Extracellular signal-regulated protein kinase activation during reoxygenation is required to restore ischaemia-induced endothelial barrier failure

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During an ischaemic insult, oedema formation occurs as a consequence of increased vascular permeability. To study mechanisms leading to vascular barrier failure, endothelial cells were exposed to ischaemia (1 % O₂ in serum- and glucose-free medium) for 5 h. In *in vitro* conditions, ischaemia increased paracellular permeability, disassembled actin stress fibres, displaced focal adhesion kinase (FAK) from focal adhesions and enhanced cytoskeletal association of occludin. Reoxygenation restored paracellular barrier function, actin organization and FAK distribution. The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) was rapidly activated after 30 min, strongly inhibited after 5 h of continuous ischaemia and reactivated 3 times more than control during

reoxygenation. Inhibition of ERK activation during reoxygenation with U0126, an inhibitor of the ERK activator, MAPK/ERK kinase 1/2, prevented both barrier restoration and stress-fibre formation, but did not prevent recruitment of FAK to focal contacts. Under normoxic conditions, ERK inhibition led to barrier failure and disassembly of stress fibres only in the absence of serum. These results demonstrate that ERK activity is essential to rebuild a disrupted endothelial barrier after ischaemia and to maintain barrier function in cells exposed to non-ischaemic stress.

Key words: cytoskeleton, endothelial permeability, occludin, U0126.

INTRODUCTION

Ischaemia caused by arterial occlusion interrupts oxygen and nutrient supply in the underlying tissue. In addition, blood vessels in the ischaemic area lose tightness, as a consequence of which liquid enters the tissue to form oedema. Lack of supply and vascular leakage hinder proper organ functioning, e.g. in the nervous system it can lead to irreversible loss of neurons within a short time. Thus understanding the mechanism of vascular leakage during ischaemia will be useful to achieve organ protection.

Vascular permeability is primarily controlled by endothelial cell tight junctions, which seal the paracellular space [1–3]. In addition, adherens junctions and endothelial contacts with the extracellular matrix are required to establish a functional paracellular barrier [4,5]. Phosphorylation plays a critical role in endothelial permeability modulation. For example, incubation of brain endothelial cells with inhibitors of tyrosine phosphatases increased paracellular permeability [6]. Using umbilical-vein endothelial cells, we have demonstrated that endothelial leakage induced by inhibition of tyrosine phosphatase involves proteolysis of the tight junction protein occludin via a metalloproteinase-dependent mechanism [7]. Elevated permeability of retinal endothelial cells triggered by vascular endothelial growth factor (VEGF) is associated with phosphorylation of occludin and the tight junction protein ZO-1 [8]. Endothelial leakage induced by tumour necrosis factor involves cytoskeletal disruptions, as well as phosphorylation and redistribution of platelet endothelial cell adhesion molecule-1 ('PECAM-1') [9]. In con-

trast, angiopoietin-1 is able to locate platelet endothelial cell adhesion molecule-1 to endothelial junctions and reduce its phosphorylation [10].

In addition to phosphorylation of structural target proteins, different external stimuli control permeability via the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) in endothelial cells [11–13]. ERK is activated by the MAPK/ERK kinase (MEK-1/2) [14]. ERK can interfere with cytosolic and cell-surface proteins and also translocate into the nucleus to regulate transcription factors [15]. Basal ERK activity is required for proper organization of the actin cytoskeleton and adherens junctions in PC-12 cells [16] and inhibition of MEK-1/2 prevents barrier formation and up-regulation of the tight junction protein claudin-2 in epithelial cells [17]. During ischaemia/reoxygenation of cultured cardiac myocytes, ERK inhibition has been found to increase myocyte apoptosis [18] and exacerbates reperfusion injury in perfused heart preparations [18]. In the central nervous system, ERK inhibition blocks the neuroprotective effect of brain-derived neurotrophic factor ('BDNF') during ischaemic brain injury [19]. However, inhibition of ERK activation reduces infarct size during focal cerebral ischaemia [20] and partially prevents cell death of cultured neurons during hypoxia [21].

To investigate the role of phosphorylation of tight junction proteins and the implication of ERK in endothelial permeability control during ischaemia, we have used a well-established endothelial-permeability model. We show that during ischaemia/reoxygenation, endothelial cells undergo reversible leakage. Barrier failure is associated with actin-stress fibre reorganization and

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PKC, protein kinase C; TBS, Tris-buffered saline; TRITC, tetramethylrhodamine β -isothiocyanate; VEGF, vascular endothelial growth factor.

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ERK inactivation. Barrier reformation occurs in an ERK-dependent manner via reformation of stress fibres. Altered phosphorylation of the tight junction protein occludin appears to be a secondary effect under these conditions.

MATERIALS AND METHODS

Antibodies and reagents

Rabbit anti-phospho-p42/44 MAPK (anti-ppERK) and anti-p42/44 MAPK (anti-ERK) antibodies were obtained from Cell Signaling Technology. Rabbit anti-occludin and mouse anti-ZO-1 antibodies were from Zymed Laboratories (San Francisco, CA, U.S.A.). Rabbit anti- β -catenin and rabbit anti-pan-cadherin antibodies were from Sigma. Rabbit anti-focal adhesion kinase (FAK) and mouse anti-phosphotyrosine antibodies were purchased from Santa Cruz. Fluorescent- and horseradish peroxidase-labelled secondary antibodies and tetramethylrhodamine β -isothiocyanate (TRITC)-phalloidin conjugate were from Sigma. The MEK-1/2 inhibitor U0126 was purchased from Promega (Catalys AG, Wallisellen, Switzerland), dissolved in DMSO, stored in aliquots at -20°C and diluted in culture medium at the desired final concentration. Protein G-Sepharose was obtained from Sigma. Tissue culture-treated collagen-coated polycarbonate transwell® filters (0.4 μm pore size) were from Costar. All other tissue culture materials were from Life Technologies (Basel, Switzerland). Gel electrophoresis reagents and all standard laboratory chemicals were of the highest grade available commercially and purchased from Fluka (Buchs, Switzerland).

Cell culture

Human umbilical-vein endothelial cells (A.T.C.C., Manassas, VA, U.S.A.; clone CRL-1730) were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l glucose), supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine and 50 $\mu\text{g}/\text{ml}$ gentamicin. For experimental use, 8×10^4 endothelial cells/ cm^2 were plated on 6.5 mm collagen-coated transwell filters, gelatine-coated 12 mm glass coverslips or Petri dishes. The medium was changed every third or fourth day.

Exposure of endothelial cells to ischaemic conditions

Endothelial cells were washed once with PBS and then incubated with serum- and glucose-free medium in a humidified atmosphere of 5% CO_2 and 1% O_2 at 37°C for different time periods as described in [22]. Control cells were incubated in the same medium as ischaemic cells but under normoxic conditions (21% O_2). For reoxygenation, the medium was replaced by DMEM (4.5 g/l glucose) containing 10% foetal calf serum, and cells were cultured at normoxic conditions.

Permeability assay

Endothelial cells were grown on transwell filters to post-confluency (7–10 days) and then incubated under the described experimental conditions. For permeability studies, the medium was replaced by a medium, supplemented with 100 μM FITC-dextran (4.4 kDa) in the apical chamber (0.1 ml) and by the same medium without tracer in the basolateral chamber (0.6 ml). The cells were then incubated for 1 h at 37°C under 5% CO_2 in humidified air. Aliquots (50 μl) were taken from the basolateral chamber and the amount of diffused tracer was measured with a TD-700 fluorimeter (Witec AG, Littau, Switzerland).

Western-blot analysis

Whole cell extracts were prepared by lysing the cells in a lysis buffer [25 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1 mM sodium vanadate, 40 mM sodium fluoride, 1% (v/v) Triton X-100, 25 μM phenylarsine oxide and $1 \times$ complete® protease inhibitor mixture (Roche Molecular Biochemicals)]. Protein concentrations in the extracts were measured by a Bradford assay (Bio-Rad Laboratories, Glattpburg, Switzerland). Equal amounts of protein were mixed with SDS-sample buffer and incubated for 3 min at 100°C before loading. Proteins were resolved by SDS/PAGE and transferred on to nitrocellulose membranes (Schleicher and Schuell). The quality of the transfer was controlled by Ponceau S staining of the membrane. For blocking, the membrane was incubated in 5% non-fat dried milk in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl (Tris-buffered saline, TBS), containing 0.05% Tween 20 for 45 min at room temperature. However, the anti-phosphotyrosine immunoblots were blocked in 1% non-fat dried milk, 1% BSA, 0.05% Tween 20 in TBS for 45 min at room temperature. The filters were then incubated with the primary antibodies diluted in blocking buffer for 1 h or overnight (p42/44 MAPK and phospho-p24/44 MAPK). After washing three times with TBS containing 0.05% Tween 20 for 5 min, a horseradish peroxidase-conjugated secondary antibody in blocking buffer was added for 30 min. After washing three times with TBS containing 0.05% Tween 20 and once with TBS, immunoreactive bands were detected by ECL® (Amersham) in accordance with the manufacturer's instructions. The chemiluminescence signals were scanned from autoradiographic films (Fuji RX) and imported into Bio-Rad Molecular Analyst for densitometric measurements or Adobe Photoshop for presentation. For reprobing the blots, the filters were stripped in a solution containing 0.2 M glycine (pH 2.2), 0.1% SDS and 1% (v/v) Tween 20 for 90 min. After washing three times with PBS containing 1% Tween 20, the filters were reprobed, beginning with the blocking step.

Immunofluorescence

Cells grown on gelatine-coated glass coverslips were washed once with PBS and fixed with 3% (w/v) paraformaldehyde in PBS for 15 min at room temperature or 4°C . After incubation with 0.1 M glycine in PBS for 5 min, the cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min. After washing three times with PBS, the cells were incubated with the primary antibody or TRITC-phalloidin in 3% BSA in PBS for 1 h at room temperature. The cells were then washed again three times for 5 min with PBS and incubated with FITC- or Cy3-conjugated secondary antibody in 3% BSA in PBS for 1 h at room temperature. After washing three times for 5 min with PBS, the coverslips were drained and mounted in 70% glycerol, 5% *n*-propylgallate in 30 mM Tris/HCl (pH 9.5). The coverslips were sealed with nail polish and examined by confocal microscopy on a Leica inverted microscope DM IRB/E. For p42/44 MAPK (ERK) and phospho-p24/44 MAPK (ppERK) staining, the cells were fixed with 3% paraformaldehyde in 50 mM Tris/HCl (pH 7.4) and 150 mM NaCl (TBS) for 20 min at 4°C . The cells were then washed three times in TBS and 0.1% Triton X-100 (TBST) for 5 min at room temperature and blocked with 5.5% normal goat serum in TBST for 1 h. Incubation with primary antibody in TBS containing 3% BSA was done overnight at 4°C . After washing once with TBS for 15 min and once with TBS and 0.1% BSA for 15 min, the cells were incubated with secondary antibody in TBS containing 3% BSA for 1 h. Cells were then washed twice with TBST for 5 min and once with TBS for 5 min and mounted and examined as described above.

Immunoprecipitation

For immunoprecipitation, cell extracts were prepared with ice-cold lysis buffer. Extracts were incubated for 30 min on ice and homogenized by pressing several times through a 21-gauge needle. After centrifugation at 10000 *g* for 20 min, the protein concentration in the supernatant was determined and adjusted to 0.5 mg/ml. Extracts (0.5 ml) were precleared with 25 μ l of 10% suspension of Protein G–Sepharose (Sigma) for 1 h at 4 °C on a rotating wheel. After centrifugation, the lysates were incubated for 4 h or overnight at 4 °C with 25 μ l of Protein G–Sepharose conjugated to the desired antibodies [previously prepared by incubating 25 μ l of 10% Protein G–Sepharose suspension with 2 μ g of the desired antibodies for 1 h at 4 °C in 1 ml of 50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 1 mM EDTA]. Immunoprecipitates were washed five times for 5 min with lysis buffer, resuspended and boiled for 8 min in 40 μ l of 2 $\times$ SDS-sample buffer and then used for SDS/PAGE analysis.

RESULTS

Ischaemia, but not hypoxia alone, leads to reversible increase in endothelial permeability

To investigate the impact of ischaemia and reoxygenation on the permeability of a barrier-forming endothelial monolayer, cells grown on transwell filters were exposed to ischaemic conditions (1% O₂, 5% CO₂, 94% N₂, serum-free DMEM without glucose) for up to 5 h and subsequently reoxygenated (readdition of glucose-containing complete medium and exposure to 21% O₂) for 2 h. During incubation, under normoxic conditions in the absence of glucose, barrier function was maintained (Figure 1). Also, hypoxic conditions alone (glucose still present) were not sufficient to induce barrier leakage. In contrast, ischaemia led to a 70% increase in paracellular permeability for 4 kDa FITC–dextran. Reoxygenation restored the initial permeability level. Longer exposure to ischaemia resulted in more than 3-fold increased permeability, which, however, was not fully reversed during reoxygenation when exceeding 10 h and resulted in the detachment of cells (results not shown). These findings indicate

Figure 1 Ischaemia, but not hypoxia or aglycaemia alone, is required to increase permeability of endothelial cell monolayers

Endothelial cells grown on transwell filters for 7 days were exposed to ischaemia (1% O₂, serum-free medium without glucose) with or without subsequent reoxygenation (re-addition of serum- and glucose-containing medium and exposure to 21% O₂) or normoxic conditions (21% O₂) in the presence or absence of glucose for the indicated time periods. Permeability of the monolayers to FITC–dextran (4.4 kDa) was measured by collecting aliquots from the bottom chamber. Under all experimental conditions, cell viability remained above 95%, as assessed by Trypan Blue exclusion (results not shown). Data are given as means $\pm$ S.D. from three individual cultures each.

Figure 2 Ischaemia induces stress fibre loss and reinforces cytoskeleton association of occludin in endothelial cells

(A) Cells grown on coverslips were exposed to ischaemia or normal conditions for 5 h and immunostained for β -catenin, occludin and ZO-1. (B) Endothelial cells were kept under normal or ischaemic conditions for 5 h and then protein extracts were prepared with 1% Triton X-100. The insoluble material was washed with PBS and then boiled in SDS-sample buffer. The immunoblot shows occludin and cadherin using a pan-cadherin antibody. (C) Endothelial cells grown on transwell filters for 7 days were exposed to ischaemia or normoxic conditions in the presence or absence of the specific PKC inhibitor chelerythrine chloride (chel. Cl) at 600 nM for the indicated time periods. Permeability to FITC–dextran was determined as described in Figure 1. Data are given as means $\pm$ S.D. from three cultures each. (D) Protein extracts from endothelial cells were grown as described in (C). The immunoblot was probed with the anti-occludin antibody. (E) Cells grown on coverslips were exposed to control conditions, ischaemia or ischaemia with subsequent reoxygenation respectively, and the actin cytoskeleton was stained with TRITC–phalloidin. Pictures show confocal images of the basal region of the cells. In (A, E), scale bars are 10 μ m.

that only the combined deprivation of glucose and oxygen is sufficient to increase endothelial permeability.

Ischaemia changes the distribution of proteins of tight and adherens junctions only subtly, but strongly disturbs actin organization

To gain insight into the mechanism of ischaemia-induced barrier failure, we assessed the distribution of junctional proteins, since we had previously shown that inhibition of tyrosine phosphatase activity increased endothelial permeability by a mechanism of metalloproteinase-dependent cleavage and redistribution of the tight junction protein occludin [7,23]. We exposed endothelial

Figure 3 Ischaemia results in transient activation and redistribution of ERK and subsequent inactivation

Cells were exposed to ischaemic conditions between 0 and 5 h with or without subsequent reoxygenation for 2 h as indicated. (A) Immunoblot showing the effect of ischaemia/reoxygenation on ERK and phosphorylated ERK (ppERK). (B) Densitometric analysis of two experiments as shown in (A). Data are given as means $\pm$ S.D. (C) Confocal images of the basal regions of immunofluorescent staining of ppERK (a, c, e, g) and phase-contrast images (b, d, f, h) of the same cell area. Data are indicated for control cells (a, b), cells exposed to ischaemia for 1 h (c, d) or 5 h (e, f) and cells exposed to ischaemia for 5 h followed by 2 h of reoxygenation (g, h). Arrows in (g) indicate peripheral localization of ERK. In (h), scale bar is 10 μ m.

cells to 5 h ischaemia. This treatment did not significantly alter the pericellular distribution of ZO-1, β -catenin, occludin (Figure 2A) and cadherin (results not shown). In well-established intercellular junctions *in vitro*, junctional proteins have a significant Triton X-100-insoluble pool, indicative of protein complex formation with the underlying cytoskeleton [24], or with membrane microdomains [25]. Since ischaemia enhanced paracellular permeability, but did not significantly influence redistribution of tight and adherens junction proteins, we examined the Triton X-100 solubility in the transmembrane proteins occludin and cadherin. Ischaemia increased the proportion of detergent-insoluble occludin, but did not alter the detergent extractability of cadherin (Figure 2B). The retarded migration of detergent-resistant occludin (Figure 2B) is presumably due to hyperphosphorylation [24,26].

Changes in the extent of occludin phosphorylation have been shown to occur after VEGF exposure [8] and are associated with tight junction permeability after protein kinase C (PKC) activation [27]. We therefore exposed endothelial cells to chelerythrine chloride, a selective PKC inhibitor, during normoxic and ischaemic incubation. As shown in Figure 2(C), PKC inhibition did not influence endothelial permeability, but increased the retarded migration of occludin during ischaemic exposure and the fraction of detergent-insoluble form after both normoxic and ischaemic exposure (Figure 2D).

An intense structural reorganization during ischaemia was observed at the level of actin-stress fibres (Figure 2E). Under normoxic conditions, barrier-forming endothelial cells had well-

developed stress fibres, indicating firm attachment. Ischaemia (5 h) led to the complete loss of stress fibres and the accumulation of actin at the cell periphery. Subsequent reoxygenation for 2 h was sufficient to regenerate these stress fibres in a manner indistinguishable from the stress fibres present under control conditions.

Modulation of ERK activity during ischaemia and reoxygenation

Since ERK is involved in cytoskeleton rearrangements, ERK activity was assessed by determining the level of ERK phosphorylation, which has been shown to correlate with ERK activity. Endothelial cells were exposed to standard ischaemic conditions for up to 5 h and subsequently reoxygenated for 2 h. Within 30 min after the onset of ischaemia, ERK phosphorylation increased approx. 2.5 times more than the level found in resting cells, and then continuously decreased to 15 % of the initial level after 5 h (Figures 3A and 3B). Reoxygenation (2 h) led to rephosphorylation of ERK approx. 3 times more than the basal level (Figures 3A and 3B). Neither ischaemia nor reoxygenation influenced the total amount of ERK protein in endothelial cells (Figure 3A). To follow ERK *in situ*, cells exposed to ischaemic and reoxygenation conditions were fixed and immunostained with the anti-ppERK antibody. In control cells, ppERK was regularly distributed throughout the cell (Figure 3C). After 1 h of ischaemia, ppERK immunoreactivity was more intense at the cell periphery. Only background immunostaining was detected after 5 h of ischaemia, consistent with the immunoblot analysis. After reoxygenation, ppERK immunoreactivity reappeared again, with a condensed distribution indicative of nuclear accumulation and minor immunoreactivity at the cell periphery (Figure 3C, arrows). No visible changes in cell morphology occurred during ischaemic treatment (Figure 3C), consistent with the minimal alterations in the distribution of junctional proteins (Figure 2A).

Inhibition of ERK-1/2 activation prevents barrier recovery during reoxygenation and induces barrier dysfunction under normoxic conditions in the absence of serum

The previous results led us to hypothesize that ERK-dependent actin reorganization is the driving force to increase endothelial permeability during ischaemia. We therefore exposed endothelial cells to U0126, a specific inhibitor of the upstream MAPK, MEK-1/2 [28], which in turn controls ERK activation. Normoxic exposure of cells to U0126 for 5 h did not alter the functional integrity of the endothelial barrier (Figure 4A). However, in the absence of serum under normoxic conditions, U0126 increased permeability, comparable with ischaemia-induced barrier dysfunction (Figures 4A and 4B). The absence of serum alone had no influence on control cells. During reoxygenation after ischaemia, U0126 prevented barrier recovery (Figures 4A and 4B). Titration of U0126 revealed that concentrations above 5 μ M were required to increase permeability under normoxic conditions in the absence of serum and to prevent barrier reformation after ischaemia (Figure 5B). Similarly, the same concentrations repressed stress fibre formation (results not shown). The ability of U0126 to suppress ERK phosphorylation was tested by immunoblot analysis. As seen in Figure 4(C), incubation of endothelial cells with U0126 under the conditions shown in Figure 4(A) led to complete suppression of ERK phosphorylation.

Analysis of the actin cytoskeleton under all conditions shown in Figure 4(A) revealed that U0126 also prevented the reformation of the initial cytoskeletal distribution during reoxygenation. Figure 4(D) shows that stress fibres were well developed throughout the cells under control conditions without U0126

Figure 4 Inhibition of ERK activation after ischaemia with the MEK-1/2 inhibitor U0126 prevents endothelial barrier and stress fibre formation during reoxygenation

(A) Permeability of endothelial cell monolayers. Endothelial cells grown on transwell filters for 7 days were exposed to ischaemia for 5 h, followed by reoxygenation for 2 h in the presence or absence of 60 μM U0126 as indicated. The influence of serum was evaluated by comparing serum-free conditions with conditions in the presence of serum. The permeability for FITC-dextran (4.4 kDa) was measured. Data are expressed as means ± S.D. from three individual cultures. (B) Dose-dependent effect of U0126 on permeability under normoxic conditions in the absence of serum and during reoxygenation after 5 h of ischaemia. (C) Effect of U0126 on ERK phosphorylation. Normoxic cells were incubated with or without 60 μM U0126 for 2 h and cell lysates were used for immunodetection of ERK and ppERK. (D) Staining of the actin cytoskeleton in endothelial cells. Cells grown on coverslips were exposed to the same conditions as in (A) and the actin cytoskeleton was stained with TRITC-phalloidin. Pictures show cells held at normoxic conditions for 5 h without additives (a), with 60 μM U0126 (b), without serum (c), without serum in the presence of 60 μM U0126 (d), cells held in normoxic conditions for 5 h followed by reoxygenation for 2 h without additives (e), with

Figure 5 FAK is removed from focal adhesion contacts during ischaemia

(A) Endothelial cells grown on coverslips were exposed to normal conditions for 5 h (a), to ischaemia for 5 h (b), to ischaemia for 5 h with subsequent reoxygenation for 2 h (c) and to ischaemia for 5 h with subsequent reoxygenation for 2 h in the presence of 60 μM U0126 during reoxygenation (d). FAK was revealed with a polyclonal anti-FAK antibody. Pictures show confocal images of the basal plane of the cells. (B) Effect of ischaemia on tyrosine phosphorylation of FAK. Cells were exposed to normal or ischaemic conditions, and cell extracts were used for FAK immunoprecipitation. FAK protein and FAK phosphotyrosine (P-Tyr) were detected in FAK immunoprecipitates. In (A), scale bar is 10 μm.

(D, part a), with U0126 (D, part b) and without serum (D, part c). Consistent with the increased permeability, U0126 caused disruption of stress fibres under normoxic conditions in the absence of serum (D, part d). Ischaemia (5 h) induced the reorganization of the actin cytoskeleton (D, part g). Reoxygenation allowed complete reformation of stress fibres in the absence (D, part h), but not in the presence of U0126 (D, part i). The inhibitor had no influence on the actin structure of control cells, which were kept under normoxic conditions for 5 h and then incubated with U0126 during reoxygenation in the presence of serum (D, parts e, f). Taken together, these results demonstrate that ERK activation is required to restore endothelial barrier function after ischaemia and that the reversible loss of stress fibres, induced during ischaemia, correlates with the reversible barrier dysfunction and depends on ERK activation.

Loss of stress fibres correlates with disappearance of FAK

Loss of stress fibres during ischaemia is a strong evidence for diminished focal adhesion. To provide evidence for this assumption, we followed the distribution of FAK since this enzyme has been shown to be recruited to focal adhesion sites in well-attached cells. Endothelial cells kept under normal or ischaemic

60 μM U0126 during reoxygenation (f), cells held at ischaemia for 5 h (g) and cells held at ischaemia followed by reoxygenation without (h) and with 60 μM U0126 during reoxygenation (i). Pictures show confocal images of the basal plane of the cells. In (i), scale bar is 10 μm.

conditions for 5 h were stained for FAK by indirect immunofluorescence. A dashed pattern was observed in control cells, but not in ischaemic cells (Figure 5A). Reoxygenation restored the original distribution. In the presence of U0126 during reoxygenation, FAK was also relocated at focal adhesions, indicating that FAK distribution occurs independent of ERK activity. In addition to its redistribution, FAK was also dephosphorylated during ischaemia [Figure 5B; $49 \pm 6\%$ compared with control ($n = 2$)]. These results are consistent with a reversible hypophosphorylation period during an extended ischaemic insult.

DISCUSSION

In the present study, we found that ischaemia, but not hypoxia alone, was required to enhance paracellular permeability. Changes induced by ischaemia included the virtually complete loss of actin stress fibres. Alterations at the level of tight and adherens junctions were only subtle and involved increased cytoskeleton association of occludin. During ischaemia, ERK was transiently activated, redistributed to the cell periphery and subsequently inactivated. Within 2 h of reoxygenation, ERK was rapidly reactivated and partially translocated to the nucleus. Inhibition of ERK reactivation during reoxygenation by the MEK-1/2 inhibitor U0126 prevented barrier and stress-fibre reformation. In the absence of serum under normoxic conditions, ERK inhibition also resulted in barrier dysfunction. These findings indicate that the regeneration of endothelial barrier function after ischaemic injury requires ERK activation. Furthermore, ERK plays a barrier protective role in endothelial cells under non-ischaemic stress conditions.

Barrier properties of a cellular monolayer depend on structural integrity of tight junctions, adherens junctions and actin cytoskeleton. Disruption of these junctions, e.g. through redistribution of junctional molecules from their circumcellular localization [7,29] and also disruption of F-actin [26,30], causes barrier breakdown. Our system of 5 h ischaemic insult allowed complete reformation of paracellular barrier function within 2 h of reoxygenation. Under these conditions, major structural changes were observed at the level of actin fibres. In addition, high-molecular-mass hyperphosphorylated forms of occludin were found in Triton X-100 insoluble material after 5 h of ischaemia. The ability of the PKC inhibitor to increase occludin phosphorylation, but to be ineffective in permeability changes, suggests however that enhanced occludin phosphorylation is not directly related to barrier failure during ischaemia.

Longer ischaemic periods of up to 10 h resulted in the progressive disappearance of occludin and ZO-1 from the cell periphery (results not shown), yet under these conditions barrier reformation was not complete. This suggests that ischaemia-induced endothelial leakage involves multiple steps, beginning with cytoskeletal reorganization and post-translational modification of occludin, followed by redistribution of occludin. Consistent with such a sequence, ATP-depletion studies in barrier-forming epithelial cells revealed gradual changes at the cytoskeletal level beginning with cortical actin, followed by stress fibre loss and changes in the actin belt at the level of tight and adherens junctions [31]. The redistribution of occludin only after extended ischaemic exposure suggests that tight junction maintenance is less susceptible to energy depletion. Supporting this view is the observation that ATP depletion can eliminate the gate function of tight junctions, but preserve the fence function [32]. Minimal relocation of tight junction proteins, altered biochemical solubility and loss of stress fibres have also been found during

chemically induced short-term ischaemia with 2-deoxy-D-glucose and antimycin A [26,33].

During ischaemia/reoxygenation-induced actin reorganization, ERK was not only hyperphosphorylated transiently, but in addition changed its subcellular distribution. When maximally activated during ischaemia, ERK was predominantly concentrated at the cell periphery, whereas activation during reoxygenation revealed mostly nuclear accumulation with only minor distribution at the periphery. This finding suggests that ERK participates in distinct signalling cascades either during early ischaemia, when the endothelial barrier is still intact, or during reoxygenation, when the barrier must be reformed. Consistent with this assumption, we observed genistein-dependent inhibition of ERK activation during ischaemia, but not during reoxygenation (M. Wachtel and S. M. Gloor, unpublished work). A contribution of ERK during barrier and stress fibre maintenance was additionally observed in endothelial cells kept under normoxic conditions in the absence of serum, but not in its presence. This finding suggests that ERK activation is involved in cellular responses after stress situations, whereas under normal conditions, ERK activity does not significantly contribute to basal endothelial cell function, consistent with the idea that ERK and other MAPK act as important transducers of environmental stimuli [15]. The implication of ERK during cellular attachment and stress-fibre formation has been recognized in different studies. ERK is activated during formation of focal adhesions, initiated by binding of integrins to extracellular-matrix proteins [34,35]. Similarly, integrin engagement to collagen induces the transformation of small F-actin punctuated spots in the basal region of the cells into F-actin bundles lining the basal membrane, and inhibition of ERK prevents this reorganization [36]. An ERK-dependent stress fibre formation has also been observed in endothelial cells after protein kinase A inhibition [37].

Consistent with a major impact of ischaemia on actin stress fibre organization and possibly focal adhesion, FAK was delocalized from focal adhesions during ischaemia and relocalized during subsequent reoxygenation. Since FAK relocation during reoxygenation was not inhibited by U0126, FAK distribution occurs independent of ERK activity. A similar recruitment of FAK during stress-fibre formation has been observed in endothelial cells after treatment with VEGF [38]. Whether FAK is an essential upstream element of ERK-dependent stress fibre formation after ischaemia is not known. However, there is evidence that FAK is, at least partially, implicated in ERK-dependent signal transduction [39,40].

Taken together, our results show that MAPK/ERK plays a critical role during ischaemia-induced alterations in endothelial barrier function. The major structural targets of ERK activity appear to be actin stress fibres, whose state is controlled by ERK during stress conditions, but not under normal conditions. Thus a protective function of ERK during ischaemia-induced barrier breakdown could be induced by prolongation of ERK activation. This could be achieved by identifying the phosphatases involved in ERK dephosphorylation in endothelial cells; a candidate is vaccinia H1-related ('VHR') phosphatase [41,42]. Interestingly, MAPK phosphatase-1 is induced under hypoxic conditions in solid tumour tissues [43], suggesting, at least partially, co-ordinated regulation of MAPKs and phosphatases under certain conditions.

We thank Professor K. Winterhalter for helpful comments and continuous support. We are grateful to Dr Hugo Marti for critically reading the manuscript. This work was supported by grants from the Swiss National Foundation (grant nos. 4038-043986 and 3100-065100.01), Novartis Foundation, Roche Foundation, Olga Mayenfisch Foundation, Swiss Life and Cancer League of the Kanton Zurich.

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Received 10 May 2002/22 July 2002; accepted 23 July 2002

Published as BJ Immediate Publication 23 July 2002, DOI 10.1042/BJ20020746