The protein kinase MEK1/2 mediate vascular endothelial growth factor- and histamine-induced hyperpermeability in porcine coronary venules

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Mitogen-activated protein kinases (MAPKs) have been implicated in the signal transduction of the endothelial response to growth factors and inflammatory stimuli. The objective of this study was to test the hypothesis that the p42/44 MAPK pathway plays a common role in mediating the microvascular hyperpermeability response to vascular endothelial growth factor (VEGF) and histamine. The apparent permeability coefficient of albumin was measured in isolated and perfused coronary venules. Application of VEGF induced a rapid increase in venular permeability, and the effect was blocked by PD98059 and UO126, selective inhibitors of the mitogen-activated protein kinase kinase MEK1/2, in a dose-dependent pattern. The same MEK1/2 inhibitors dose-dependently attenuated the increase in venular permeability caused by histamine. In addition, the increases in venular permeability caused by agents that are known to activate the nitric oxide pathway, including the calcium ionophore ionomycin, the nitric oxide donor *S*-nitroso-*N*-acetylpenicillamine, and the protein kinase G activator 8-bromo-cGMP, were significantly attenuated in venules pretreated with the MEK1/2 inhibitors. Furthermore, transfection of venules with active MEK1 increased baseline permeability. In contrast, transfection of active ERK1, a downstream target of MEK1/2, did not significantly alter the basal permeability of venules. Moreover, inhibition of ERK1/2 with a specific inhibiting peptide did not prevent the hyperpermeability response to VEGF or histamine. The results suggest that activation of MEK1/2 may play a central role in the signal transduction of microvascular hyperpermeability in response to growth factors and inflammatory mediators.

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The mitogen-activated protein kinase (MAPK) family consists of a network of signalling cascades that mediate diverse cellular responses to growth factors, physical and chemical stress, and inflammatory cytokines (Pearson *et al.* 2001; Kyriakis & Avruch, 2001). In mammalian cells, extracellular signal-related kinases (p42/44 MAPK or ERK1/2), c-jun N-terminal kinases (JNK), and p38 MAPK are terminal kinases of three different MAPK cascades that have been well characterized (Chang & Karin, 2001). It is known that the p42/44 MAP kinase kinase MEK1/2 and its downstream target ERK1/2 can be activated by mitogens and morphogens, thereby controlling cell proliferation and differentiation, respectively (Pearson *et al.* 2001). Within this context, the importance of ERK1/2 in the signal transduction of angiogenesis induced by vascular endothelial growth factor (VEGF) has been established (Berra *et al.* 2000; Zachary & Gliki, 2001). Recent evidence indicates that certain inflammatory mediators, including histamine, thrombin, and intracellular calcium-elevating agents, are able to activate the MEK–ERK cascade in cultured endothelial cells (Wheeler-Jones & Pearson, 1995; Fleming *et al.* 1995; Katoch & Moreland, 1995; Gorenne *et al.* 1998; Koch *et al.* 2000; Robinson & Dickenson, 2001; Bates *et al.* 2001). While the precise molecular mechanisms underlying these reactions remain elusive, the fact that hyperpermeability is a common form of the venular response to both VEGF and histamine raises the possibility that the activation of MAPK cascades is involved in the regulation of endothelial barrier function in postcapillary venules. Another important consideration is the demonstration in cultured endothelial monolayers that ERK1/2 activation may serve as a mechanism to increase barrier permeability (Verin *et al.* 2000; Breslin *et al.* 2003). The involvement of ERK1/2 and p38 MAPK in

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the regulation of basal and VEGF-stimulated permeability in endothelial monolayers has also been documented (Lal *et al.* 2001; Varma *et al.* 2002).

The endothelial lining of microvessels provides a semi-permeable barrier to the transvascular flux of plasma fluid and proteins. The permeability of the microvascular endothelium can be increased by an array of mediators, including VEGF and histamine, resulting in microvascular leakage and tissue oedema (Lum & Malik, 1994; Ferrara & Davis-Smyth, 1997; Dvorak *et al.* 1999; Yuan, 2000). This process has been implicated in angiogenesis, ischaemic heart disease, inflammation, trauma, sepsis, and many other pathological conditions. Tremendous effort has been devoted to identifying key signalling molecules that are responsible for the hyperpermeability reaction. Our previous investigations (Yuan *et al.* 1993*b*; Wu *et al.* 1996, 1999) suggest that VEGF- and histamine-induced microvascular hyperpermeability are both mediated by a signalling cascade triggered by receptor binding and transduced by a serial activation of intracellular enzymes, including phospholipase C (PLC), endothelial nitric oxide synthase (eNOS), soluble guanylate cyclase (sGC), and protein kinase G (PKG). Subsequently, the VEGF-activated NO–PKG pathway was linked to ERK1/2-mediated proliferation of cultured endothelial cells via phosphorylation and activation of the upstream p42/44 MAPK cascade component RAF by PKG (Hood & Granger, 1998). However, whether the same mechanism is also involved in regulating endothelial barrier function in postcapillary venules from porcine heart has yet to be determined. Therefore, the purpose of this study was to examine the potential contribution of the p42/44 MAPK cascade to microvascular hyperpermeability in response to VEGF and histamine. The results suggest that MEK1/2 activation serves as a common signal downstream of the NO–PKG cascade in mediating coronary venular hyperpermeability elicited by VEGF and histamine.

Methods

Materials

An albumin-physiological salt solution (APSS) was used as a bathing solution while the microvessels were being dissected. It had the following composition (mM): NaCl 145.0, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and 3-*N*-morpholinopropanesulphonic acid buffer 3.0. After adding 1% bovine serum albumin, the solution was buffered to a pH of 7.40 at 4°C and then filtered through a Millex-PF 0.22 μm filter unit (Millipore, Bedford, MA, USA). The APSS used to perfuse the vessels during permeability measurements had the same composition as mentioned above, but was buffered to a pH of 7.40 at 37°C. The chemicals used to make the perfusate, including fluorescein isothiocyanate (FITC)-albumin, were purchased from Sigma (St Louis, MO, USA). Bovine serum albumin was obtained from United States Biochemical (Cleveland, OH, USA). Cell culture supplies including the culture media Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Gibco (Gaithersburg, MD, USA). VEGF was from R & D Systems (Minneapolis, MN, USA) and histamine was from Sigma. The calcium ionophore ionomycin, the nitric oxide donor *S*-nitroso-*N*-acetylpenicillamine (SNAP), the protein kinase G activator 8-bromo-cGMP, and the selective MEK1/2 inhibitors PD98059 and UO126 were purchased from Calbiochem (San Diego, CA, USA). The cell-permeable ERK1/2-inhibiting peptide stearyl-MPKKPTPIQLNP was ordered from SynPep (Dublin, CA, USA). Human recombinant active MEK1 and ERK1 were acquired from Upstate (Lake Placid, NY, USA) and Calbiochem, respectively.

Animal preparation

Yorkshire pigs weighing 9–13 kg were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, I.V.) and heparinized (250 units kg^{-1}, I.V.). Following tracheotomy and intubation, animals were ventilated with room air. A left thoracotomy was performed and the heart was electrically fibrillated, excised, and placed in 4°C physiological saline. The pigs were killed by the surgical removal of the heart. The animals were housed and handled in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Texas A & M University, and Scott and White Hospital.

Isolation and perfusion of coronary venules

The coronary sinus was cannulated, and India ink–gelatin–physiological salt solution was infused to clearly define venular microvessels. This solution was prepared by adding 0.2 ml of India ink (Koh-I-Noor, Bloomsbury, NJ, USA) and 0.35 g of porcine skin gelatin to 10 ml of warm physiological salt solution and filtered through P8 filter paper (Fisher Scientific, Pittsburgh, PA, USA). Information regarding the validation and limitation of the ink-perfusion procedure has been provided in our previous publication (Yuan *et al.* 1993*a*). The method for isolating and cannulating coronary venules has also been described in detail in that study. Briefly, a suitable venule (length 0.8–1.2 mm, diameter 20–60 μm) was dissected from the surrounding myocardium in a dissecting chamber containing APSS at 4°C with the aid of a Zeiss stereo dissecting microscope. The vessel was transferred to a cannulating chamber which was mounted on a Zeiss axiovert microscope. The isolated vessel was cannulated with an inflow and

outflow micropipette on each end and secured with 11-0 suture (Alcon, Fort Worth, TX, USA). A third smaller pipette was inserted into the inflow pipette. The vessel was perfused with either APSS through the outer inflow pipette or APSS containing FITC-albumin through the inner inflow pipette. Each cannulating micropipette was connected to a reservoir and the vessel was perfused at a relatively constant intraluminal pressure and flow rate. The bath solution in the chamber was maintained at 37°C and pH 7.4 throughout the experiments. The image of the vessel was projected onto a Hamamatsu charged coupled device-intensified camera and was displayed on a high resolution monochromatic video monitor and recorded onto a VHS video recorder. The diameter of the vessel was measured on-line with a video caliper (Cardiovascular Research Institute, College Station, TX, USA).

Measurement of venular permeability

The permeability of the vessel was measured with a fluorescence ratioing technique (Huxley *et al.* 1987; Yuan *et al.* 1993*a*). Using an optical window of a video photometer positioned over the venules and adjacent space on the monitor, the fluorescent intensity from the window was measured and digitized on-line. In each measurement, the isolated venule was first perfused with APSS through the outer inflow pipette to establish a baseline intensity. The venular lumen was then rapidly filled with APSS containing FITC-albumin by switching the perfusion to the inner inflow pipette. This produced an initial step increase, followed by a gradual increase, in fluorescence intensity. There was a step decrease of intensity when the fluorescently labelled molecules were washed out of the vessel lumen by switching the perfusion back to the outer inflow pipette. The apparent solute permeability coefficient of albumin (P_a) was calculated using the equation $P_a = (1/\Delta I_f)(dI_f/dt)_o(r/2)$, where ΔI_f is the initial step increase in fluorescence intensity, $(dI_f/dt)_o$ is the initial rate of gradual increase in intensity as the fluorescently labelled solutes diffuse out of the vessel into the extravascular space, and r is the venular radius.

In each experiment, the cannulated venule was perfused at a constant perfusion pressure of 20 cmH_2O. The preparation was equilibrated for 45–60 min after cannulation and the measurements were conducted at 36–37°C and a pH of 7.35–7.45. A limited number (< 3) of interventions were applied to each vessel. Between interventions the preparation was washed three times and allowed to equilibrate for 10–15 min. In some vessels, the permeability was monitored over 6 h to ensure that the barrier property of the venules was not significantly altered with time.

VEGF (10^{-10} M) or histamine (10^{-5} M) was topically added into the suffusion bath and the permeability of the vessels was measured before and after exposure to the mediators up to 15 min. To study the role of MEK in hyperpermeability responses, venules were preincubated for 30 min with selective MEK inhibitors PD98059 (10^{-7}–10^{-5} M) or UO126 (10^{-7}–10^{-4} M) (Davies *et al.* 2000). The P_a values were then determined before and after adding VEGF or histamine for 15 min in the presence of the inhibitor. To further verify that the p42/44 MAPK signal is located downstream of the NO pathway, the same experiment was repeated in separate vessels that were treated with the MEK inhibitors before stimulation by the NO cascade activators ionomycin (10^{-5} M), SNAP (10^{-5} M), and 8-bromo-cGMP (10^{-5} M).

In order to specify the effect of the MEK downstream target, ERK1/2, we measured the permeability response to VEGF and histamine in venules that were treated with a specific ERK1/2-inhibiting peptide (50 μM) (Kelemen *et al.* 2002). Furthermore, we tested the direct effects of MEK and ERK on venular permeability by transfecting active MEK1 or active ERK1 into venular endothelium. The MEK1 protein is a recombinant full-length human MEK1 expressed in *E. coli* that is activated by *in vitro* phosporylation with active RAF1 (Alessi *et al.* 1995). The ERK1 protein is a recombinant human MAPK expressed in *E. coli* activated by phosphorylation with a constitutively active MEK1 bound to glutathione agarose beads (Charest *et al.* 1993). The technique of transfecting proteins into intact microvascular endothelium has been described and evaluated in detail in our previous publications (Tinsley *et al.* 1998, 2001). Venules were perfused for 1 h with a transfection mixture containing the polyamine reagent TransIT (Pan Vera) at 10 μl ml^{-1} and active MEK1 (0.1 U ml^{-1}) or ERK1 (5 μg ml^{-1}). After transfection, the vessels were washed with the regular perfusate and then subjected to chemical stimulation as described above.

Data analysis

In the intact vessel studies, P_a was measured two to three times in each venule at each experimental intervention and the values were averaged. For each experimental condition, the values of P_a from different vessels were averaged, normalized to the basal values obtained before drug treatments, and reported as a percentage of the basal value as mean ± S.E.M. For all experiments n is given as the number of vessels studied, with each vessel representing a separate animal. Analysis of variance was used to evaluate the significance of intergroup differences. A value of $P < 0.05$ was considered significant for the comparisons.

Results

VEGF induced a significant increase in venular permeability (Fig. 1). In agreement with our previous observations (Wu *et al.* 1996, 1999), the hyperpermeability response was rapid, peaking at 3–5 min and recovering within 15 min. At 10^{-10} M VEGF, P_a

rose to a peak value of $232 \pm 17\%$ of the basal permeability (P_a from $(2.45 \pm 0.20) \times 10^{-6}$ cm s^{-1} to $(5.69 \pm 0.62) \times 10^{-6}$ cm s^{-1}, $n = 8$). The MEK inhibitors did not affect the basal barrier property of the venules, but blocked VEGF-induced hyperpermeability in a dose-dependent fashion. Neither the time course to peak response nor the duration of recovery was significantly altered by the inhibitors. As shown in Fig. 1, the peak P_a values after treatment with VEGF (10^{-10} M) were reduced to $189 \pm 12\%$ of basal in the presence of PD98059 at 10^{-7} M. The peak P_a value was further reduced to $164 \pm 26\%$ at 10^{-6} M and $120 \pm 9\%$ at 10^{-5} M, respectively. Similarly, UO126-treated venules decreased VEGF-stimulated P_a to $162 \pm 5\%$ at 10^{-7} M, $148 \pm 6\%$ at 10^{-6} M, $125 \pm 3\%$ at 10^{-5} M, and $120 \pm 15\%$ at 10^{-4} M, respectively. The results indicated that the activation of MEK was involved in the mechanism of VEGF-induced hyperpermeability.

Treatment of the venules with histamine rapidly increased venular permeability by $268 \pm 34\%$ over the basal value ($P_a = (2.55 \pm 0.11) \times 10^{-6}$ cm s^{-1} before histamine, and $P_a = (6.85 \pm 0.96) \times 10^{-6}$ cm s^{-1} after histamine, $n = 4$). Similar to the VEGF response, the histamine response peaked at 3–5 min and dissipated after 15–20 min. The hyperpermeability was greatly attenuated during inhibition of MEK without a significant change in the duration or time of peak response (Fig. 2). Specifically, PD98059 reduced the peak P_a upon histamine to $237 \pm 11\%$ over the basal value at 10^{-7} M, $180 \pm 17\%$ at 10^{-6} M and $133 \pm 15\%$ at 10^{-5} M, respectively. In the group of vessels pretreated with UO126, the permeability responses to histamine were attenuated to $171 \pm 13\%$ at 10^{-7} M, $146 \pm 15\%$ at 10^{-6} M, $131 \pm 12\%$ at 10^{-5} M and $128 \pm 7\%$ at 10^{-4} M, respectively.

Previous studies indicated that the p42/44 MAPK cascade is downstream of the NO–PKG pathway (Hood & Granger, 1998), which has been shown to mediate VEGF- and histamine-elicited hyperpermeability responses in venules. In control venules, the calcium ionophore ionomycin, the exogenous NO donor SNAP, and the PKG activator 8-bromo-cGMP produced a hyperpermeability effect with a time course and peak response comparable to those of histamine. The MEK inhibitors PD98059 and UO126 displayed a dose-related inhibition of the peak response to all of these agonists without affecting the time course of hyperpermeability (Figs 3–5). In Fig. 3, ionomycin (10^{-5} M) increased P_a to $234 \pm 33\%$ from its basal value in the absence of the inhibitors (P_a was from $(2.38 \pm 0.31) \times 10^{-6}$ cm s^{-1} to

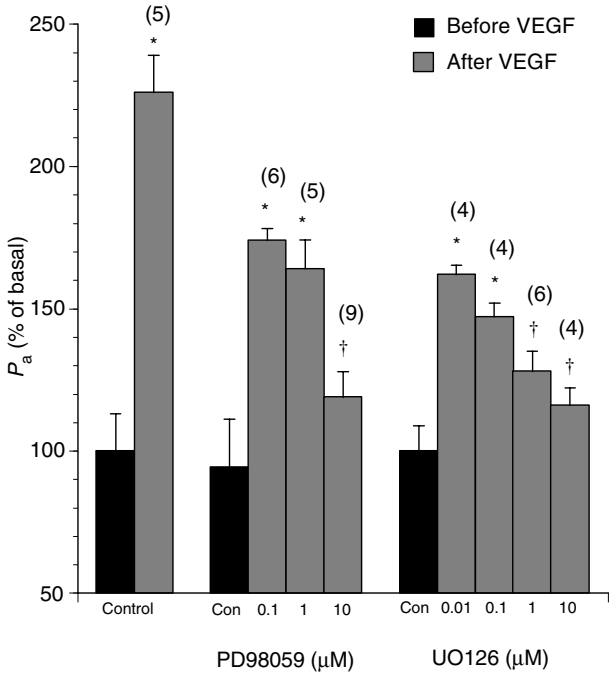

Figure 1. Changes in the apparent permeability coefficient of albumin upon treatment with vascular endothelial growth factor (VEGF; 10^{-10} M) in venules pretreated with the MEK1/2 inhibitors PD98059 or UO126

Controls show the effects of vehicle (0.1% DMSO) alone. Numbers in parentheses represent the numbers of vessels studied. * Significant difference ($P < 0.05$) *versus* the basal P_a value in untreated venules. † Significant difference ($P < 0.05$) *versus* the P_a value after VEGF without PD98059 or UO126 treatment.

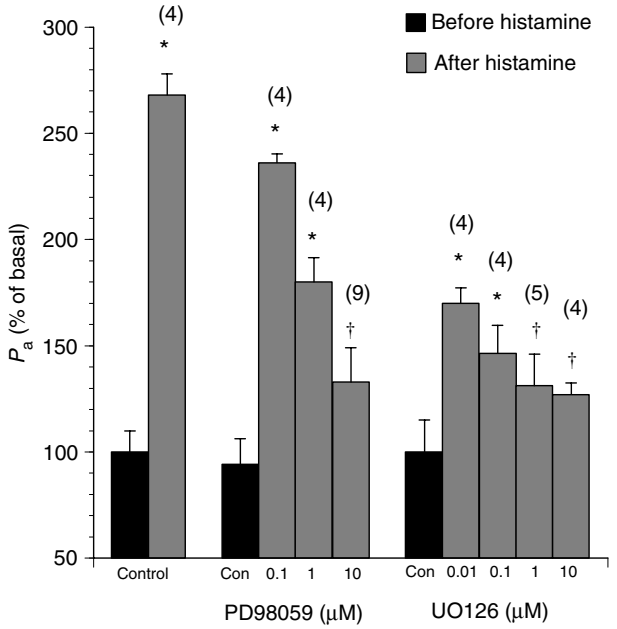

Figure 2. Changes in the apparent permeability coefficient of albumin (P_a) upon treatment with histamine (10^{-5} M) in venules pretreated with the MEK1/2 inhibitors PD98059 or UO126

Controls show the effects of vehicle (0.1% DMSO) alone. Numbers in parentheses represent the numbers of vessels studied. * Significant difference ($P < 0.05$) *versus* the basal P_a value in untreated venules. † Significant difference ($P < 0.05$) *versus* the P_a value after histamine without PD98059 or UO126 treatment.

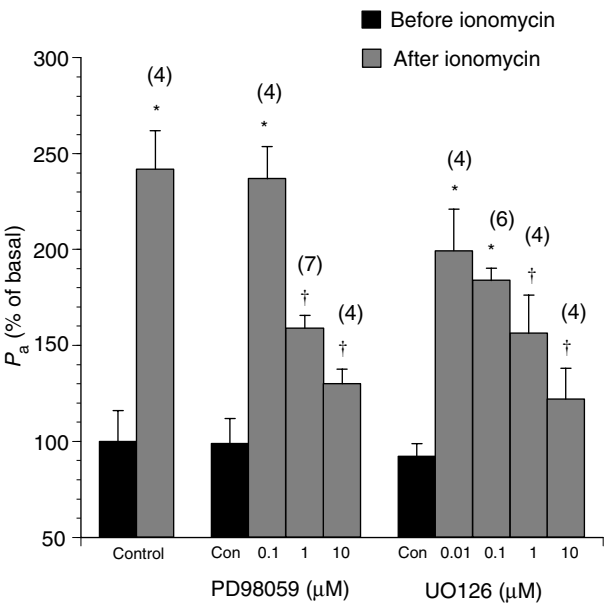

Figure 3. Changes in the apparent permeability coefficient of albumin upon treatment with the calcium ionophore ionomycin (10^{-5} M) in venules pretreated with the MEK1/2 inhibitors PD98059 or UO126

Controls show the effects of vehicle (0.1% DMSO) alone. Numbers in parentheses represent the numbers of vessels studied. * Significant difference ($P < 0.05$) *versus* the basal P_a value in untreated vessels. † Significant difference ($P < 0.05$) *versus* the P_a value after ionomycin without PD98059 or UO126 treatment.

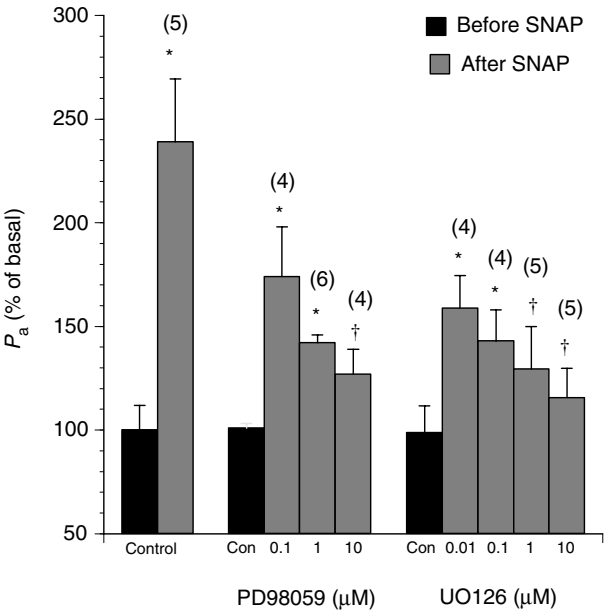

Figure 4. Changes in the apparent permeability coefficient of albumin upon treatment with the exogenous NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-5} M) in venules pretreated with the MEK1/2 inhibitors PD98059 or UO126

Controls show the effects of vehicle (0.1% DMSO) alone. Numbers in parentheses represent the numbers of vessels studied. * Significant difference ($P < 0.05$) *versus* the basal P_a value in untreated venules. † Significant difference ($P < 0.05$) *versus* the P_a value after SNAP without PD98059 or UO126 treatment.

(5.27 ± 0.38) $\times 10^{-6}$ cm s^{-1}, $n = 4$). In the PD98059-treated group, the increases were reduced to $184 \pm 32\%$ at 10^{-7} M, $177 \pm 13\%$ at 10^{-6} M and $128 \pm 18\%$ at 10^{-5} M, respectively. Similarly, ionomycin-induced increases in P_a were attenuated, in the presence of UO126, to $199 \pm 22\%$ at 10^{-7} M, $185 \pm 6\%$ at 10^{-6} M, $156 \pm 20\%$ at 10^{-5} M and $122 \pm 17\%$ at 10^{-4} M, respectively.

The NO donor SNAP increased the basal permeability to $240 \pm 31\%$ (Fig. 4). The effect of SNAP on P_a was significantly attenuated to $127 \pm 12\%$ in the presence of PD98059 (10^{-5} M) and to $140 \pm 21\%$ in the presence of UO126 (10^{-5} M). In addition, the increase in P_a induced by 8-bromo-cGMP was attenuated during MEK inhibition (Fig. 5). In control venules, 8-bromo-cGMP elevated P_a to $231 \pm 39\%$ of the basal value. In contrast, the P_a value in response to 8-bromo-cGMP was $137 \pm 15\%$ in PD98059 (10^{-5} M)-treated venules. In UO126 (10^{-5} M)-treated venules, P_a was $147 \pm 17\%$ of basal after 8-bromo-cGMP.

Pretreatment of venules with an ERK1/2-specific inhibiting peptide (50 μM ERKi) did not alter the basal permeability, nor did it prevent VEGF- and histamine-induced hyperpermeability (Fig. 6*A*). However, transfection of venules with active MEK1 (0.1 U ml^{-1}, which equals an enzymatic activity of 0.3 nmol phosphate (mg myelin basic protein)$^{-1}$ min^{-1})

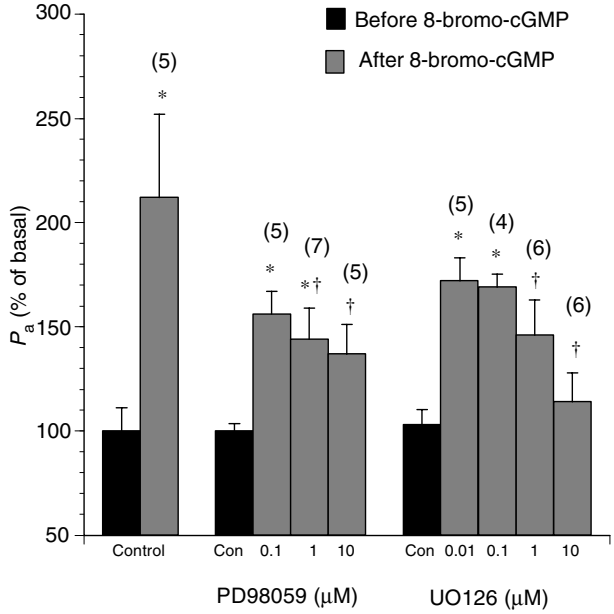

Figure 5. Changes in the apparent permeability coefficient of albumin upon treatment with the protein kinase G (PKG) activator 8-bromo-cGMP (10^{-5} M) in venules pretreated with the MEK1/2 inhibitors PD98059 or UO126

Controls show the effects of vehicle (0.1% DMSO) alone. Numbers in parentheses represent the numbers of vessels studied. * Significant difference ($P < 0.05$) *versus* the basal P_a value in untreated venules. † Significant difference ($P < 0.05$) *versus* the P_a value after cGMP without PD98059 or UO126 treatment.

significantly increased P_a by 161 ± 22% (Fig. 6*B*). In contrast, transfection with active ERK1 (5 μg ml^{-1}, an enzymatic activity of 0.5 nmol phosphate (mg myelin basic protein)$^{-1}$ min^{-1}) did not elevate the permeability (Fig. 6*B*). The transfection procedure *per se* did not alter the endothelial barrier function as indicated by an unchanged basal permeability.

Discussion

Previous studies on isolated, perfused postcapillary venules from the porcine heart support the convergence of histamine (Yuan *et al.* 1993*b*; Huang & Yuan, 1997) and VEGF (Wu *et al.* 1996, 1999) signalling on the PLC–eNOS–sGC–PKG cascade. As illustrated in Fig. 7, histamine activates the β1 isoform of PLC through the association of the ligated H2 receptor with the G protein Gq. VEGF binding to the flk-1/KDR receptor tyrosine kinase results in phosphorylation and activation of PLCγ1. Both PLC isoforms catalyse the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylgyclerol (DAG) from phosphatidylinositol 4,5-bisphosphate molecules imbedded in the inner leaflet of the plasma membrane. DAG activates protein kinase C, and IP_3 stimulates the release of calcium from internal stores. The subsequent rise in cytosolic calcium activates eNOS, and facilitates the stimulation of PKC by DAG. Activated PKC phosphorylates eNOS and further stimulates NO production by eNOS. In turn, NO turns on soluble guanylate cyclase, thereby increasing the production of the second messenger cyclic GMP. The cyclic nucleotide binds to protein kinase G, resulting in stimulation of catalytic activity. The operation of this common signalling cascade in porcine coronary venules has been established through the use of inhibitors of PLC, eNOS, sGC and the binding and catalytic domains of PKG. Moreover, exposure of the venules to ionomycin, NO donors and cell-permeable cGMP analogues leads to hyperpermeability responses that are blocked by inhibitors that bind to catalytic sites on PKG. Finally, hyperpermeability due to increased shear rate may also converge on this signalling cascade since inhibition of NO production blocks the response in coronary venules (Yuan *et al.* 1992).

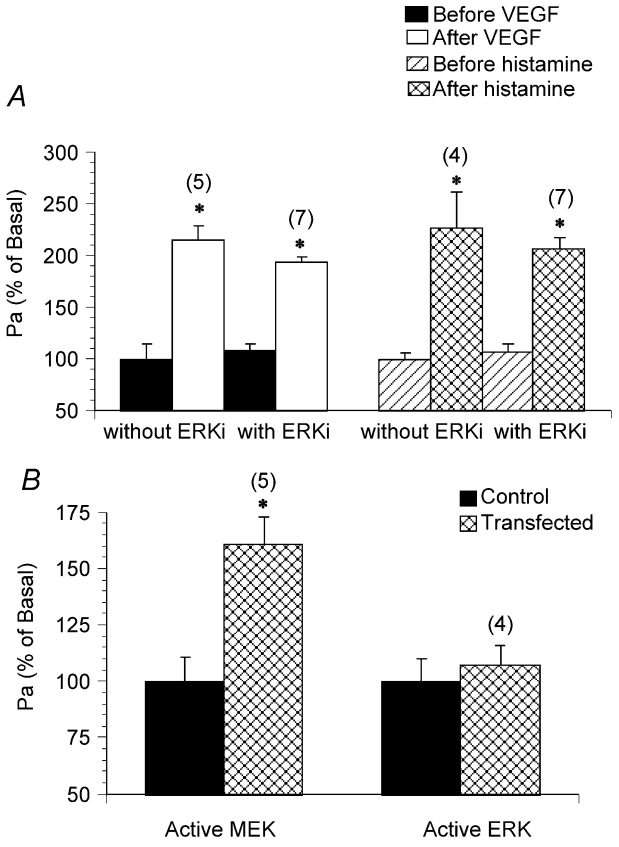

Figure 6. Changes of venular permeability after inhibition of ERK1/2 and transfections of active MEK and ERK
Changes in the apparent permeability coefficient of albumin upon treatment with VEGF (10^{-10} M) and histamine (10^{-5} M) in venules pretreated with an ERK1/2-inhibiting peptide (ERKi, 50 μM) (*A*). *B*, effects of transfection of active MEK1 (0.1 U ml^{-1}) and active ERK1 (5 μg ml^{-1}) on venular permeability. Controls show the effects of vehicle (the transfection reagent TransIT) alone. Numbers in parentheses represent the numbers of vessels studied. * Significant difference ($P < 0.05$) *versus* the basal P_a value in untreated venules.

The present study represents the first attempt to map the hyperpermeability signalling pathway in coronary venules beyond the PLC–eNOS–sGC–PKG cascade. Because previous work from this laboratory demonstrated that PKG phosphorylates and activates RAF1 in cultured endothelial cells (Hood & Granger, 1998), we hypothesize that the hyperpermeability signals are transmitted from PKG and other kinases to the RAF–MEK cascade. Figure 7 shows our hypothetical model of permeability signalling in postcapillary venules. Multiple pathways are proposed, based on our studies with intact isolated venules (Yuan *et al.* 1993*b*; Wu *et al.* 1996, 1999; Huang & Yuan, 1997; Yuan, 2000) and results from other laboratories using intravital microscopy (Bates & Harper, 2003; Aramoto *et al.* 2004), as well as *in vitro* experiments with cultured endothelial cell monolayers (Lum *et al.* 1999; Lal *et al.* 2001; Varma *et al.* 2002). Additional support for this hypothesis in the literature includes: (1) histamine (Huang & Yuan, 1997; Robinson & Dickenson, 2001) and VEGF (Hood & Granger, 1998; He *et al.* 1999; Takahashi *et al.* 1999; Lal *et al.* 2001; Varma *et al.* 2002) activate PKG, PKC and/or Src, three known stimulators of RAF1 kinase activity and the p42/44 and p38 MAPK cascades in cultured endothelial cells; (2) other hyperpermeability stimuli such as shear stress (Jo *et al.* 1997; Traub *et al.* 1997), hyperosmolarity (Duzgun *et al.* 2000), and hydrogen peroxide (Kevil *et al.* 2000) elicit transient phosphorylation of ERK; (3) MEK and ERK phosphorylation transients mirror coronary venular hyperpermeability responses, peaking at 3–5 min and dissipating within 15–30 min

(Parenti *et al.* 1998; Takahashi *et al.* 1999; Breslin *et al.* 2003); and (4) activation of PKA, a known inhibitor of RAF1 kinase activity, by cAMP in cultured endothelial cells leads to a reduction in both p42/44 MAPK cascade activity and monolayer permeability (Lum *et al.* 1999; Liu *et al.* 2001).

At the micromolar concentrations used in this study, PD98059 and UO126 exert potent inhibitory effects on MEK1 and MEK2 by preventing association with their activating partner RAF1; however, they do not affect any other protein kinase (Davies *et al.* 2000). Furthermore, at the concentrations used in this study, neither of these inhibitors act on MEKs associated with JNK or p38 kinase, the two other major MAP kinases. The use of two different types of inhibitor for MEK1/2 minimized the risk of studying non-specific effects. The current study demonstrates the importance of MEK1/2 activation as a common signal in the transduction of the venular hyperpermeability response to VEGF and histamine. Moreover, hyperpermeability responses elicited by ionomycin, SNAP and 8-bromo-cGMP, all putative activators of PKG, are also blocked by UO126 and PD98059. Furthermore, the upstream p42/44 MAPK component RAF1 is a major participant in the transmission of the hyperpermeability signal elicited by VEGF, histamine, NO and cyclic GMP. This view is supported by the ability of PD98059 and UO126, inhibitors of RAF1 binding to MEK1/2, to reduce the permeability responses to these mediators.

In addition to the pharmacological approach, we examined the effect of ERK1/2 as a potential downstream hyperpermeability target of MEK1/2 by treating venules with a specific ERK1/2-inhibiting peptide. The cell-permeable inhibitory peptide, stearyl-MPKKKPTPIQLNP, is derived from the amino terminus sequence of MEK1 and prevents ERK1 and ERK2 activation by interfering with MEK binding to ERK at the docking site. This inhibition of ERK activity is concentration dependent with an IC_{50} of 2.5 μM *in vitro* and 13 μM *in vivo* (Kelemen *et al.* 2002). The peptide fails to inhibit JNK and p38 protein kinases, even at concentrations of 100 μM *in vitro* (Kelemen *et al.* 2002). At a concentration equal to 4 times the *in vivo* IC_{50}, the specific ERK1/2-inhibiting peptide failed to alter baseline permeability and to diminish the hyperpermeability elicited by VEGF. This finding suggests that the hyperpermeability signal is not transmitted to the terminal component of the p42/44 MAPK cascade.

Although others have shown reduced hyperpermeability reactions in endothelial monolayers chronically transfected with ERK antisense oligonucleotides (Verin *et al.* 2000; Breslin *et al.* 2003), acute insertion of active ERK protein into coronary venules did not elevate baseline albumin transport above control. In contrast, baseline albumin transport was augmented after introducting active MEK1 into the

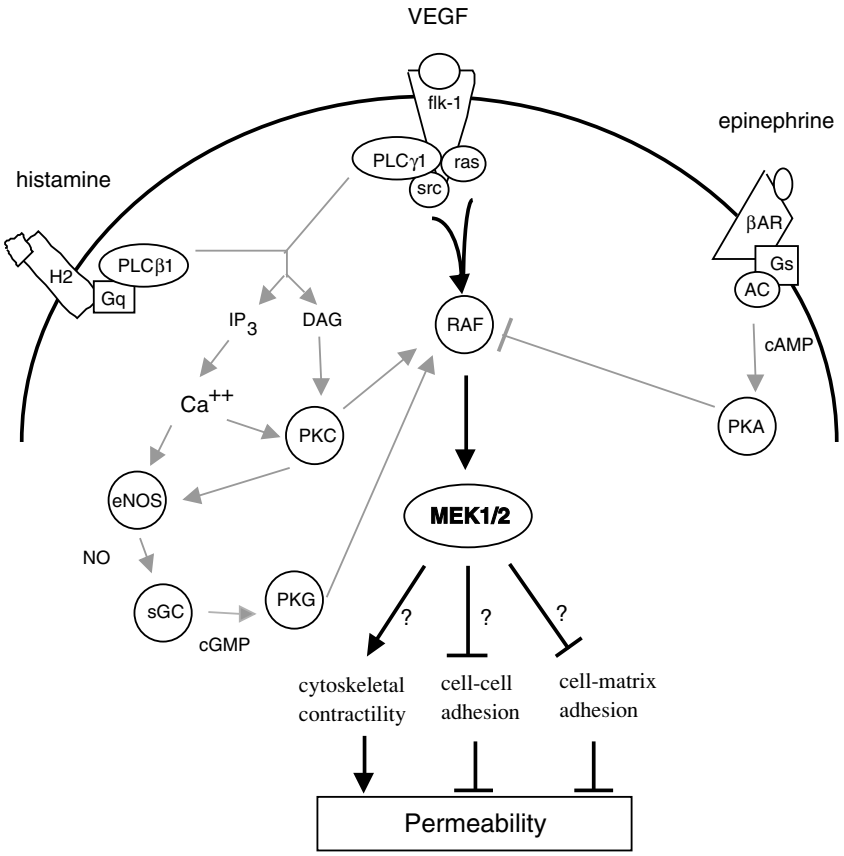

Figure 7. Hypothetical model of permeability signalling in postcapillary venules of porcine heart
In the centre, the MAPK cascade integrates positive (lines with filled arrow) and negative (lines with perpendicular at tip) influences at the level of RAF. Phosphorylation of RAF by src, PKC or PKG increases its kinase activity and leads to activation of MEK1/2. MEK1/2 may affect endothelial barrier function by altering the cytoskeletal arrangement or cell–cell and cell–matrix adhesions. Phosphorylation of RAF by PKA inhibits the activity of the MAPK cascade and results in reduced permeability. Not shown are phosphodiesterases that catalyse cGMP and cAMP degradation, and phosphatases that dephosphorylate RAF, MEK, ERK and other phosphoproteins. βAR, β-adrenergic receptor; Gs, stimulatory.

endothelial cells of isolated postcapillary venules. Taken together, these findings using active forms of MEK1 and ERK1 support the notion that the hyperpermeability signal associated with histamine and VEGF travels through RAF1 and MEK1/2 but does not extend to ERK1/2. In other words, the hyperpermeability signal under these specific circumstances exits the p42/44 MAP kinase cascade at MEK1/2 in coronary venules. By contrast the mitogenic signal has been shown by many others to continue through ERK1/2.

A search of potential MEK targets using the consensus phosphorylation motif TXY produces important proteins associated with endothelial permeability including paxillin, focal adhesion kinase (FAK), β4 integrin, ZO1, VE-cadherin, catenin, glycogen synthase kinase-3β, F-actin, endothelial actin-binding protein, vimentin and caveolin; all have been linked to the mechanisms underlying paracellular or transcellular permeability. Within this context, our recent study (Wu *et al.* 2003) showed that VEGF stimulated FAK phosphorylation and focal adhesion redistribution with a time course similar to the increase in venular permeability; the biochemical morphological and physiological responses were all attenuated by a specific FAK inhibitor. Thus, FAK activation may serve as an important signalling event downstream of MEK1/2 in VEGF regulation of endothelial barrier function. The precise molecular interaction between MEK and FAK remains to be elucidated. We do not rule out the possibility that FAK participates in VEGF signalling at an initial stage upstream from MEK1/2 activation.

The permeability of microvascular endothelium is maintained by an equilibrium between the contractile force generated by the endothelial cytoskeleton and adhesive forces produced at endothelial cell–cell junctions and cell–matrix focal contacts (Lum & Malik, 1994; Garcia & Schaphorst, 1995; Moy *et al.* 2000). Over the past few years, multiple signalling cascades have been proposed to be involved in the regulation of these structural elements. For example, the cAMP–PKA cascade has been shown to maintain barrier function through an adhesion-dependent mechanism (Lampugnani *et al.* 1990). Activation of myosin light chain kinase or the Rho GTPases is known to cause endothelial cell contraction (Yuan, 2000; van Nieuw Amerongen *et al.* 2000). The PKC pathway has been linked to stress fibre formation and junction dissociation (Siflinger-Birnboim & Johnson, 2003). The results from this study, along with our previous findings, lead to the delineation of the PLC–eNOS–sGC–PKG–RAF–MEK cascade. While the current study demonstrates a common role for MEK1/2 in the hyperpermeability response to two different types of stimuli, cross-talk between MEK1/2 and other pathways could occur at different levels and redundancy may exist. We hypothesize that MEK1/2 activation is required for conveying the hyperpermeability signal to final adhesion, cytoskeletal or membrane effectors in coronary venules (Fig. 7). Further studies are necessary not only to establish the importance of the MEK pathway relative to others, but also to identify the routes of transendothelial movement of fluid and proteins modulated by this signalling cascade. Within this context, the effects of VEGF on hydraulic conductivity, reflection coefficient, and diffusive permeability have all been investigated in intact vessels (Bates & Harper, 2003). However, the findings in this study using measurements of apparent permeability coefficient for albumin offer limited information on the relative roles of different modes of transport activated by the p42/44 MAPK cascade.

The involvement of MEK1/2 as potential common mediators for venular permeability signals has important implications. First, the findings imply a direct linkage between mitogenic and hyperpermeability signals in vascular endothelium. We have previously shown that NO and cyclic GMP are mitogenic in cultured venular and venous endothelial cells (Ziche *et al.* 1993*a*; Morbidelli *et al.* 1996; Hood *et al.* 1998), as well as acting as hyperpermeability factors in isolated coronary venules (Yuan *et al.* 1993*b*). Moreover, agents normally considered as purely hyperpermeability factors, such as histamine, bradykinin and substance P, are capable of stimulating endothelial cell proliferation in culture through NO-dependent mechanisms (Marks *et al.* 1986; Ziche *et al.* 1993*b*, 1994; Parenti *et al.* 2001). Second, our results suggest that any growth factor capable of turning on MEK1/2 is a potential mediator of venular hyperpermeability. In this regard, only VEGF has received significant attention as a hyperpermeability factor. Previous studies have failed to demonstrate acute opening of the endothelial barrier after exposure to FGF, PDGF and other tyrosine receptor kinases capable of activating the p42/44 MAPK cascade (Murohara *et al.* 1998; Rissanen *et al.* 2003). However, recent experiments from our laboratory (authors' unpublished data) suggest that both FGF and PDGF are capable of increasing the permeability of isolated coronary venules from the porcine heart. Thus, a consistent linkage between mitogenic and permeability signalling through the p42/44 MAPK pathway amongst endothelial growth factors has not yet been conclusively established. Finally, our findings hint at the potential for new mechanisms of signalling hyperpermeability, namely, through modulation of the phosphatases that terminate the actions of RAF and MEK1/2 through dephosphorylation (Haneda *et al.* 1999). For example, signalling pathways that lead to inhibition of these phosphatases could lead to hyperpermeability responses of greater magnitude and greater duration. By contrast, activation of the phosphatases could prevent the subsequent actions of inflammatory mediators that

normally signal hyperpermeability reactions via the p42/44 MAPK pathway.

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