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Hypoxia-inducible factor and nuclear factor kappa-B activation in blood–brain barrier endothelium under hypoxic/reoxygenation stress

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Abstract

This investigation focuses on transcription factor involvement in blood–brain barrier (BBB) endothelial cell-induced alterations under conditions of hypoxia and post-hypoxia/reoxygenation (H/R), using established *in vivo*/*ex vivo* and *in vitro* BBB models. Protein/DNA array analyses revealed a correlation in key transcription factor activation during hypoxia and H/R, including NFκB and hypoxia-inducible factor (HIF)1. Electrophoretic mobility shift assays confirmed NFκB and HIF1 binding activity *ex vivo* and *in vitro*, under conditions of hypoxia and H/R. Hypoxia- and H/R-treated BBB endothelium showed increased HIF1α protein expression in both cytoplasmic and nuclear fractions, in *ex vivo* and *in vitro* models. Co-immunoprecipitation of HIF1α and HIF1β was shown in the nuclear fraction under conditions of hypoxia and H/R in both models. Hypoxia- and H/R-treated BBB endothelium showed increased expression of NFκB-p65 protein in both cytoplasmic and nuclear fractions. Co-immunoprecipitation of NFκB-p65 with NFκB-p50 was shown in the nuclear fraction under conditions of hypoxia and H/R in the *ex vivo* model, and after H/R in the *in vitro* model. These data offer novel avenues in which to alter and/or investigate BBB activity across model systems and to further our understanding of upstream regulators during hypoxia and H/R.

Keywords: blood-brain barrier, hypoxia, hypoxia-inducible factor, nuclear factor kappa-B, reoxygenation.

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The blood–brain barrier (BBB) is a physical and metabolic barrier that separates the peripheral circulation from the CNS, and serves to regulate and protect the microenvironment of the brain. Disruption of the BBB occurs in a number of pathological conditions, including inflammatory disorders, Alzheimer's disease, diabetes, multiple sclerosis and stroke/reperfusion (Hawkins *et al*. 1991; Banks *et al*. 1997; Abbruscato and Davis 1999; Kalaria 1999; Huber *et al*. 2001). Hypoxia and reoxygenation changes associated with various disease states significantly influence the BBB, with subsequent effects on more sensitive neural tissue. Clarifying the extent and manner of BBB alterations induced by hypoxia and/or post-hypoxic reoxygenation (H/R) remains a considerable task. Regulation and activation of transcription factors (TFs) under such conditions is vital to our understanding of these BBB changes.

Cells have developed elaborate strategies to respond to increased or decreased oxygen tension. Specific cellular systems detect excessive hypoxia and associated reactive oxygen species, leading to the activation of specific TFs and expression of related target genes (D'Angio and Finkelstein 2000). The oxygen and redox-sensitive TFs hypoxia-inducible factor (HIF)1 and nuclear factor kappa-B (NFκB) are

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Abbreviations used: AP-1, activator protein 1; BBB, blood–brain barrier; BBMEC, bovine brain microvessel endothelial cell; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; HAS, hypoxia-inducible factor ancillary sequence; HBS, hypoxia-inducible factor binding site; HIF, hypoxia-inducible factor; H/R, hypoxia/reoxygenation; HRE, hypoxia reponse element; NFκB, nuclear factor kappa-B; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; RREB, Ras-responsive transcription element; TF, transcription factor; TJ, tight junction; VEGF, vascular endothelial growth factor; ZO-1, zona occluden-1.

believed to be differentially regulated by oxidative conditions. HIF1 is selectively stabilized and activated in hypoxia, coordinating adaptive responses to hypoxia. Conversely, upon activation by inflammatory signals or pro-oxidant stresses, NFκB activates genes particularly involved in the inflammatory response, as well as in modulating the cellular response to oxidative injury. Interestingly, recent reports have indicated an interrelation in HIF1 and NFκB regulation (Jung *et al*. 2003a,b; Lukiw *et al*. 2003; Zhou *et al*. 2003). Understanding the gene transcription regulators that are altered by hypoxia and H/R will provide insight into genes that may underlie the structural protein changes that lead to functional disruptions of the BBB.

HIF1 occupies the center of the hypoxia signaling pathway. It binds a consensus sequence of the hypoxia response element (HRE) in the promoter region of oxygen-responsive genes and regulates/modulates their expression (Semenza 1998). More than 40 HIF1 target genes have been identified so far, which encode proteins that play a critical role in developmental and physiological processes, including angiogenesis/vascular remodeling, erythropoiesis, glucose transport, glycolysis, iron transport and cell proliferation/survival (Semenza 2002; Welsh and Powis 2003). HIF1 activity is regulated by cellular oxygen concentration and by growth factor-stimulated signal transduction pathways (Semenza 2002). HIF1 is a heterodimer composed of an inducibly expressed HIF1α subunit and a constitutively expressed HIF1β subunit (aryl hydrocarbon receptor nuclear translocator) (Wang and Semenza 1995). Functional HIF1 exists as an αβ heterodimer, the activation of which is dependent upon stabilization of the oxygen-sensitive degradation domain of the α-subunit by the ubiquitin–proteasome pathway (Huang *et al*. 1998). Studies indicate that HIF1 induces vascular endothelial cell growth factor (VEGF) expression under hypoxic stress, which can lead to increased paracellular permeability (Dvorak *et al*. 1995; Carmeliet and Storkebaum 2002; Harmey and Bouchier-Hayes 2002).

NFκB is activated in response to various stimuli, including infection and oxidative stress, which leads to expression of genes mediating inflammatory responses. NFκB transactivates a number of pro-inflammatory, apoptotic and oncogenic genes that collectively function to foster cellular adaptation to stress (Pahl 1999). NFκB forms homodimers and heterodimers with members of the NFκB/Rel family including RelA (p65), RelB, c-Rel, p50 (NFκB1) and p52 (NFκB2). The most prominent of these is the dimer p65/p50. NFκB exists in the cytoplasm in an inactive form when associated with regulatory protein IκB (Mercurio and Manning 1999). NFκB activation occurs through the signal-induced proteolytic degradation of IκB in the cytoplasm, via phosphorylation of IκB, exposing the nuclear localization site of NFκB. This frees NFκB to interact with the nuclear import machinery and translocate to the nucleus, where it binds to the promoter of target genes to initiate transcription (Mercurio and Manning 1999; Rothwarf and Karin 1999; Karin and Ben-Neriah 2000). In addition, inhibition of NFκB correlates with down-regulation of VEGF mRNA (Shibata *et al*. 2002), which may be mediated via HIF1α (Jung *et al*. 2003b). The expression of NFκB has also been positively correlated with increases in paracellular permeability, associated with alterations in tight junction (TJ) proteins (Brown *et al*. 2003).

The regulation of both HIF1 and NFκB activity shares similar features, but there are also specific/differential effects. Their regulation is very complex, involving numerous steps, a large portion of which are oxidant sensitive. The microvascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissues, is a major target of oxidant stress, playing a critical role in the pathophysiology of several vascular diseases and disorders. Intracellular mechanisms induced within endothelial cells during hypoxia and H/R alter endothelial cell functions, not only by direct cellular injury but also by eliciting signaling cascades (Lum and Roebuck 2001). Specifically, oxidant stress increases vascular endothelial permeability and promotes leucocyte adhesion, which is coupled with alterations in endothelial signal transduction and redox-regulated TFs such as HIF1 and NFκB (Lum and Roebuck 2001). In addition, HIF1 and NFκB activation leads to up-regulation of many genes involved in the recruitment, adhesion and activation of leucocytes, thus creating a positive feedback loop further enhancing vascular damage. Therapies aimed at inhibiting transactivation of endothelial cell genes that, when over-expressed, lead to endothelial dysfunction, have wide applicability to patients with heart disease and other forms of ischemia/reperfusion injury (Boyle *et al*. 1999).

Our laboratory has previously reported alterations in the cerebral microvasculature by demonstrating increased paracellular permeability associated with hypoxia and H/R, correlated with alterations in key TJ proteins (Abbruscato and Davis 1999; (Mark and Davis 2002; Mark *et al*. 2003; Witt *et al*. 2003). The response to hypoxia and H/R involves decreases in cortical actin band, increased stress fibers, increased endothelial surface adhesiveness (up-regulation of luminal adhesion molecules), as well as loss/disassembly of tight and adherens junctions (Lum *et al*. 1992; Kevil *et al*. 1998, 2000; Lum and Roebuck 2001). We hypothesize that BBB TJ breakdown under conditions of hypoxia and H/R (Abbruscato and Davis 1999; Mark and Davis 2002; Mark *et al*. 2003; Witt *et al*. 2003) is regulated through NFκB and/or HIF1 activation. To date, there are few reports investigating TF alterations within cerebral microvessel endothelial cells under these stressors. Here we use DNA/protein array analyses to assess the activation of multiple inflammatory/oxidative stress-associated TFs induced by hypoxia and H/R, using both *in vivo*/*ex vivo* and *in vitro* BBB models. We further examine HIF1 and NFκB TF expression and activation resulting from hypoxia and H/R.

Materials and methods

Materials

Bovine fibronectin, bovine serum albumin (BSA), equine serum, minimal essential medium and Ham's F-12 medium were purchased from Sigma Chemical (St Louis, MO, USA). Rat tail collagen (type I) was purchased from Collaborative Biomedical Products (Medford, MA, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Indianapolis, IN, USA). Primary mouse anti-HIF-1α monoclonal antibody (Novus Biologicals, Littleton, CO, USA), mouse anti-HIF-1β monoclonal antibody (Novus Biologicals) and rabbit anti-NFκB-p50 and anti-NFκB-p65 (Zymed, San Francisco, CA, USA) were used in these experiments. Conjugated anti-rabbit and anti-mouse IgG–horseradish peroxidase was purchased from Amersham Life Science Products (Springfield, IL, USA). All other nutrients, salts and antibiotics used in the culture media or assay buffers were of cell culture quality from Sigma Chemical.

In vivo hypoxic and reoxygenation treatment

In vivo hypoxic treatment conditions were created as described previously (Witt *et al*. 2003). Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Female adult Sprague–Dawley rats (Harlan, Inc., Indianapolis, IN, USA), weighing 250–300 g, were used for all experiments. Rats were housed under standard 12-h light/dark conditions and received food and water *ad labium*.

Anesthetized rats were subjected to hypoxia (6% O_2 for 1 h) or H/R [6% O_2 for 1 h, with 10 min reoxygenation (21% O_2)] in an oxygen-controlled normobaric hypoxic chamber (COY Laboratory, Grass Lake, MI, USA). Normoxic controls consisted of rats kept in room air (21% O_2) over an identical time course. Rats received 1.0 mL/kg i.m. anesthetic cocktail (ketamine 78.3 mg/ml, xylazine 3.1 mg/ml and acepromazine 0.6 mg/ml). Body temperature was maintained at 37°C with a heating pad (Harvard Apparatus, South Watwick, MA, USA).

Ex vivo rat microvessel isolation

After treatment the anesthetized rat was decapitated, and the brain was removed ($n = 3$ per set). Meninges and choroid plexus were excised and the cerebral hemispheres were homogenized in 4 mL microvessel isolation buffer [103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, 2.5 mM $NaHCO_3$, 10 mM D-glucose, 1 mM sodium pyruvate, dextran (MW 64 000; 10 g/l), pH 7.4] with complete protease inhibitor (Roche, Diagnostics, Indianapolis, IN, USA). Some 3 ml ice-cold 26% dextran was added to the homogenate, which was then vortexed and centrifuged at 5600 *g* for 10 min followed by aspiration of the supernatant. Pellets were resuspended in 10 mL microvessel isolation buffer and passed through a 70-μm filter (Falcon; Becton Dickinson, Franklin, NJ, USA). The filtered homogenates were centrifuged at 3000 *g* in isolation buffer, leaving a microvascular pellet. The pellet, consisting of the microvessels from the grey mater of three rats, was used for nuclear and cytoplasmic protein extraction. Protein concentrations were determined by BCA protein assay with BSA as a standard.

Isolation and culturing of bovine brain microvessel endothelial cells (BBMECs)

Fresh bovine brains were obtained from the University of Arizona Animal Services. Primary BBMECs, used as an *in vitro* BBB model, were collected from gray matter of bovine cerebral cortices by a combination of enzymatic digestion and centrifugal separation as described previously (Miller *et al*. 1992). BBMECs were seeded (50 000 cells/cm^2) on collagen-coated, fibronectin-treated six-well culture plates. Culture medium contained 45% minimal essential medium, 45% Ham's F-12 nutrient mix, 10 mM HEPES, 13 mM $NaHCO_3$, 50 μg/mL gentamicin, 10% equine serum, 2.5 μg/mL amphotericin B and 100 μg/mL heparin. Cell cultures were grown in a humidified 37°C incubator with room air−5% CO2, and culture medium was replaced every other day until the BBMEC monolayers reached confluency (approximately 12–14 days).

In vitro hypoxia and reoxygenation treatment

Confluent BBMEC monolayers were subjected to normoxia, hypoxia or H/R (Mark and Davis 2002). Briefly, BBMEC monolayers were exposed to reduced oxygen (1% O_2) for 24 h at 37°C in an oxygen-controlled hypoxic workstation. Following 24 h of hypoxia, cells in the HR group were returned to normoxic conditions for 2 h at 37°C. Changes in BBMEC monolayers following these treatment protocols were measured with respect to control monolayers incubated under normoxic conditions (room air−5% CO_2) for 24 h at 37°C.

Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic extracts were prepared using a modification of the method described by Dignam *et al*. (1983). Extracts from confluent BBMECs or isolated rat brain microvessel endothelial cells (gray matter from three brains per group) were used for examination of protein expression in western blots, electrophoretic mobility shift assays (EMSAs) and DNA/TF protein microarray analyses in normoxia (control), hypoxia and H/R groups. Lysis and extraction of cultured endothelial cells or rat microvessels was performed under the same oxygenation conditions as the respective pretreatment conditions.

Briefly, cells were washed in isolation buffer (see above), and 500 μL hypotonic buffer (20 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% glycerol and 20% Triton X-100) containing protease inhibitor was added to the pellet. The pellet was briefly vortexed and then disrupted by sonication for 20 min on ice, after which the cells were centrifuged at 1000 *g* for 5 min. The supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in 100 μL extraction buffer (20 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% glycerol, 20% Triton X-100 and 420 mM NaCl) with protease inhibitor. The pellet was vortexed and shaken vigorously for 20 min and then centrifuged at 12 000 *g* for 10 min. This supernatant was collected as the nuclear fraction. Protein concentrations were determined by BCA protein assay with BSA as a standard.

DNA/TF binding

The array assay was carried out in accordance with the procedure in the TranSignal protein/DNA array kit user manual (Panomics Inc., Redwood City, CA, USA) and as described by Wheeler *et al*. (2003). This protein/DNA array is a semiquantitative technology that represents a high-throughput method for the characterization of TF binding and allows multiple TFs to be profiled simultaneously. Probes containing 54 or 96 DNA-binding elements coinciding with inflammatory/oxidative stress-associated TFs were spotted on to a

nylon-based membrane. All DNA-binding elements were spotted in duplicate. Nuclear extract from both rat microvessels (*ex vivo*) and BBMECs (*in vitro*) in control, hypoxia and H/R groups were assessed simultaneously. Briefly, 20 μg nuclear extract was mixed with probe mix and incubated at 15°C for 30 min. The entire content of the mixture was loaded on a 6% agarose gel and electrophoresed at 120 V in 0.5% TBE (Tris Boric acid EDTA) for 20 min. The gel area from above the dye front to the loading well, which represents the migration distance of TF protein/DNA complexes, was excised from the agarose gel. The DNA probes were recovered from the protein/DNA complexes and denatured at 95°C for 3 min before being hybridized to the array membrane at 42°C overnight. The membrane was washed twice in 2 × saline sodium citrate buffer (SSC)/0.5% sodium dodecyl sulfate (SDS) at 42°C for 20 min and then twice in 0.1 × SSC/0.5% SDS at 42°C for 20 min. The membrane was then blocked with 1% blocking reagent at 25°C for 30 min. The biotin-labeled probe was detected with streptavidin–horseradish peroxidase diluted 1 : 1000. After washing three times and equilibrating in buffer, the membrane was overlaid with lumino/enhancer and substrate for 5 min. The image was visualized on X-ray film (Eastman Kodak Co., Rochester, New York, UK) and Kodak Image Station (440CF) was used for analysis of DNA/TF protein expression.

EMSA

EMSA was performed for HIF1 and NFκB, using a chemiluminescent 'Gel shift' kit from Panomics Inc. Briefly, 10 μg nuclear extract from rat microvessels or BBMECs was incubated with 5 × binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM ammonium sulfate, 1 mM dithiothreitol, 30 mM KCl and 0.2% Tween-20), 1 μg poly(dI–dC) and 5.0 μL distilled water for 5 min on ice. Biotin-labeled probe was then added [HIF1: AGC TTG CCC TAC GTG CGT TCT CAG A (Yamashita *et al*. 2001); NFκB: AGT TGA GGG GAC TTT CCC AGG C (Harada *et al*. 1994)]. The reactions were incubated at 15°C for 30 min. A competition control was set up by adding excess non-biotin-labeled 'cold' probe with an identical sequence to the reaction. The samples were electrophoresed on a 6% DNA retardation gel (Invitrogen, Carlsbad, CA, USA) in 0.5% TBE for 55 min at 120 V. The samples were then transferred in 0.5% TBE on to a nylon membrane at 240 mA for 50 min. After transfer, the samples were fixed on the membrane by Stratalinker® UV cross-linking (Stratagene, La Jolla, CA, USA). For detection the membrane was first blocked with 1% blocking reagent at room temperature for 15 min. The biotin-labeled probe was then detected with streptavidin–horseradish peroxidase diluted 1 : 1000. After washing three times and equilibrating in buffer, the membrane was overlaid with chemiluminescence solution for 5 min. The image was visualized on X-ray film and Kodak Image Station (440CF) was used for semiquantitative analysis of protein expression. The results were normalized to the amount of protein loaded. Data are presented as mean ± SEM.

Immunoprecipitation and immunoblotting

Nuclear and cytoplasmic isolates were analyzed for expression of HIF1α and NFκB-p65, and co-immunoprecipitation of HIF1β and NFκB-p65 (with NFκB-p50 antibody immunoprecipitation) respectively. Immunoprecipitation studies were performed to determine cytoplasmic and nuclear expression changes resulting from hypoxia and H/R (compared with normoxic control). In brief, 100 μg protein/sample was diluted with 250 μL immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl and 1% Triton X-100) and incubated at room temperature for 45 min with 50 μL rec-protein G–Sepharose beads (Zymed) at 37°C. After incubation, each sample was centrifuged at 10 000 *g* for 3 min. The supernatant was decanted into 100 μL immunoprecipitation buffer with 5% BSA and 4 μg respective antibody, and incubated at 4°C for 2 h with shaking. Some 50 μL rec-protein G–Sepharose beads were added, and incubated for 10 min. Samples were pelleted, washed twice with immunoprecipitation buffer and washed once with non-detergent buffer (10 mM Tris-HCl, pH 7.5 and 5 mM EDTA), centrifuging at 10 000 *g* for 3 min between washes. Samples were resuspended in SDS buffer and heated to 96°C for 2 min before electrophoresis.

Immunoprecipitants were resolved on 10% Tris-HCl gels (Bio-Rad, Hercules, CA, USA) for 90 min at 125 V and transferred to a nitrocellulose membrane for 45 min at 240 mA. Membranes were blocked in Tris-buffered saline (TBS) (141 mM NaCl, 20 mM Tris base, and 0.1% Tween 20; pH 7.6) with 5% non-fat milk for 4 h, and incubated with secondary antibody for 1 h. Co-immunoprecipitation was determined by incubating membranes in primary antibody (HIF1β and NFκB-p65 after immunoprecipitation of HIF1α and NFκB-p50 respectively) for 2 h, rinsing with TBS with 5% non-fat milk for 1 h, and incubating with secondary antibody for 1 h. Membranes were developed using an enzyme chemiluminescence method (ECL^{plus}; Amersham) and bands were visualized on X-ray film (Kodak). Kodak Image Station (440CF) was used for semiquantitative analysis of protein expression and the results were normalized to the amount of protein loaded. Data are presented as mean ± SEM.

Results

DNA/TF binding and EMSA

These experiments provided evidence for regulation of key TFs under conditions of hypoxia and H/R in both *in vivo*/*ex vivo* and *in vitro* BBB models. To investigate the activity associated with hypoxia and H/R, a DNA sequence array was used to allow for increased screening of hypoxia-induced TFs. With this array-based assay, DNA binding activities of multiple TFs can be elucidated. EMSA analyses of this technology have previously confirmed the accuracy of the methodology (Jiang *et al*. 2003).

Figure 1 shows the TF protein/DNA arrays of nuclear extracts from rat microvessels (*ex vivo*) and BBMECs (*in vitro*) following normoxia (control), hypoxia and H/R. A significant increase in TF activation was observed within nuclear extracts, of key regulators involved in hypoxia and H/R stress (Table 1). Within the cluster of oxygen response genes, several have been described previously to be induced in a HIF1- and NFκB-dependent manner.

Using both DNA arrays and EMSA, TF activation of HIF1 was shown in both *ex vivo* microvessels (Figs 1 and Fig. 2a) and *in vitro* endothelial cells (Figs 1 and Fig. 2b) in hypoxia and H/R groups. The *ex vivo* microvessels showed a substantial increase in HIF1 TF activation in both the H

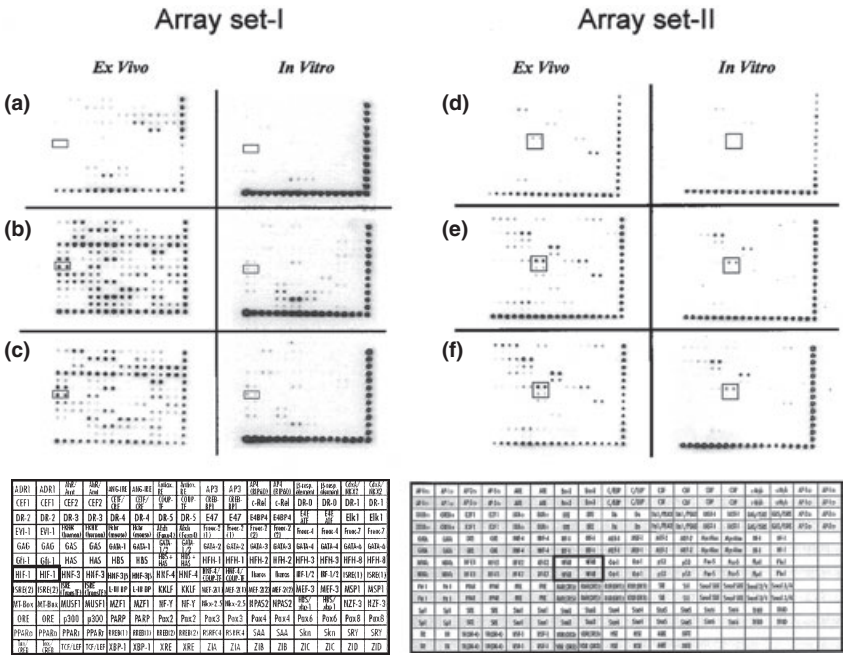

Fig. 1 TF activation on protein arrays (array set I and II) from nuclear extracts of rat microvessels (*ex vivo*) (left column) and BBMECs (*in vitro*) (right column), respective to array set. Each blot shows activation during (a, d) normoxic, (b, e) hypoxic or (c, f) H/R conditions. All blots were run simultaneously and DNA sequences were spotted in duplicate. Protein/DNA binding differences for HIF (array set-I) and NFκB (array set-II) are identified by a box. Schematic diagram of the TranSignal™ Protein/DNA Array layout is shown beneath.

Table 1 TF activation

		Ex vivo			*In vitro*		
TF	Description/alternative name	C-H	C-H/R	H-H/R	C-H	C-H/R	H-H/R
AP-1	Fos, FosB, Fra1, Fra2, Jun, JunB	↑ 28.9	↑ 46.5	↑ 1.6	↑ 8.8	↑ 15.3	↑ 1.7
AP-2	AP2 (activating enhancer binding protein 2)	↑ 15.2	↑ 21.3	↑ 1.4	↑ 4.6	↑ 9.5	↑ 2.0
AhR/Arnt	Aryl hydrocarbon receptor nuclear translocator binding element	↑ 7.9	↑ 9.7	↑ 1.2	↑ 6.3	↑ 11.5	↑ 1.8
CREB	cAMP responsive element binding protein 1	↑ 14.1	↑ 19.6	↑ 1.4	↑ 3.9	↑ 9.3	↑ 2.4
EGR	Early growth response	↑ 2.9	↑ 5.9	↑ 2.0	↑ 4.4	↑ 6.1	↑ 1.4
Gfi-1	Growth factor independent 1	↑ 209	↑ 120	↓ 1.7	↑ 6.2	↑ 7.2	↑ 1.2
HAS	HIF-1 ancillary sequence (cis element for VEGF)	↑ 18.4	↑ 6.1	↑ 3.0			
HBS	HIF-1 binding site (cis- element regulating VEGF)	↑ 16.7	↑ 8.7	↓ 1.9			
HBS + HAS	HBS and HAS sequences combined	↑ 18.8	↑ 11.8	↓ 1.6	↑ 5.2	↑ 4.2	↓ 1.2
HIF1	Hypoxia-inducible factor 1	↑ 144	↑ 115	↓ 1.3	↑ 5.3	↑ 8.6	↑ 1.6
MEF1	Myocyte enhancing factor 1	↑ 6.1	↑ 6.0	1	↑ 2.0	↑ 3.7	↑ 1.8
NFkB	Nuclear factor of kappa B	↑ 3.3	↑ 3.9	↑ 1.2	↑ 6.2	↑ 6.2	1
ORE	Osmotic response element	↑ 55.5	↑ 23.4	↑ 2.4	↑ 2.3	↑ 2.8	↑ 1.2
p53	TP53: tumor protein p53	↑ 14.1	↑ 14.1	1			
p300	p300 coactivator	↑ 15.3	↑ 2.9	↓ 5.2			
RREB (1)	Ras-responsive transcription element	↑ 15.7	↑ 3.3	↓ 4.7	↑ 5.3	↑ 1.7	↓ 3.1
Smad3/4	MADH3/4: MAD	↑ 1.2	1	↓ 1.2	↑ 2.2	↑ 2.9	↑ 1.3
XRE	Xenobiotic response element	↑ 13.8	↑ 2.4	↓ 5.7	↑ 3.0	↑ 1.1	↑ 3.3

TF activation from nuclear extracts of *ex vivo* rat microvessels and *in vitro* BBMECs, as determined from DNA/protein array (Fig. 1). Respective fold-changes shown (C-H, control vs. hypoxic; C-H/R, control vs. H/R; H-H/R, hypoxic vs. H/R; MADH3/4, MAD mothers against decapentaplegic homolog3/4), with arrows indicating the relative increase (↑) or decrease (↓).

and H/R groups; the *in vitro* endothelial cells showed a similar trend. Analysis of the VEGF gene promoter has shown that a HIF1 binding site (HBS) and its downstream HIF1 ancillary sequence (HAS) within the HRE are required as *cis*-elements for the transcriptional activation of VEGF by hypoxia (Kimura *et al*. 2000, 2001). These data support our TF array analysis, which showed an increase in HBS, HAS, and HBS + HAB binding activity during hypoxia and H/R in

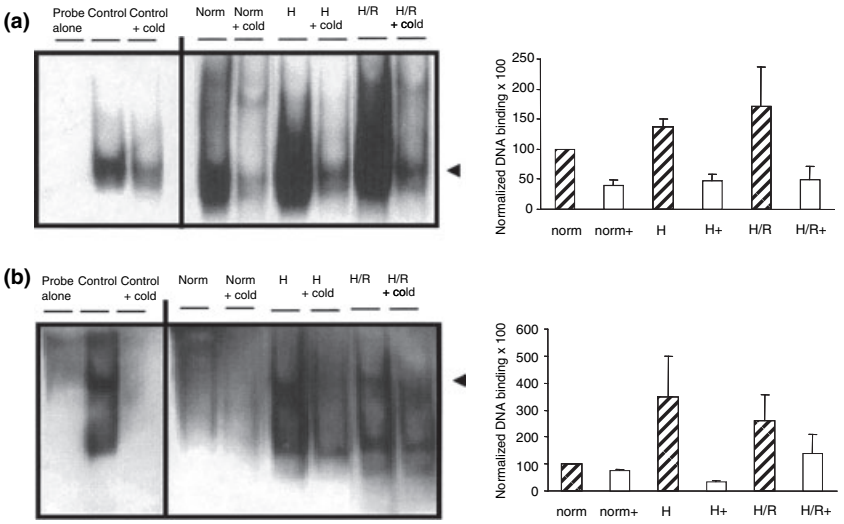

Fig. 2 Representative EMSA of HIF1 from nuclear extracts of (a) rat microvessels (*ex vivo*) and (b) BBMECs cells (*in vitro*). Each blot shows DNA binding of HIF1 after normoxia (norm), hypoxia (H) or H/R, and in the presence (+) of a 'cold' probe of identical sequence after each treatment. Bar graphs to the right of each blot show the change in expression with respect to the normoxic group in the nuclear fraction (striped bars) and in the presence of 'cold' probe (open bars). Data are mean ± SEM.

the *ex vivo* BBB model (Fig. 1 and Table 1). Although the individual binding activity of HBS and HAS was not detectable *in vitro*, the combined HBS + HAS sequence was shown to increase (Fig. 1 and Table 1). The individual and combined attributes of the HBS and HAS within the VEGF gene promoters, as well as the regulation of VEGF activity and expression, is not yet fully understood. Analysis of the VEGF promoter has revealed that deletion of the HREs completely abolishes VEGF induction by nitric oxide and hypoxia (Semenza and Wang 1992). Further analysis of HREs showed that not only the HBS, but also its downstream HAS, is essential for induction by these stimuli and that the activator protein (AP)-1 site is required for its optimal response (Semenza and Wang 1992). AP-1 is another TF shown to be altered under our conditions of hypoxia and H/R (Fig. 1 and Table 1). There is evidence that under specific experimental conditions, both oxidants and antioxidants can lead to activation of the AP-1 TF (Gius *et al*. 1999). Additionally, c-Fos and c-Jun (subunits of AP-1) are capable of physically interacting with NFκB-p65 through the Rel homology domain (Stein *et al*. 1993). This complex of NFκB-p65 and Jun or Fos exhibits enhanced DNA binding and biological function via both the κB and AP-1 response elements (Stein *et al*. 1993).

Using EMSA and DNA array analysis, TF activation of NFκB was shown in both *ex vivo* microvessels (Fig. 1 and Fig. 3a) and *in vitro* endothelial cells (Fig. 1 and Fig. 3b) in hypoxia and H/R groups. Both the *ex vivo* microvessels and *in vitro* endothelial cells showed a substantial increase compared with control in NFκB TF activation in both hypoxia and H/R groups.

There is also a close association between the activity of NFκB and the level of p53. Over-expression of p53 inhibits NFκB activity, and a high level of NFκB suppresses p53 transactivation (Webster and Perkins 1999; Shao *et al*. 2000). In our study p53 activation was increased in the *ex vivo* model (Fig. 1 and Table 1), indicating its potential regulation of NFκB during hypoxia and H/R. Although increasing evidence has appeared to suggest that an interaction occurs between p53 and NFκB, information in cerebral microvessel endothelial cells is still lacking.

In addition, a number of factors potentially involved in endothelial regulation [early growth response (EGR)1, myocyte enhancing factor (MEF)1, Ras-responsive transcription element (RREB), xenobiotic response element (XRE), osmotic response element (ORE) and growth factor independent (Gfi)-1], but not previously shown to be activated in the BBB, were demonstrated in BBB models in the present study (Fig. 1 and Table 1). Examination of hypoxia and H/R insults also revealed an increased activation of several other TFs within both BBB models. The RREB showed increased activity under the hypoxic insult, with a subsequent decrease upon reoxygenation. The Ras signaling cascade is a well characterized intracellular signaling pathway between receptor tyrosine kinases and respective downstream cell regulator genes. Although Ras has been implicated in transduction of hypoxic signaling and occurs upstream of HIF1 (Richard *et al*. 1999; Zhong *et al*. 2000; Sheta *et al*. 2001), little is known about the effect of RREB TF. Interestingly, NFκB and AP-1 act synergistically to augment Ras-related gene expression (Denhardt 1996). The XRE, shown to be mediated by HIF1 under hypoxic conditions (Gassmann *et al*. 1997), also demonstrated increased activity under hypoxic insult in our BBB models. Although transcriptional activity cannot be linked to structural and/or functional changes, our observations are important as to the correlations (i.e. similar trends in TF regulation) that can be derived between *in vivo* and *in vitro* models under hypoxia and H/R stress. Smad, AP-2 and p300, which were also observed in these DNA/TF arrays (Fig. 1 and Table 1) have been

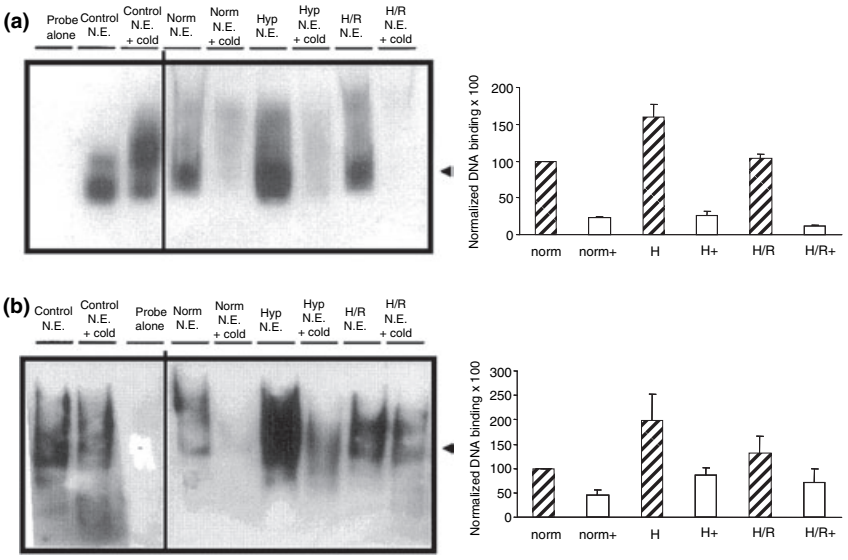

Fig. 3 Representative EMSA of NFκB from nuclear extracts (N.E.) of (a) rat microvessels (*ex vivo*) and (b) BBMECs (*in vitro*). Each blot shows DNA binding of NFκB after normoxia (norm), hypoxia (Hyp, H) or H/R, and in the presence of a 'cold' probe of identical sequence. Bar graphs to the right of each blot show the change in expression relative to the normoxic group within the nuclear fraction (striped bars) and in the presence (+) of 'cold' probe (open bars). Data are mean ± SEM.

implicated in VEGF gene expression (Josko and Mazurek 2004).

Immunoprecipitation and immunoblotting

The *ex vivo* study showed a relative increase in expression of HIF1α in both the nuclear and cytoplasmic fractions in the hypoxia and H/R groups compared with the control group (norm) (Fig. 4a). Furthermore, increased co-immunoprecipitation of HIF1β with HIF1α within the nuclear fraction was observed in the hypoxia and H/R groups relative to the control group (Fig. 4b). *In vitro* there was a similar, but less definitive, change in HIF1α (Fig. 5a) expression and HIF1β co-immunprecipitation (Fig. 5b). The relative increase in expression of HIF1α within the nuclear fraction, in association with HIF1β, is consistent with the regulation and activation of the HIF complex during hypoxia and H/R (Huang *et al*. 1998; Semenza 1998). This is further supported by our EMSA data that showed an increase in HIF1 nuclear protein binding to the conserved HRE sequence in both *ex vivo* and *in vitro* BBB models (Fig. 2).

Ex vivo experiments showed a relative increase in expression of the NFκB-p65 subunit in the nuclear and

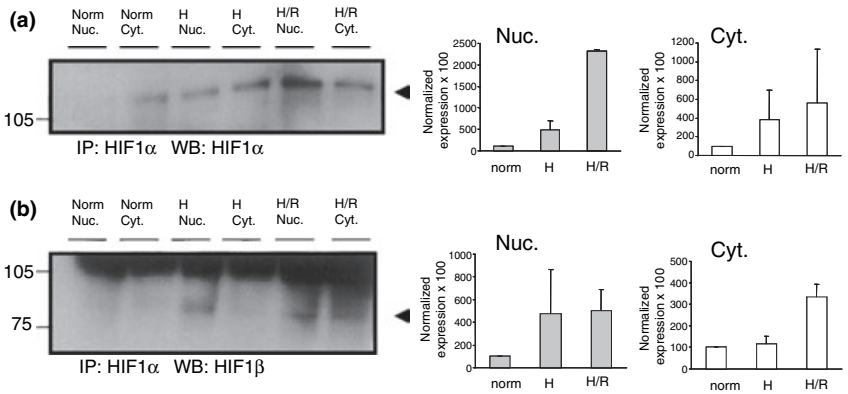

Fig. 4 Representative western blots showing levels of (a) HIF1α and (b) co-immunoprecipated HIF1β after immunoprecipitation of HIFα in rat microvessel endothelial cells (*ex vivo*). Each blot shows expression of proteins in nuclear (Nuc.) and cytosolic (Cyt.) fractions after normoxia (norm), hypoxia (H) or H/R. Antibodies used for immunoprecipitation (IP) and probing western blots (WB) are shown beneath each blot. Bar graphs to the right of each blot show the change in expression, normalized to levels in the normoxia group. Data are mean ± SEM.

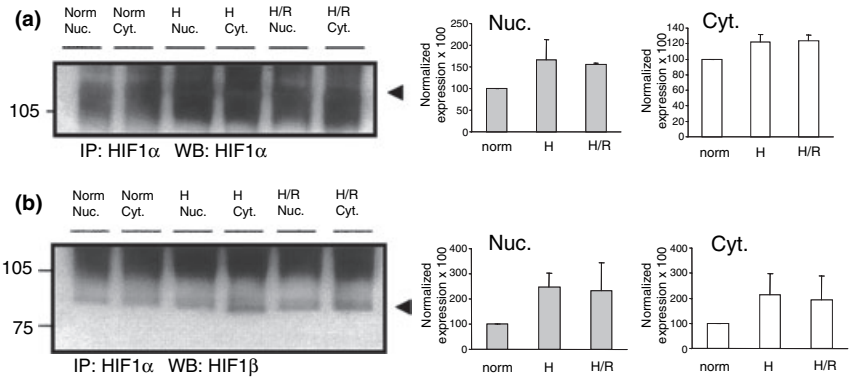

Fig. 5 Representative western blots showing levels of (a) HIF1α and (b) co-immunoprecipated HIF1β after immunoprecipitation of HIFα in BBMECs (*in vitro*). Each blot shows expression of proteins in nuclear (Nuc.) and cytosolic (Cyt.) fractions after normoxia (norm), hypoxia (H) or H/R. Antibodies used for immunoprecipitation (IP) and probing western blots (WB) are shown beneath each blot. Bar graphs to the right of each blot show the change in expression, normalized to levels in the normoxia group.

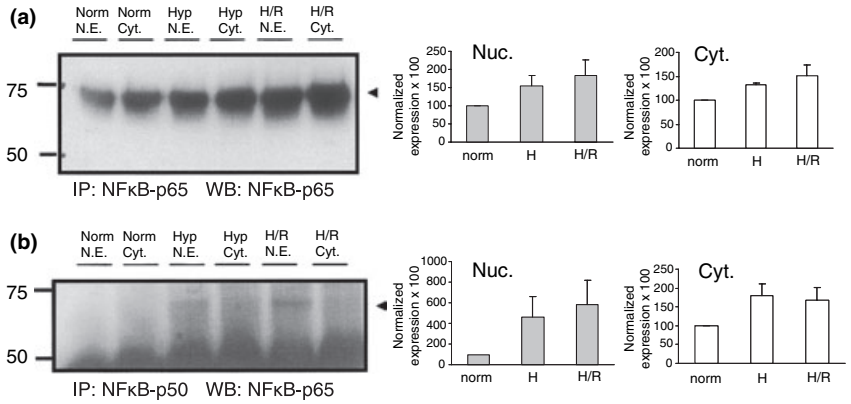

Fig. 6 Representative western blots showing levels of (a) immunoprecipitated NFκB-p65 and (b) NFκB-p65 co-immunoprecipated with NFκB-p50 in rat microvessel endothelial cells (*ex vivo*). Each blot shows expression of proteins in nuclear (N.E.,Nuc.) and cytosolic (Cyt.) fractions after normoxia (norm), hypoxia (Hyp, H) or H/R. Antibodies used for immunoprecipitation (IP) and probing western blots (WB) are shown beneath each blot. Bar graphs to the right of each blot show the change in expression, normalized to levels in the normoxia group. Data are mean ± SEM.

cytoplasmic fractions in both hypoxia and H/R groups compared with control levels (Fig. 6a). In addition, there was an increase in NFκB-p65 co-immunoprecipition with NFκB-p50 in the nuclear and cytoplasmic fractions in the hypoxia and H/R groups compared with controls (Fig. 6b). A relative increase in the *in vitro* expression of NFκB-p65 (Fig. 7a) was observed in the nuclear and cytoplasmic fractions in the hypoxia group, and in the cytoplasm in the H/R group, compared with control. There was increased co-immunoprecipitation of NFκB-p65 with NFκB-p50 in the nuclear and cytoplasmic fractions in the H/R group compared with the control (Fig. 7b). The expression change of the NFκB-p50/p65 dimer within the nuclear fraction is consistent with the regulation and activation of the NFκB complex during hypoxia and H/R (Mercurio and Manning 1999). This is further supported by our EMSA data that showed an increase in NFκB nuclear protein binding to the conserved HRE sequence in both *ex vivo* and *in vitro* BBB models (Fig. 3). Several research groups have reported changes in expression and/or activation of NFκB upon H/R in various experimental paradigms and tissue types (Howard *et al*. 1998; Royds *et al*. 1998; Natarajan *et al*. 2002). Although research supports the hypothesis that NFκB activation is stimulated by inflammatory or oxidative stress, debate continues as to the exact mechanism that leads to its activation with respect to stressor. Reports have indicated that a basal level of NFκB activation is necessary for basic cell activity, suggesting subtle nuances in TF activation (Birbach *et al*. 2002). The observations in the present study are supported by recent reports examining NFκB in retinal endothelial cells exposed to hypoxia (Lukiw *et al*. 2003). Although experimental conditions (hypoxic exposure and duration) were different,

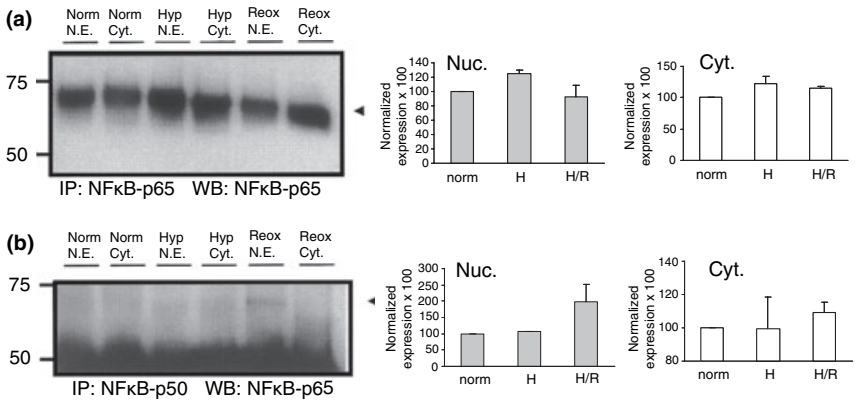

Fig. 7 Representative western blots showing levels of (a) immunoprecipitated NFκB-p65 and (b) NFκB-p65 co-immunoprecipated with NFκB-p50 in BBMECs (*in vitro*). Each blot shows expression of proteins in nuclear (N.E., Nuc.) and cytosolic (Cyt.) fractions after normoxia (norm), hypoxia (Hyp, H) or H/R (Reox). Antibodies used for immunoprecipitation (IP) and probing western blots (WB) are shown beneath each blot. Bar graphs to the right of each blot show the change in expression, normalized to levels in the normoxia group. Data are mean ± SEM.

increases in nuclear fraction of the TFs HIF1 and NFκB, but not AP-1, were observed in monkey choroid retina endothelial cells (Lukiw *et al*. 2003). Furthermore, increased nuclear expression of p65 NFκB subunit has been reported in human umbilical vein endothelial cells following H/R (Collard *et al*. 1999).

An increase in *ex vivo* VEGF expression (data not shown), which coincides with HIF1α expression and HIF1 binding, supports the theory that HIF1α accumulation during hypoxia leads to transcriptional activation of HIF1 target genes, such as VEGF. This observation is in agreement with other studies showing up-regulation of VEGF in the whole brain of mice and rats exposed to hypoxia (Xu and Severinghaus 1998; Kuo *et al*. 1999; Chavez *et al*. 2000).

Discussion

The TFs HIF1 and NFκB have been identified as a critical component of the cellular and systemic response to hypoxic stress. They mediate oxygen-dependent transcription of target genes involved in the regulation of adaptive responses, such as changes in energy metabolism and angiogenesis. Hypoxic and H/R insults have been shown to induce cytoskeletal disruption and decreased TJ integrity, which underlie the induced paracellular permeability in cerebral microvessel endothelial cells (Mark and Davis 2002; Witt *et al*. 2003). In the present study we used both *in vivo*/*ex vivo* and *in vitro* models to investigate mechanisms underlying the BBB alteration associated with hypoxia and H/R.

The models developed within our laboratory were specifically created to examine dynamic alterations at the BBB (Abbruscato and Davis 1999; Mark and Davis 2002; Mark *et al*. 2003; Witt *et al*. 2003). Although we concede that direct correlations between *in vivo* and *in vitro* models are often tenuous, the parameters of these models were first set to a common functional criteria of altered paracellular permeability (Mark and Davis 2002; Witt *et al*. 2003). The *in vivo* model allows an acute hypoxic event to be studied within the whole animal, thereby providing an assessment of changes in an intact neurovascular unit. This model does not result in severe or necrotic tissue damage, allowing for dynamic alterations to be monitored. The *in vitro* model comprises cerebral microvessel endothelial cells alone cultured on a collagen–fibronectin matrix, and allows assessment of the primary barrier cells of the BBB, without interference from support cells or peripheral circulating factors. The purpose of this study was to assess expression and activation of both the HIF and NFκB TFs to further our understanding of hypoxia-mediated response mechanisms of the BBB. There was an impressive degree of correlation between the BBB models, which lends credence to the observed alterations in permeability and TF activity.

Interestingly, recent reports have focused on HIF1α expression regulated at the translational level under normoxic conditions (Laughner *et al*. 2001), through an NFκB-dependent pathway (Jung *et al*. 2003a,b; Lukiw *et al*. 2003). VEGF expression can be regulated through dual independent mechanisms involving HIF1 directly (via the HIF1–VEGF promoter) and indirectly through NFκB mediation of cyclo-oxygenase-2 and PGE_2 (Lukiw *et al*. 2003). The overlap in HIF1 and NFκB activity observed within the present study, are supported by these data (Jung *et al*. 2003a,b; Lukiw *et al*. 2003). Although HIF1 activity is generally associated with hypoxia, and NFκB is associated with reoxygenation–reperfusion, our study reveals similarities between HIF1 and NFκB activities in both our BBB models, under both conditions of hypoxia and H/R. Although it might be argued that observed alterations in the *in vivo*/

ex vivo model might be the result of disparity in oxygenation, the independent *in vitro* endothelium showed an identical trend. It is plausible that HIF1 and NFκB activities are not completely independent, with respect to hypoxia and H/R, but interact in their regulation of the key cellular pathways, which may involve VEGF regulation.

An understanding of the regulation of TJ protein alteration under conditions of hypoxia and H/R, via HIF1 and NFκB, is emerging, which is supported by the present study and previous work (Abbruscato and Davis 1999; Mark and Davis 2002; Mark *et al*. 2003; Witt *et al*. 2003). We believe that HIF1 and NFκB are upstream mediators of TJ protein alterations under conditions of hypoxia and H/R, which may involve VEGF induction and expression. *In vivo* and *in vitro* investigations of VEGF positively correlate with changes in TJ redistribution of zona occluden-1 (ZO-1) and occludin, as well as with alterations in the actin cytoskeleton (Antonetti *et al*. 1998, 1999; Wang *et al*. 2001; Pedram *et al*. 2002). Phosphorylation signaling pathways lying downstream of VEGF induction (Wu *et al*. 1999; Breslin *et al*. 2003) have been hypothesized to modulate the barrier function of endothelial cell TJ proteins (Antonetti *et al*. 1999; Lippoldt *et al*. 2000; Pedram *et al*. 2002; Fischer *et al*. 2004). Localization and expression of key TJ proteins ZO-1 and occludin have been shown to be altered during hypoxia and H/R stress in our *in vivo*/*ex vivo* and *in vitro* BBB models, and correlated with increased paracellular permeability and edema (Abbruscato and Davis 1999; Mark and Davis 2002; Mark *et al*. 2003; Witt *et al*. 2003). We have also reported a band shift indicative of occludin phosphorylation, which correlated with increased paracellular permeability in our *in vivo*/*ex vivo* model (Witt *et al*. 2003). However, despite the number of significant correlations in HIF and/or NFκB regulation of VEGF, and subsequent VEGF-regulated changes in TJ proteins, the precise path by which VEGF modulates TJ proteins is still a matter of debate. The Flk-1 tyrosine kinase domain receptor (KDR/VEGF-R2) has been proposed to participate in VEGF-mediated permeability (Murohara *et al*. 1998; Wu *et al*. 1999). VEGF binding leads to receptor dimerization followed by autophosphorylation of the cytosolic domains of receptors, which results in the stimulation of several intracellular kinases (Risau 1997). Flk-1 receptors activate membrane-associated kinases, such as Src and phosphatidylinositol 3-kinase (PI3K) (Murohara *et al*. 1998). VEGF-induced Src was found to be essential for alterations in vascular permeability that occur with cerebral edema, following ischemic stroke (Eliceiri *et al*. 1999; Paul *et al*. 2001). Src activation has also been implicated in VEGF stimulation of extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) mitogen-activated protein (MAP) kinases, as well as activation of PI3K/Akt (Pedram *et al*. 2002). Furthermore, phosphorylation of ZO-1 and occludin downstream of JNK and PI3K/Akt results in morphological actin structure and TJ protein localization alterations with enhanced permeability (Pedram *et al*. 2002). The protein kinase C (PKC) family has also been associated with TJ protein alterations, regulating both the subcellular localization and the phosphorylation states of TJ proteins (Stuart and Nigam 1995; Lippoldt *et al*. 2000; Avila-Flores *et al*. 2001; Chen *et al*. 2002), and has been shown to be involved in VEGF-induced effects on paracellular permeability (Wu *et al*. 1999). The particular PKC isoform(s) associated with paracellular permeability are currently under investigation, but preliminary studies suggest PKC-ε involvement, which is induced in both our *in vivo*/*ex vivo* and *in vitro* BBB models (data not shown).

The findings of the present study support the hypothesis that HIF1 and NFκB are activated under conditions of hypoxia and H/R stress at the BBB. With the use of dual *in vivo*/*ex vivo* and *in vitro* BBB modeling we were able to show similar activation of HIF1 and NFκB during hypoxia and H/R. To our knowledge, this is the first examination of these key TFs at the BBB, as well as the first correlative examination between BBB models under these conditions. A detailed examination of HIF1- and NFκB-mediated mechanisms, including a broader time course, is necessary to elucidate alterations shown in the tight junctional proteins of the BBB under conditions of hypoxia and H/R. Understanding the molecular mechanisms induced by hypoxia and H/R that result in altered BBB permeability will advance the development of pharmacotherapeutics for the treatment of cerebrovascular disease, as well as provide early markers for more accurate diagnoses.

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