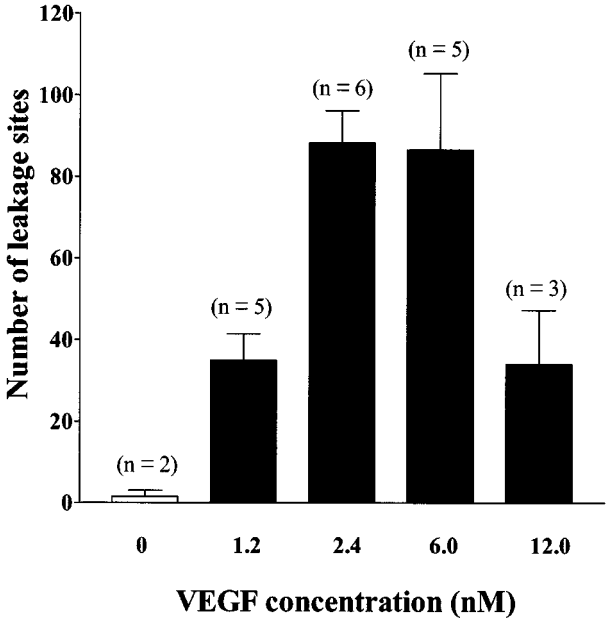

Figure 1 Effect of $VEGF_{165}$ in the hamster cheek pouch. Each pouch was subjected to a single administration of VEGF. Data are shown as means$\pm$s.e.mean, n represents the number of animals studied.

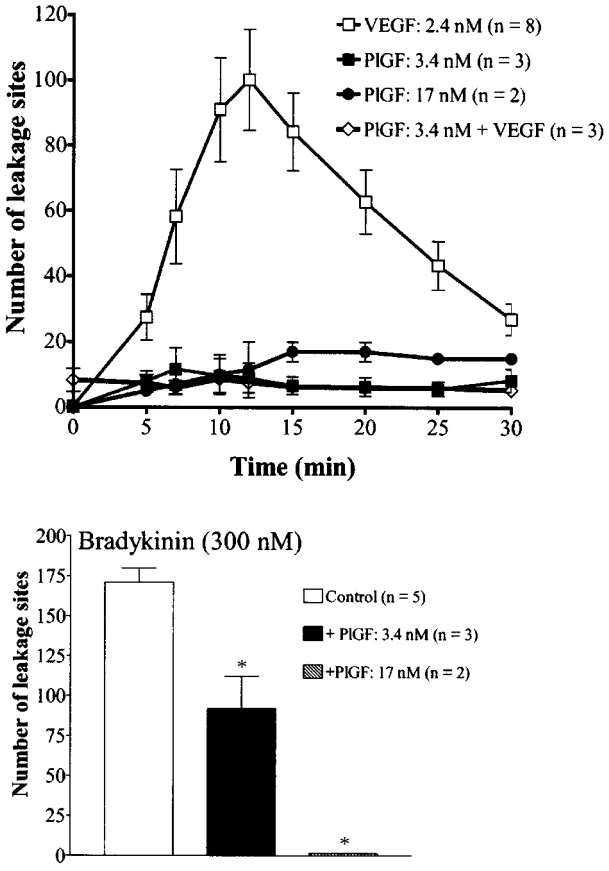

Figure 2 Top panel: Time-dependent changes in the number of microvascular leakage sites induced by $VEGF_{165}$ (2.4 nM), PlGF-1 (3.4 and 17 nM) and VEGF (2.4 nM) in presence of PlGF (3.4 nM). PlGF did not produce any significant changes in plasma extravasation. When compared to control the response to VEGF in presence of PlGF (3.4 nM) was significantly different at each time point. Bottom panel shows the maximum number of leakage sites produced by bradykinin (300 nM) in presence or not of PlGF-1 (3.4 and 17 nM). The asterisk indicates a statistically significant inhibition of the bradykinin response ($P<0.05$). Data are shown as means$\pm$s.e.mean, n represents the number of animals studied.

significantly affect the increase in plasma extravasation produced by bradykinin (300 nM).

Genistein (5 and 10 μM), a tyrosine kinase inhibitor, LY 294002 (10 and 30 μM), a phosphatidylinositol-3-kinase inhibitor and bisindolylmaleimide (1 μM), a protein kinase C inhibitor, produced a partial inhibition of the VEGF-induced increase in plasma extravasation. The maximum numbers of leakage sites were statistically smaller at each concentration tested, with the exception of the compound LY 294002 which did not produce any significant changes at the lowest concentration studied. In contrast, the responses to bradykinin (300 nM) were not significantly affected by these various inhibitors (Table 1). Indomethacin (3 μM), a cyclo-oxygenase inhibitor, and L-nitro-arginine (10 μM), a nitric oxide synthase inhibitor, virtually abolished the response to VEGF. Indomethacin did not significantly affect the response to bradykinin (300 nM; Table 1). None of these inhibitors, at the concentration tested, induced alone an increase in plasma extravasation during the 30 min pre-incubation period. The increase in plasma extravasation produced by VEGF was not

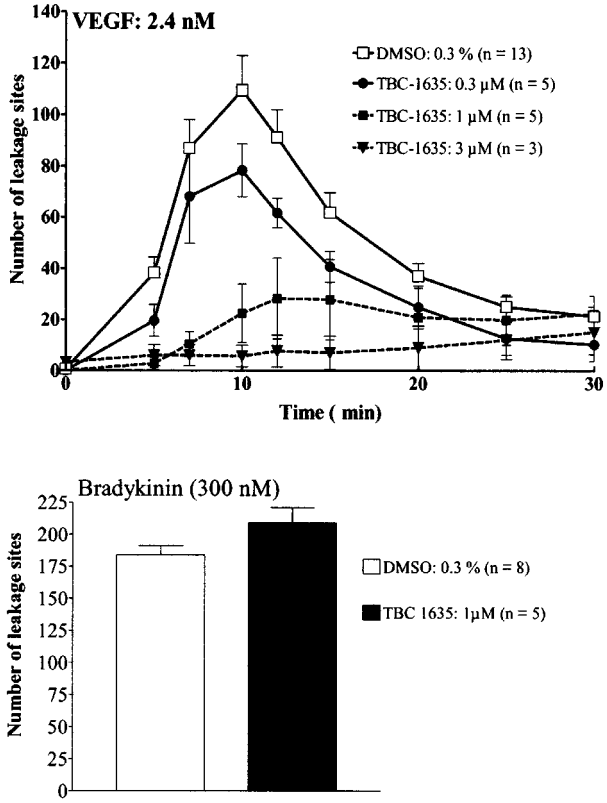

Figure 3 Top panel: Time-dependent changes in the number of microvascular leakage sites induced by $VEGF_{165}$ (2.4 nM), in presence of not of the heparin-binding growth factor antagonist TBC 1635 (0.3, 1 and 3 μM). The effects of TBC 1635 were statistically significant for the two highest concentrations studied. Bottom panel shows the maximum number of leakage sites produced by bradykinin (300 nM) in presence or not of TBC 1635 (1 μM). Data are shown as means $\pm$ s.e.mean, *n* represents the number of animals studied.

inhibited by pretreatment with HOE 140 (a bradykinin B2 receptor antagonist: 5 nmol kg^{-1} iv) at a concentration that virtually abolished the response to bradykinin (300 nM; data not shown).

Discussion

The present study demonstrates that, in the hamster cheek pouch, topically applied VEGF is a very potent inducer of vascular leakage (approximately 100 and 1000 times more potent than bradykinin and histamine respectively: Svenjo & Arfors, 1979; Félétou *et al*., 1996). This involves the stimulation of a tyrosine kinase receptor, possibly VEGF-R2 (Flk-1/KDR), and the activation of phosphatidylinositol-3-kinase and protein kinase C. The production of both nitric oxide and prostaglandin is required to observe an increase in vascular leakage.

VEGF is a vasodilator and releases nitric oxide from the endothelium of various vascular beds (Ku *et al*., 1993; van der Zee *et al*., 1997; He *et al*., 1999). NO is involved in the increase in plasma extravasation of the hamster cheek pouch in response to numerous mediators such as bradykinin, substance P, PAF, ADP or histamine (Mayhan *et al*., 1992; 1994; Ramirez *et al*., 1995; Félétou *et al*., 1996). The present study is in agreement with other works showing that VEGF produces a NO-dependent increase in permeability (Wu *et al*., 1996; Murohara *et al*., 1998; Tilton *et al*., 1999). However, in the hamster cheek pouch, in contrast to the changes in plasma extravasation produced by bradykinin, VEGF-induced vascular leakage is also dependent on cyclo-oxygenase activation (Félétou *et al*., 1996). VEGF stimulates prostacyclin production in isolated endothelial cells (Wheeler Jones *et al*., 1997) and in mice the increase in permeability produced by intradermal injection of the growth factor is also sensitive to either NO-synthase or cyclo-oxygenase inhibition (Murohara *et al*., 1998). In the murine model, the changes in permeability have been linked to the induction of the iNO-synthase and COX2 (Fujii *et al*., 1997). In the hamster cheek pouch, the formation of vascular leaks is more likely to be attributed to the stimulation of the constitutive enzymes as the maximum changes in plasma extravasation are observed within 10 to 15 min of VEGF application. The VEGF-stimulated NO-dependent vasodilatation is rapidly tachyphylactic (Lopez *et al*., 1997), while the increase in hydraulic conductivity produced by VEGF in isolated perfused microvessels is not (Bates & Curry, 1997). In the present study, the formation of vascular leakage sites was also tachyphylactic but the development of diffuse plasma extravasation along the venules and arterioles was observed during prolonged application of VEGF, though this latter phenomenon was not studied in detail in the present work. Nevertheless, these observations are consistent with the fact that VEGF can induce both short-term and long-term effects. For example, this growth factor evokes an acute increase in NO production during acute exposure and genomic changes with an increase in NOS expression during prolonged exposure (Papapetropoulos *et al*., 1997; Feng *et al*., 1999a,b). The reason why VEGF-induced changes in plasma extravasation required both the activity of cyclo-oxygenase and NO-synthase while bradykinin for instance required only the latter is not known at present. In the murine model, dermal injection of a NO donor and of a prostacyclin analogue produced an increase in permeability when applied together but not when administered individually (Murohara *et al*., 1998). Whether or not the inhibition of VEGF-induced increase in plasma extravasation by the NO-synthase and cyclo-oxygenase inhibitors is linked to alteration in blood flow is not know at present. L-nitro-arginine vasconstricts the hamster cheek pouch, a phenomenon that contributes to its inhibitory effect against bradykinin-induced plasma extravasation (Félétou *et al*., 1996). However, indomethacin does not affect the diameter of the blood vessels in the hamster cheek pouch (Suzuki *et al*., 1996).

In the present study, VEGF- but not bradykinin-induced changes in plasma extravasation were inhibited in a concentration-dependent manner by a non-selective heparin-binding growth factor antagonist, TBC 1635, at concentrations that are consistent with the published value of IC_{50} (Stephan *et al*., 1998). Furthermore, HOE 140, the bradykinin B_2 selective receptor antagonist, did not inhibit VEGF-induced vascular leakage and there was no evidence for cross-desensitization between VEGF and bradykinin, suggesting that VEGF-induced vascular leakage was mediated by a specific receptor. $VEGF_{165}$ is a ligand of VEGF-R1, VEGF-R2, and neuropilin1 and 2 (Neufeld *et al*., 1999). In the

Table 1 Inhibitors of VEGF signal transduction and the maximum increase in leakage sites in the hamster cheek pouch

		VEGF (2.4 nM)		*Bradykinin (300 nM)*	
	n	*Number of leaks*	*Inhibition (%)*	*Number of leaks*	*Inhibition (%)*
DMSO (0.2%)	11	123±8		162±9	
Genistein: 5 μM	6	82±10*	−33	183±13	+13
Genistein: 10 μM	6	92±13*	−25	161±6	−0.01
DMSO (0.3%)	11	125±17		173±8	
LY 294002: 10 μM	4	69±26	−45	175±23	+1
LY 294002: 30 μM	7	74±10*	−41	165±13	−5
DMSO (0.2%)	5	80±10		135±11	
Bisindolylmaleimide: 1 μM	5	28±8*	−65	146±14	+8
Indomethacin: 3 μM	5	10±2*	−88	152±16	+13
Saline	3	83±7		ND	
L-NA:	4	4±2*	−95	ND	

Data are shown as means±s.e.mean and *n* indicates the number of hamsters studied. *Indicates a statistically significant difference between a treated group and its respective contemporary control ($P<0.05$). ND: not determined in the present study.

present study, genistein, a tyrosine kinase inhibitor, at a concentration that has been shown to be effective in this model (Kim & Duran, 1995), partially inhibited the response to VEGF, confirming earlier studies (Fujii *et al*., 1997; Murohara *et al*., 1998; Cohen *et al*., 1999). However, the inhibition observed represented only 30% of the total response, suggesting either that the experimental conditions used to study genistein were not optimal (concentration, duration of the administration) or that another pathway besides tyrosine kinase is involved in the transduction of the VEGF signal. However, this inhibition was apparently selective as the increase in plasma extravasation produced by bradykinin was not affected. The tyrosine kinase activation suggests that either VEGF-R1 or -R2 mediates the response to VEGF since the involvement of VEGF-R3 in the changes in microvascular permeability has been definitively ruled out by other groups (Joukov *et al*., 1998; Wise *et al*., 1999; Ogawa *et al*., 1998). PlGF-1, a specific agonist of VEGF-R1 (Park *et al*., 1994; Sawano *et al*., 1996; Neufeld *et al*., 1999), did not produce any significant changes in plasma extravasation, indicating that VEGF-R1 is not involved in the VEGF effects (Sawano *et al*., 1996; Muruhora *et al*., 1998). Therefore, the present study confirms previous works strongly suggesting that VEGF-R2 is involved in the changes in permeability produced by VEGF (Murohara *et al*., 1998). This hypothesis is substantiated by the observation that a mutant of VEGF-C that lacks binding affinity towards VEGF-R2 lost its ability to induce vascular permeability (Joukov *et al*., 1998) and by the fact that monoclonal antibodies toward VEGF-R2 inhibit the VEGF-induced hyperpermeability in isolated venules (Wu *et al*., 1999). Additional indirect evidence supports the implication of VEGF-R2. The increase in endothelial intracellular calcium (Cunningham *et al*., 1999), a process required for an increase in permeability (Bates & Curry 1997), as well as the activation of endothelial NO-synthase and cyclo-oxygenase depend on VEGF-R2 stimulation (Wheeler-Jones *et al*., 1997; Feng *et al*, 1999a,b; He *et al*., 1999). However, Stacker *et al*. (1999) have shown than a mutant form of VEGF that lacks VEGF-R2 activation retains the ability to induce vascular permeability suggesting that VEGF-R2 is not the only explanation. Two possible hypotheses could be evoked to explain these discrepancies. A yet unknown receptor with a high affinity for VEGF could be responsible for the changes in plasma extravasation. Alternatively, a heterodimeric complex including VEGF-R2 and another receptor which provides the high affinity interaction with VEGF, possibly neuropilin 1 or 2, is involved (Stacker *et al*., 1999; Neufeld *et al*., 1999). These two hypotheses are not inconsistent with our data and could explain the partial inhibition observed even with a high concentration of genistein and also the inhibitory effect of PlGF-1 on VEGF-induced changes in plasma extravasation. High concentrations of PlGF-1 can inhibit the binding of PlGF-2 and $VEGF_{165}$ to neuropilin-1 without affecting the binding of the latter to VEGF-R2 (Migdal *et al*., 1998). Nevertheless, the inhibitory effect of PlGF on bradykinin-induced changes in plasma extravasation, which remains unexplained at present, suggests that PlGF could produce unspecific inhibitory effects.

In the present study, the changes in plasma extravasation produced by VEGF, but not those produced by bradykinin, were inhibited by LY 294002 and bisindolylmaleimide, inhibitors of phosphatidylinositol-3-kinase and protein kinase C, respectively. These observations are in agreement with the signal transduction pathways for VEGF-R2 (Xia *et al*., 1996; Thakker *et al*., 1999; Takahashi & Shibuya, 1997; Gerber *et al*., 1998). Inhibitors of protein kinase C and especially protein kinase C β-isoform inhibit the changes in permeability produced by VEGF *in vivo* and *in vitro* (Aiello *et al*., 1997; Wu *et al*., 1999) and inhibitors of phosphatidylinositol-3-kinase inhibit VEGF-induced NO release in endothelial cells (Papapetropoulos *et al*., 1997). The hamster cheek pouch is therefore an appropriate model to improve our understanding of the mechanisms of VEGF-induced changes in permeability and to study new drugs that may antagonize its action at various levels of the signalling pathway.

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