Protection against hypoxia-induced increase in blood-brain barrier permeability: role of tight junction proteins and NFκB

Rachel C. Brown, Karen S. Mark, Richard D. Egleton, Jason D. Huber, Amanda R. Burroughs and Thomas P. Davis*

Department of Pharmacology, The University of Arizona College of Medicine, Tucson, AZ, USA
*Author for correspondence (e-mail: davistp@u.arizona.edu)

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Summary

Co-culture with glial cells and glia-conditioned media can induce blood-brain barrier properties in microvessel endothelial cells and protect against hypoxia-induced blood-brain barrier breakdown. We examined the effect of two types of glia-conditioned media on brain microvessel endothelial cell permeability and tight junction protein expression, and studied potential mechanisms of action. We found that C6-glioma-conditioned media, but not rat astrocyte-conditioned media, protected against an increase in permeability induced by exposure to 1% oxygen for 24 hours. This hypoxic stress caused an increase in the expression of tight junction proteins claudin-1 and actin, particularly in cells treated with C6-conditioned media. We found that C6-conditioned media has a significantly higher level of both basic fibroblast growth factor and vascular endothelial growth factor. Treatment with C6-conditioned media for 1 or 3 days protects against hypoxia-induced permeability increases, and this protective effect may be mediated by signal transduction pathways terminating at the transcription factor NFκB.

Key words: Basic fibroblast growth factor, Vascular endothelial growth factor, Claudin-1, Actin, Hypoxic stress, NFκB

Introduction

The blood-brain barrier (BBB) is a metabolic and physical barrier separating the microenvironment of the central nervous system (CNS) from the peripheral circulation. The BBB is crucial for normal CNS function, owing to its ability to regulate ion flux and the supply of nutrients to the brain. The BBB is located at the level of the cerebral microvessel endothelial cells and characterized by limited paracellular diffusion, reduced fluid-phase endocytosis and the presence of specific transporters for ions, peptides and nutrients (Takakura et al., 1991; Banks, 1999), which allow for strict regulation of CNS homeostasis. BBB characteristics are regulated by complex interactions between capillary endothelial cells, the basement lamina and astrocytic endfeet processes. Disruption of the BBB occurs in a number of pathological conditions, including stroke, Alzheimer's disease, diabetes, multiple sclerosis and inflammatory pain (Hawkins et al., 1991; Banks et al., 1997; Abbruscato and Davis, 1999; Kalaria, 1999; Huber et al., 2001b).

The restrictive nature of the BBB is due in part to tight junctions (TJ) formed between adjacent endothelial cells (Reese and Karnovsky, 1967; Kneisel and Wolburg, 2000). TJ allow for regulation of ion flux and paracellular diffusion through the development of high transendothelial electrical resistances (TEER), in the range of 1500-2000 $\Omega \cdot cm^2$ (Butt et al., 1990). A number of TJ protein components have been identified and extensively characterized, including the claudin family (Furuse et al., 1998), occludin (Furuse et al., 1993) and zonula occludens-1, -2 and -3 (ZO-1, ZO-2 and ZO-3), which interact with claudins and occludin (Itoh et al., 1999; Mitic et al., 1999). Claudin-1 and occludin have been found at the BBB (Huber et al., 2001b; Mark and Davis, 2002). Both of these proteins have multiple transmembrane domains and form homodimeric bridges with adjacent cells, creating a physical blockade to paracellular diffusion (Tsukita and Furuse, 1999). Stabilization of the TJ complex involves a network of occludin and claudins linked to the actin cytoskeleton via the ZO proteins. ZO proteins mediate this linkage by binding actin to the cytoplasmic tails of occludin and claudin (Huber et al., 2001a), in a manner similar to the cadherin-catenins-actin interaction at the adherens junction (Brown and Davis, 2002). ZO proteins are members of the membrane-associated guanylate kinase (MAGUK) family; they have a conserved guanylate kinase domain, an SH3 domain and multiple PDZ domains (Huber et al., 2001a), suggesting that ZO proteins participate in signal transduction cascades.

In order to examine mechanistic and molecular events in the BBB that occur under different pathological conditions and to assess drug delivery to the CNS, in vitro models have been developed using primary cultures of brain microvessel endothelial cells (BMEC). There is a great deal of evidence that astrocytes are important for inducing and maintaining certain BBB characteristics in vitro. The characteristics affected by astrocytes or astrocyte products include high TEER, low paracellular diffusion and low transcellular endocytosis (Wolburg et al., 1994). Co-culture studies show

that astrocytes induce BBB-like characteristics in endothelial cells isolated from various sources, including bovine aortic endothelial cells (Isobe et al., 1996), immortalized bovine BMEC (Sobue et al., 1999) and immortalized rat BMEC (El Hafny et al., 1997). The influence of astrocytes on barrier permeability is mediated by factors released from astroglioma cells (Raub et al., 1992) or mixed cultures of rat astrocytes (Dehouck et al., 1994). Several factors have been identified that may account for these BBB-inducing properties of astrocytes, including basic fibroblast growth factor (bFGF) (Sobue et al., 1999) and glia-derived neurotrophic factor (Igarashi et al., 1999). Furthermore, glial co-culture protects in vitro BBB models against breakdown induced by hypoxic stress (Kondo et al., 1996; Fischer et al., 2000). However, there is little information as to how co-culture with different glial cell types affects expression levels of TJ proteins under normal and pathological conditions. In this study, we compared the effects of conditioned media generated from C6 glioma and primary rat astrocytes on permeability and TJ protein expression, both under normoxic conditions and after a 24 hour hypoxic stress, to determine if the previously described protective effect of glial cell co-culture may be due to changes in the expression of key TJ components.

Materials and Methods

Cell culture

Fresh bovine brains were obtained from the University of Arizona Meat Laboratory, and bovine brain microvessel endothelial cells (BBMEC) were isolated from the gray matter of the cerebral cortex and cryo-preserved as previously described (Abbruscato and Davis, 1999; Mark and Davis, 2002). Isolated cells were seeded at a density of 50,000 cells/cm^2 onto collagen/fibronectin-coated Transwell filters, allowing for access to both the apical and basolateral sides of the BBMEC monolayers. All BBMEC used for these studies were primary cultured cells from passage zero, which have been shown to maintain excellent BBB characteristics in vitro (Weber et al., 1993; Brownson et al., 1994; Abbruscato et al., 1997). BBMEC were grown in MEM/F12 with 50 μg/ml gentamicin, 10% equine serum, 50 mg/ml sodium heparin and 10 mg/ml Amphotericin B.

Astrocytes were derived from two sources. C6 glioma cells (American Type Culture Collection, Rockville, MD) from passages 6-10 were grown in MEM/F12 with 50 μg/ml gentamicin, 10% equine serum and 2.5% fetal bovine serum. Primary rat astrocytes (RA) were isolated from newborn rat brains by the method of Frangakis and Kimelberg (Frangakis and Kimelberg, 1984). Briefly, newborn rat pups (<24 hours old) were anesthetized and their brains removed. Cerebral cortices were minced and incubated with trypsin/EDTA for 30 minutes at 37°C. The supernatant was removed, and cells were seeded in tissue culture flasks coated with 0.01% poly-L-lysine. Cells were allowed to grow for 4 days in astrocyte growth media (MEM with 10% fetal bovine serum, 20 mM glucose, 0.5 mM L-glutamine, 12 mg/ml penicillin, 12 mg/ml streptomycin and 5 mg/ml insulin). After 4 days, the flasks were shaken at 120 oscillations/min at 37°C for 8 hours to remove attached cells. Cells still attached to the plasticware (astrocytes) were trypsinized and frozen in liquid nitrogen until used for experiments. Conditioned media (CM) was generated by plating either C6 or RA cells at a density of 40,000 cells/cm^2 and harvesting media after 3 days.

BBMECs were exposed to conditioned media as previously described (Abbruscato and Davis, 1999). C6-conditioned media (C6-CM) or RA-conditioned media (RA-CM) was added to the basolateral side of the Transwell filters for 1-3 days before further experiments. Media was changed every 2 days to provide adequate nutrition, and there was no evidence of detaching or dying cells with this feeding regimen. On the day of the experiment, BBMEC monolayers were incubated in assay buffer (122 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM HEPES and 0.4 mM $K2HPO_4$) for permeability studies or dissolved in TRI® reagent (Sigma, St Louis, MO) for protein isolation and western blot analyses.

Permeability studies

Permeability studies with [^{14}C]-sucrose were used to determine paracellular flux across confluent BBMEC monolayers. Apical-to-basolateral flux was determined by dividing the pmoles of radioactive marker appearing in the receiver chamber by the time in minutes. The apparent permeability coefficient was calculated using the equation:

$$PC\ (cm/min)=Flux/(A*C_{Do}),$$

where flux is the slope of the line, A is the area of the membrane and C_{Do} is the initial donor concentration of radioactive marker.

Western blot protein analyses

After coculturing, protein was isolated from cell cultures using TRI® reagent (Sigma, St Louis, MO). Protein was separated from RNA and DNA by chloroform and ethanol extraction and precipitated using isopropanol. Protein pellets were washed with guanidinium chloride/95% ethanol and dissolved in 1% SDS. Protein levels were measured using the bicinchonic acid method (Pierce, Indianapolis, IN) using bovine serum albumin (BSA) as a standard. Protein samples (10-20 μg) were separated on precast 4-12% Tris-glycine gels (Invitrogen, Carlsbad, CA) at 125 V for 75-90 minutes. Proteins were transferred to polyvinylidene fluoride membranes at 240 mA at 4°C for 30 minutes. Membranes and/or gels were stained to control for variability in protein loading prior to blocking. Membranes were incubated with primary antibody (anti-claudin-1, 1:1000; anti-occludin, 1:2000; anti-ZO-1, 1:2000; anti-NFκB, from Zymed Laboratories, anti-actin, 1:1000, from Sigma, anti-HSP90α, 1:1000, from Calbiochem) in 0.5% BSA/PBS. Horseradish-peroxidase-conjugated secondary antibody in 0.5% BSA/PBS was applied for 30 minutes at room temperature. Protein bands were visualized using the enhanced chemiluminescent method (ECL^{plus}, Amersham, Piscataway, NJ). Quantification of band density was done using Scion Image (NIH, Bethesda, MD); band intensity was normalized to protein loading band density and expressed as a percentage of control values.

Growth factor immunoassays

Samples of cell culture media were assayed for levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Briefly, samples of growth media, C6-CM and RA-CM were incubated in microplates coated with anti-VEGF or anti-bFGF antibodies (R&D Systems, Minneapolis, MN). Antibody conjugate was added and incubated. A colorimetric assay was developed and absorbance was read at 450 nm, with correction for interference by subtracting readings at 540 nm. VEGF and bFGF levels in different media were determined by calculation from standard curves. The sensitivity limit of the assays was 3 pg/ml for both VEGF and bFGF.

Immunoprecipitation

Immunoprecipitation of NFκB was performed using the μMACS Microbeads protocol (Miltenyi Biotech, Auburn, CA). In brief, 4 μg of antibody was incubated with 50 μg of total protein isolated from control and co-cultured cells and 50 μl of Protein G microbeads on ice for 30 minutes and then run through columns. Columns were rinsed, and immunoprecipitated proteins were eluted by applying

95°C SDS loading buffer. Samples were run on precast 8% Tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk in Tris buffer and incubated with horseradish-peroxidase-linked anti-rabbit secondary antibody. Blots were developed as previously described for western blot analyses.

Statistics

Data are presented as means±s.e.m. for n=4-16 permeability studies or n=3-8 western blot analyses, growth factor assays or immunoprecipitations. Growth factor levels were measured in duplicate from three separate experiments. Data were analyzed by one-way (growth factor assays) or two-way (permeability studies, western blot analyses and immunoprecipitation) analysis of variance (ANOVA) followed by Tukeys posthoc test, with significance defined as P<0.05.

Results

We previously demonstrated that 24 hours of hypoxic stress increases the permeability of BBMEC monolayers in vitro and increases the expression of the TJ protein actin (Mark and Davis, 2002). In the current study, we examined the effects of normal growth media (MEM/F12), C6-conditioned media (C6-CM) and primary rat astrocyte-conditioned media (RA-CM) on permeability and expression of TJ proteins under normoxic and hypoxic conditions in BBMEC monolayers.

BBMEC permeability studies showed a significant effect of both glia-conditioned media ($F_{2,90}$=11.527, P<0.001) and hypoxic stress ($F_{1,90}$=109.822, P<0.001). Glia-conditioned media had no significant effect on basal permeability of BBMEC monolayers in the normoxic groups (Fig. 1). After 24 hours of hypoxic stress, the permeability of BBMEC monolayers in MEM/F12 increased 2.2-fold (Fig. 1, P<0.001). Incubating monolayers with C6-CM partially blocked the hypoxia-induced increase in permeability (P<0.001 versus MEM/F12, P<0.05 versus RA-CM), although permeability in the hypoxic C6-CM monolayers was significantly higher then normoxic C6-CM monolayers (P<0.001). By contrast, RA-CM had no effect on hypoxia-induced increase in permeability when compared to hypoxic MEM/F12 monolayers. Statistical analysis showed a significant interaction between culture condition and hypoxic stress ($F_{2,90}$=3.554, P=0.033).

Glia-conditioned media does not affect basal expression of TJ proteins (Table 1). Analysis of western blot expression of claudin-1 shows a significant effect owing to hypoxic stress ($F_{1,42}$=4.306, P=0.044). After 24 hours of hypoxic stress, there is a significant increase in claudin-1 expression, although there is no significant effect from glia-conditioned media, nor is there an interaction between hypoxic stress and glia-conditioned media.

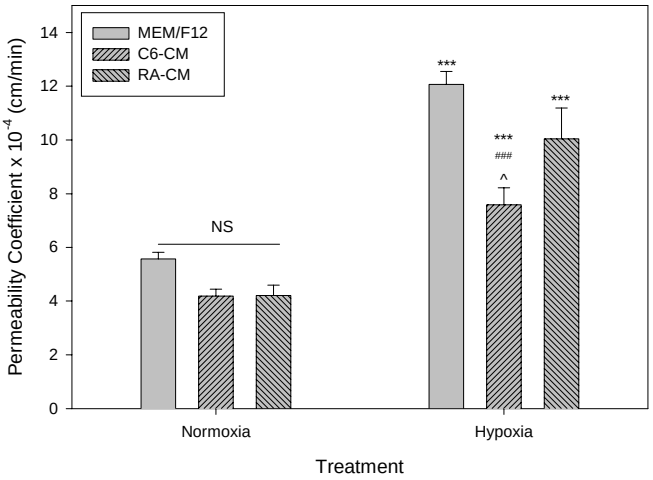

Fig. 1. C6-conditioned media protects against hypoxia-induced permeability changes in BBMEC monolayers. BBMEC monolayers were incubated for three days in C6-CM or RA-CM and then subjected to 24 hours of normoxia or hypoxic stress (1% O_2). Under normoxic conditions, neither C6-CM nor RA-CM has any effect on BBMEC monolayer permeability. However, after 24 hours of hypoxia, there is a significant increase in permeability in all three treatment groups. This increase in permeability after hypoxic stress is partially blocked by co-culture with C6-CM. ***P<0.001 versus comparable normoxic treatment group, $^{\#\#\#}P$<0.001 versus hypoxic MEM/F12, $^{\wedge}P$<0.05 versus hypoxic RA-CM, n=16.

Hypoxic treatment also significantly increased actin expression (Table 1, $F_{1,30}$=14.184, P<0.001). Similar to claudin-1 expression changes, there was no significant effect from culture conditions, although in both cases, C6-CM-treated monolayers had the largest increase in actin expression after hypoxic stress. No interaction between hypoxic stress and glia-conditioned media was observed. ZO-1 and occludin expression levels were not significantly affected by hypoxic stress or culture condition (Table 1).

We hypothesized that a glia-secreted factor might be involved in mediating the C6-CM protective effect, and we measured levels of bFGF and VEGF in glia-conditioned media. bFGF and VEGF were chosen as important factors in this protective scenario because of evidence in the literature that these factors are released from C6 glioma cells (Okumura et al., 1989; Plate et al., 1993) and can modulate endothelial cell and BBB properties (Dobrogowska et al., 1998; Sobue et al., 1999). C6-CM had 20% higher levels of bFGF (Table 2) compared to MEM/F12 and RA-CM ($F_{2,25}$=17.836, P<0.001).

Table 1. Effects of C6- and RA-conditioned media on TJ protein expression in BBMEC monolayers

	Normoxic MEM/F12	Normoxic C6-CM	Normoxic RA-CM	Hypoxic MEM/F12	Hypoxic C6-CM	Hypoxic RA-CM
ZO-1	100±10.9	123.8±16.4	124.5±15.5	96.6±14.4	125.5±18.9	68.2±22.9
Occludin	100±14.3	128.0±15.6	136.8±7.6	149.6±26.5	134.2±19.4	123.8±24.7
Claudin-1	100±26.3	123.2±20.4	127.9±41.9	159.2±18.2	197.1±18.5	141.7±29.0
Actin	100±28.7	182.1±41.9	227.4±31.2	341.9±73.0	439.8±89.6	253.5±50.6

Results are presented as the mean percentage of control (normoxic MEM/F12)±s.e.m. and were analyzed by two-way ANOVA. There is a significant effect of 24 hours of hypoxic stress on the expression of claudin-1 ($F_{1,42}$=4.306, P=0.044) and actin ($F_{1,30}$=14.184, P<0.001). n=5-8 separate blots.

Table 2. Levels of bFGF and VEGF in glial-conditioned media

	bFGF (pg/ml)	VEGF (pg/ml)
MEM/F12	32.2±1.1	59.2±5.1
C6-CM	37.8±0.5***	1384.2±137.8***
RA-CM	32.7±0.9	99.1±4.8

C6-CM has a significantly higher level of both bFGF and VEGF compared with MEM/F12 and RA-CM. There is no significant difference between MEM/F12 and RA-CM for either bFGF or VEGF. ***P<0.001 versus MEM/F12 and RA-CM, n=12-14.

Levels of VEGF were also significantly elevated in C6-CM (Table 2) compared with either MEM/F12 or RA-CM ($F_{2,35}$=229.879, P<0.001).

We hypothesized that some secreted factor in C6-CM might be involved in mediating the C6-CM protective effect. We first hypothesized, owing to the very high levels of VEGF in the C6-CM, that VEGF might be acting in the initial stages of the co-culture as a preconditioning stress. To determine if this was the case, we examined permeability of BBMEC monolayers after one and three days of co-culture with C6-CM. Statistical analysis shows a significant effect of both C6-CM ($F_{2,38}$=21.508, P<0.001) and hypoxic stress ($F_{1,38}$=62.881, P<0.001) and a significant interaction between C6-CM and hypoxic stress ($F_{2,38}$=9.804, P<0.001). Under normoxic conditions, there was no effect of C6-CM on basal permeability after either one or three days of co-culture (Fig. 2). After 24 hours of hypoxic stress, both one and three day co-culture with C6-CM protected against hypoxia-induced permeability increases, indicating that any preconditioning due to C6-CM exposure is probably occurring within a smaller time window.

In an attempt to elucidate cellular processes that might be triggered by C6-CM, we analyzed expression of heat shock protein 90α (HSP90α), a protein regulated by VEGF (Brouet et al., 2001) and by bFGF (Jerome et al., 1991). Glia-conditioned media does not affect HSP90α expression under normoxic conditions (Table 3). After 24 hours of hypoxic stress, control monolayers express significantly higher levels of HSP90α (Table 3, P<0.01). This increase was blocked by both types of glial conditioned media. C6-CM-treated monolayers showed no change in HSP90α expression. RA-CM-treated monolayers had significantly less HSP90α expression after hypoxic stress compared with both MEM/F12 and C6-CM-treated monolayers. Two-way ANOVA analysis indicates that levels of HSP90α in hypoxic C6-CM and RA-CM monolayers were not significantly different from their respective normoxic levels (P=0.498 for C6-CM-treated monolayers and P=0.065 for RA-CM-treated monolayers). Two-way ANOVA indicates a significant effect of glial conditioned media ($F_{2,12}$=16.860, P<0.001) and a significant interaction between glial conditioned media and hypoxic stress ($F_{2,12}$=9.219, P=0.004). These results indicate that heat shock protein expression can be influenced by glia-conditioned media, but this effect is probably not involved in protecting the integrity of the BBMEC monolayers.

Table 3. Effects of glial-conditioned media and hypoxic stress on HSP90α expression

	MEM/F12	C6-CM	RA-CM
Normoxic	100.0±8.4	110.2±15.3	73.1±13.0
Hypoxic	184.8±17.9**	94.5±25.4$^{\#\#,a}$	27.4±9.1$^{\#\#\#,\wedge,a}$

Results are presented as mean percentage of the control (normoxic MEM/F12)±s.e.m. Two-way ANOVA indicates a significant effect of co-culture treatment ($F_{2,12}$=16.860, P<0.001) and a significant interaction between co-culture treatment and hypoxic stress ($F_{2,12}$=9.219, P=0.004). **P<0.01 versus normoxic MEM/F12, $^{\#\#}P$<0.01 versus hypoxic MEM/F12, $^{\#\#\#}P$<0.001 versus hypoxic MEM/F12, ^P<0.05 versus hypoxic C6-ACM, [a]not significantly different from comparable normoxic treatment, n=3.

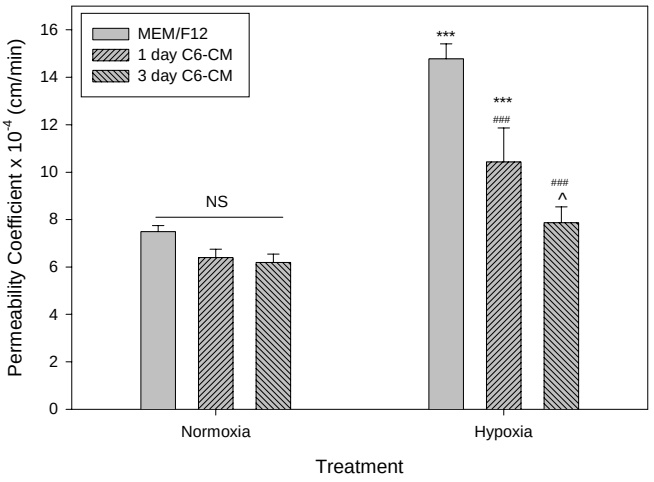

Fig. 2. C6-CM protective effect is time dependent. BBMEC monolayers were incubated with MEM/F12 or C6-CM for 1 or 3 days of exposure. Under normoxic conditions, neither 1 nor 3 days of exposure to C6-CM had any effect on monolayer permeability. However, after 24 hours of hypoxic stress, both 1 and 3 days of C6-CM exposure protected against hypoxia-induced monolayer breakdown. This effect was time dependent, with 1 day exposure being partly protective, and 3 day exposure resulting in almost complete protection against hypoxic stress. ***P<0.001 versus a comparable normoxic treatment group, $^{\#\#\#}P$<0.001 versus hypoxic MEM/F12, ^P<0.01 versus hypoxic 1 day C6-CM, n=4-8.

Growth factor modulation of BBB endothelial cell function can occur through signaling cascades resulting in activation of transcription factors such as NFκB (Selzman et al., 1999; Sasaki et al., 2000). Immunoprecipitation with anti-NFκB antibody reveals that both C6-CM and RA-CM treatment significantly increased NFκB expression under normoxic conditions (Fig. 3). After 24 hours of hypoxia, the levels of NFκB in MEM/F12 and RA-CM-treated samples significantly increased, whereas levels in C6-CM-treated samples did not change. There was a significant effect of hypoxic stress ($F_{1,30}$=12.633, P=0.001), as well as a significant interaction between culturing condition and hypoxic stress ($F_{2,30}$=3.967, P=0.03).

Discussion

One of the major issues in BBB research has been the development of representative in vitro models to examine cellular and molecular mechanisms of BBB function under normal and pathological conditions. Previous studies have demonstrated that coculturing with astrocytes or glioma cells can increase transendothelial electrical resistance (Raub et al., 1992; Kondo et al., 1996; Abbruscato and Davis, 1999) and

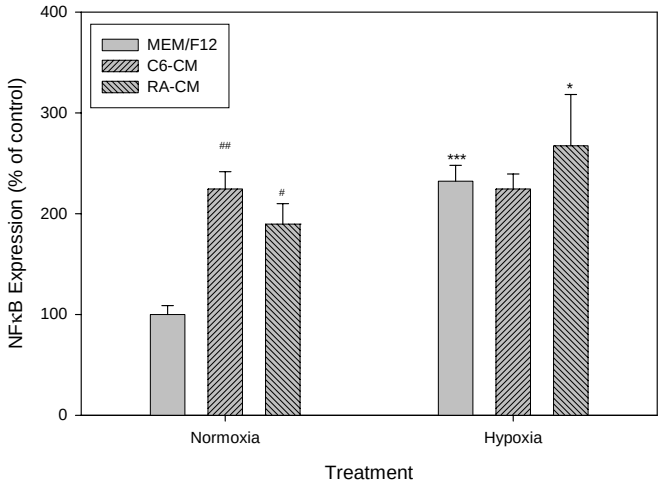

Fig. 3. C6-conditioned media increases NFκB expression in BBMEC monolayers. BBMEC monolayers were incubated for three days in C6-CM or RA-CM and then subjected to 24 hours of normoxia or hypoxic stress (1% O_2). NFκB was immunoprecipitated and detected by western blotting. C6-CM significantly increases NFκB expression under normoxic conditions; RA-CM also significantly increases NFκB expression compared with MEM/F12. After 24 hours of hypoxia, NFκB levels increase in both MEM/F12 and RA-CM-treated samples. There is no significant change in NFκB expression in hypoxic C6-CM-treated samples compared to normoxic samples. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus normoxic MEM/F12, $^{*}P<0.05$ and $^{***}P<0.001$ versus comparable normoxic treatment, $n=3$.

decrease paracellular permeability in BBB model systems (Raub et al., 1992; Isobe et al., 1996; Sobue et al., 1999). We chose to compare the BBB-inducing capabilities of C6 glioma cells and primary rat astrocytes using glia-conditioned media with [^{14}C]-sucrose as a marker for paracellular diffusion. BBMEC monolayers were cultured for 10 days in vitro. Confluent monolayers were incubated with either C6-CM or RA-CM for three days. Co-culture with glial-conditioned media did not significantly decrease basal normoxic [^{14}C]-sucrose permeability. When monolayers were subjected to 24 hours of hypoxic stress, permeability increased 2.2-fold in the untreated samples, which is similar to results previously described (Mark and Davis, 2002). C6-CM, but not RA-CM, was able to attenuate this hypoxia-induced increase in permeability. Furthermore, there is a significant interaction between culture condition and hypoxic stress. This interaction suggests that some factor or factors secreted by C6 glioma cells exert a protective effect on BBMEC monolayers undergoing hypoxic stress.

We examined expression levels of four TJ proteins to determine if glial conditioned media alone had any direct effect on TJ, which are critical for maintaining BBB properties. Glial conditioned media had no effect on basal levels of TJ protein expression. After hypoxic stress, there was no significant change in protein expression levels of ZO-1 or occludin. Claudin-1 expression was increased by hypoxic stress, and co-culture with C6-CM caused a greater increase in claudin-1 expression then MEM/F12 or RA-CM. Actin levels were also increased by hypoxic stress, with the greatest increase seen with C6-CM treatment. Together, these results suggest the protection of monolayer integrity is potentially due to strengthening of existing TJ or formation of new TJ complexes (Fig. 4). It remains to be seen if the higher expression levels of claudin-1 and actin are due to newly synthesized proteins or to the removal of already existing protein components from other subcellular locations. Previously, we have found that claudin-1 expression and localization is unlikely to be altered after insult, in both in vivo (Huber et al., 2001b) and in vitro (Mark and Davis, 2002) BBB model systems, although paracellular permeability is significantly disrupted. Claudin-1 is considered to be the critical transmembrane protein required for TJ structure and function, and the dramatic changes seen in this system seem to indicate an alteration of the TJ protein regulation. We have recently demonstrated that 24 hour hypoxic stress does cause a dissociation of other TJ components, with the removal of occludin and ZO-1 from their normal membrane localization and the formation of actin stress fibers (Mark and Davis, 2002). It may be that under our C6-CM-treated conditions, ZO-1 and occludin released initially from the dissolution of TJ are able to associate with increased levels of claudin-1 and actin and make new TJ to prevent increased paracellular permeability (Fig. 4). However, we can not rule out a mechanism by which C6-CM protects already existing TJ during hypoxic stress.

To identify factors that may be involved in the C6-CM protective effect and account for the interaction between culture condition and hypoxic stress seen in the permeability data, we assayed two angiogenic growth factors known to have effects on the BBB. Basic fibroblast growth factor (bFGF) has been shown to tighten in vitro BBB models (Sobue et al., 1999) and may be important for promoting proliferation in hypoxic endothelial cells (Kuwabara et al., 1995). Vascular endothelial growth factor (VEGF) is a well characterized angiogenic factor that increases BBB permeability (Nag et al., 1997; Dobrogowska et al., 1998) and may be directly involved in increasing BBB permeability by modulating TJ after hypoxia (Fischer et al., 2002). We found that C6-CM had significantly higher concentrations of both bFGF and VEGF compared with MEM/F12 alone and RA-CM, which were not significantly different from each other.

A possible mechanism by which C6-CM might be protective is a 'preconditioning' effect. Previous studies show that brief insults or stressors can be protective against later, more sustained insults. These preconditioning stressors are typically brief hypoxic exposures or heat shock (Gobbel et al., 1995; Pohlman and Harlan, 2000), which can prevent later hypoxia-induced endothelial cell damage. We hypothesize that high levels of VEGF present in C6-CM potentially act as a preconditioning stress, causing an initial breakdown of the BBB in the early stages of co-culture. After three days exposure to high VEGF levels, BBMEC monolayers adapt to the stress and the barrier retightens. However, when we examined permeability of BBMEC monolayers at one and three days after exposure to C6-CM, there was no increase in permeability at one day, as was expected. Furthermore, after 24 hours pf hypoxic stress, both one and three days of exposure to C6-CM were protective, with three days showing the greatest degree of protection. As there is an increase in NFκB levels seen with C6-CM exposure, indicating that the cells have been stressed, this suggests that any transient increase in permeability that may occur upon initial exposure to C6-CM has been recovered from by the time points examined. Previous

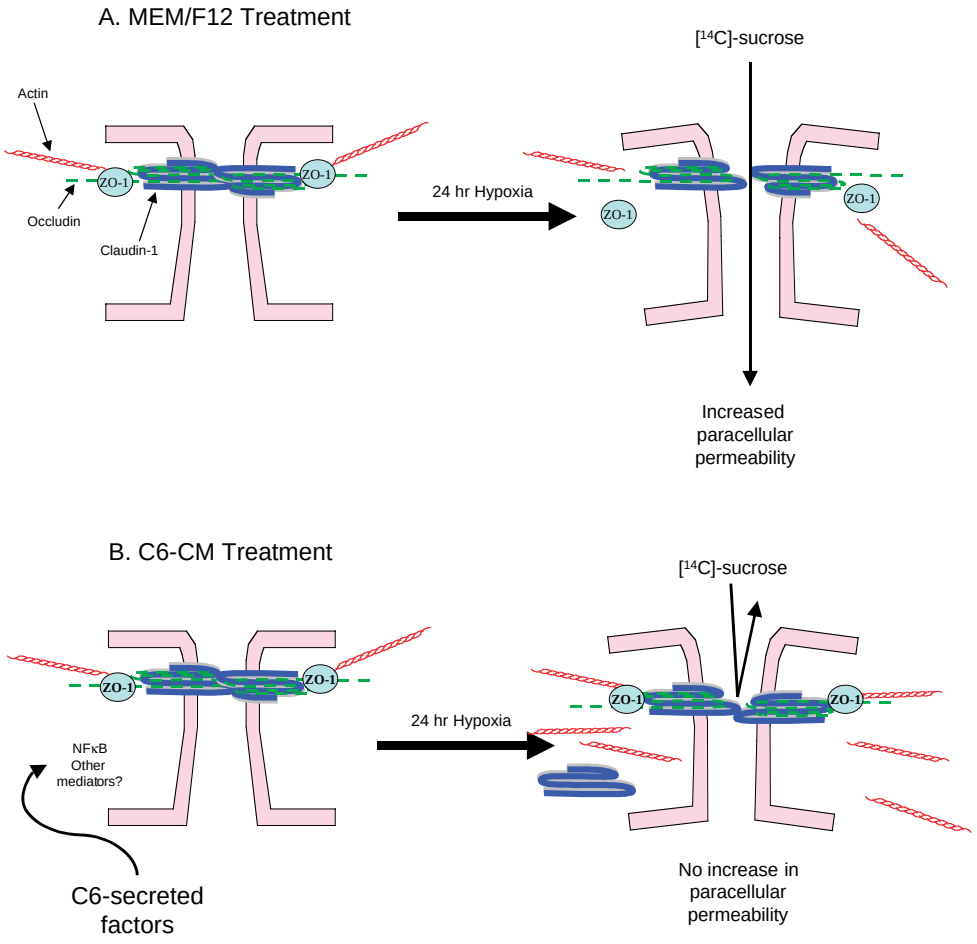

Fig. 4. A potential model for C6-conditioned media protection against hypoxia-induced changes in paracellular permeability. (A) Cells grown in MEM/F12 and subjected to a 24-hour hypoxic stress undergo a breakdown of the TJ, with a resulting increase in paracellular permeability as measured by [^{14}C]-sucrose flux. This breakdown of the TJ is probably caused by some dissociation of the component proteins, with resulting formation of actin stress fibers and removal of occludin and ZO-1 from their normal membrane-associated subcellular locations (Mark and Davis, 2002). We hypothesize that under C6-CM co-culture conditions (B), secreted factors in C6-CM trigger the activation of signal transduction mechanisms, linked to NFκB or other as yet unidentified pathways. This treatment allows for an adaptive response in the BBMEC when they are exposed to 24 hour hypoxic stress, such that they respond by increasing their expression of claudin-1 and actin. These increases in claudin-1 and actin enable the BBMEC to build additional TJ, thereby preventing the increase in paracellular permeability seen under MEM/F12 conditions. However, C6-CM treatment may also protect via the maintenance of already existing TJ; the exact mechanisms remain to be elucidated.

studies have shown relatively quick transient increases in BBB permeability ranging from 15 minutes with bradykinin analogues (Mackic et al., 1999), to 2-4 hours after exposure to TNFα (Mark et al., 2001) or inflammatory pain (Huber et al., 2001b).

In support of this data ruling out a preconditioning effect of VEGF in C6-CM-mediated protection, we found no correlation between HSP90α levels and glial conditioned media treatment, although there was a significant effect of culture condition and a significant interaction between culturing condition and hypoxic stress. HSP90α is important in mediating VEGF activity on endothelial cells, primarily by interacting with endothelial nitric oxide synthase and increasing nitric oxide production (Garcia-Cardena et al., 1998; Brouet et al., 2001). Although C6-CM did prevent the hypoxia-induced increase in HSP90α, RA-CM significantly lowered HSP90α levels after hypoxia. It may be that treatment with RA-CM causes a downregulation of HSP90α under hypoxic conditions that prevents activation of a pathway necessary for TJ maintenance, but further studies are required to determine the contribution of HSP90α to TJ integrity.

Another potential mediator of growth factor effects on endothelial cells is the transcription factor NFκB. NFκB is a major transcription factor involved in the inflammatory process and is linked to cell adhesion molecule expression in endothelial cells (Kupatt et al., 1997). NFκB is also activated by hypoxic stress. The finding that C6-CM and RA-CM increased basal levels of NFκB in our BBMEC monolayers suggests that the co-culture conditions alone constitute a stressor. However, when subjected to hypoxic stress, the levels of NFκB in the C6-CM treated cells did not change significantly, whereas increased expression was observed in both the MEM/F12- and RA-CM-treated cultures. The fact that NFκB expression did not increase after hypoxic stress, whereas expression in the other two treatment groups did increase, suggests that the C6-CM-treated monolayers are not further stressed by the removal of oxygen or that they are unable to respond above a maximum level of NFκB response. Although we can not directly link NFκB activation to protection of paracellular permeability, it may be an important trigger for downstream events involved in this protection. NFκB has been linked to the expression of cell-cell adhesion molecules involved in inflammatory processes, such as ICAM-1 (Kupatt et al., 1997), as well as to the expression of VEGF (Sasaki et al., 2000), various cytokines and immunoreceptors (Li and Stark, 2002) and important proteins involved in reactive oxygen species homeostasis (Chiarugi et al., 1999), but it may also alter TJ protein expression, and this remains to be determined.

In conclusion, we have demonstrated that the protective effect of culturing BBMEC monolayers with C6-CM occurs in a time-dependent manner, with as little as one day of exposure to C6-CM causing a significant protection against hypoxic stress. This protection may be due to an enhancement of TJ protein expression (claudin-1 and actin) after hypoxic stress.

C6-CM has significantly higher levels of VEGF and bFGF then MEM/F12 or RA-CM, but, surprisingly, VEGF does not appear to be acting as a preconditioning stress under the conditions examined. The C6-CM-mediated protection does appear to involve NFκB signaling. Although not a direct physiological paradigm except in the case of hypoxic stress and/or brain tumors, this model system may be useful for examining (1) changes in TJ protein expression at the BBB under various stressors or pathological incidents and (2) may provide a system in which the exact interactions between astrocyte-secreted growth factors and the endothelial cells of the BBB may be better studied.

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References

Abbruscato, T. J., Thomas, S. A., Hruby, V. J. and Davis, T. P. (1997). Blood-brain barrier permeability and bioavailability of a highly potent and mu-selective opioid receptor antagonist, CTAP: comparison with morphine. *J. Pharmacol. Exp. Ther.* **280**, 402-409.

Abbruscato, T. J. and Davis, T. P. (1999). Combination of hypoxia/aglycemia compromises in vitro blood-brain barrier integrity. *J. Pharmacol. Exp. Ther.* **289**, 668-675.

Banks, W. A. (1999). Physiology and pathology of the blood-brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders. *J. Neurovirol.* **5**, 538-555.

Banks, W. A., Jaspan, J. B. and Kastin, A. J. (1997). Effect of diabetes mellitus on the permeability of the blood-brain barrier to insulin. *Peptides* **18**, 1577-1584.

Brouet, A., Sonveaux, P., Dessy, C., Balligand, J. L. and Feron, O. (2001). Hsp90 ensures the transition from the early Ca^{2+}-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. *J. Biol. Chem.* **276**, 32663-32669.

Brown, R. C. and Davis, T. P. (2002). Calcium modulation of adherens and tight junction function: A potential mechanism for blood-brain barrier disruption after stroke. *Stroke* **33**, 1706-1711.

Brownson, E. A., Abbruscato, T. J., Gillespie, T. J., Hruby, V. J. and Davis, T. P. (1994). Effect of peptidases at the blood brain barrier on the permeability of enkephalin. *J. Pharmacol. Exp. Ther.* **270**, 675-680.

Butt, A. M., Jones, H. C. and Abbott, N. J. (1990). Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *J. Physiol.* **429**, 47-62.

Chiarugi, V., Magnelli, L., Chiarugi, A. and Gallo, O. (1999). Hypoxia induces pivotal tumor angiogenesis control factors including p53, vascular endothelial growth factor and the NFkappaB-dependent inducible nitric oxide synthase and cyclooxygenase-2. *J. Cancer Res. Clin. Oncol.* **125**, 525-528.

Dehouck, B., Dehouck, M. P., Fruchart, J. C. and Cecchelli, R. (1994). Upregulation of the low density lipoprotein receptor at the blood-brain barrier: intercommunications between brain capillary endothelial cells and astrocytes. *J. Cell Biol.* **126**, 465-473.

Dobrogowska, D. H., Lossinsky, A. S., Tarnawski, M. and Vorbrodt, A. W. (1998). Increased blood-brain barrier permeability and endothelial abnormalities induced by vascular endothelial growth factor. *J. Neurocytol.* **27**, 163-173.

El Hafny, B., Chappey, O., Piciotti, M., Debray, M., Boval, B. and Roux, F. (1997). Modulation of P-glycoprotein activity by glial factors and retinoic acid in an immortalized rat brain microvessel endothelial cell line. *Neurosci. Lett.* **236**, 107-111.

Fischer, S., Wobben, M., Kleinstuck, J., Renz, D. and Schaper, W. (2000). Effect of astroglial cells on hypoxia-induced permeability in PBMEC cells. *Am. J. Physiol. Cell Physiol.* **279**, C935-C944.

Fischer, S., Wobben, M., Marti, H. H., Renz, D. and Schaper, W. (2002). Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvasc. Res.* **63**, 70-80.

Frangakis, M. V. and Kimelberg, H. K. (1984). Dissociation of neonatal rat brain by dispase for preparation of primary astrocyte cultures. *Neurochem. Res.* **9**, 1689-1698.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S. and Tsukita, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol.* **123**, 1777-1788.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. and Tsukita, S. (1998). Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J. Cell Biol.* **141**, 1539-1550.

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A. and Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392**, 821-824.

Gobbel, G. T., Chan, T. Y. and Chan, P. H. (1995). Amelioration of hypoxic and hypoglycemic damage to cerebral endothelial cells. Effects of heat shock pretreatment. *Mol. Chem. Neuropathol.* **24**, 107-120.

Hawkins, C. P., Mackenzie, F., Tofts, P., du Boulay, E. P. and McDonald, W. I. (1991). Patterns of blood-brain barrier breakdown in inflammatory demyelination. *Brain Res.* **114**, 801-810.

Huber, J. D., Egleton, R. D. and Davis, T. P. (2001a). Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. *Trends Neurosci.* **24**, 719-725.

Huber, J. D., Witt, K. A., Hom, S., Egleton, R. D., Mark, K. S. and Davis, T. P. (2001b). Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. *Am. J. Physiol. Heart. Circ. Physiol.* **280**, H1241-H1248.

Igarashi, Y., Utsumi, H., Chiba, H., Yamada-Sasamori, Y., Tobioka, H., Kamimura, Y., Furuuchi, K., Kokai, Y., Nakagawa, T., Mori, M. and Sawada, N. (1999). Glial cell line-derived neurotrophic factor induces barrier function of endothelial cells forming the blood-brain barrier. *Biochem. Biophys. Res. Commun.* **261**, 108-112.

Isobe, I., Watanabe, T., Yotsuyanagi, T., Hazemoto, N., Yamagata, K., Ueki, T., Nakanishi, K., Asai, K. and Kato, T. (1996). Astrocytic contributions to blood-brain barrier (BBB) formation by endothelial cells: a possible use of aortic endothelial cell for in vitro BBB model. *Neurochem. Int.* **28**, 523-533.

Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M. and Tsukita, S. (1999). Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J. Cell Biol.* **147**, 1351-1363.

Jerome, V., Leger, J., Devin, J., Baulieu, E. E. and Catelli, M. G. (1991). Growth factors acting via tyrosine kinase receptors induce HSP90 alpha gene expression. *Growth Factors* **4**, 317-327.

Kalaria, R. N. (1999). The blood-brain barrier and cerebrovascular pathology in Alzheimer's disease. *Ann. N Y Acad. Sci.* **893**, 113-125.

Kneisel, U. and Wolburg, H. (2000). Tight junctions of the blood-brain barrier. *Cell. Mol. Neurobiol.* **20**, 57-76.

Kondo, T., Kinouchi, H., Kawase, M. and Yoshimoto, T. (1996). Astroglial cells inhibit the increasing permeability of brain endothelial cell monolayer following hypoxia/reoxygenation. *Neurosci. Lett.* **208**, 101-104.

Kupatt, C., Weber, C., Wolf, D. A., Becker, B. F., Smith, T. W. and Kelly, R. A. (1997). Nitric oxide attenuates reoxygenation-induced ICAM-1 expression in coronary microvascular endothelium: role of NFkappaB. *J. Mol. Cell. Cardiol.* **29**, 2599-2609.

Kuwabara, K., Ogawa, S., Matsumoto, M., Koga, S., Clauss, M., Pinsky, D. J., Lyn, P., Leavy, J., Witte, L., Joseph-Silverstein, J. et al. (1995). Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. *Proc. Natl. Acad. Sci. USA* **92**, 4606-4610.

Li, X. and Stark, G. R. (2002). NFkappaB-dependent signaling pathways. *Exp. Hematol.* **30**, 285-296.

Mackic, J. B., Stins, M., Jovanovic, S., Kim, K. S., Bartus, R. T. and Zlokovic, B. V. (1999). Cereport (RMP-7) increases the permeability of human brain microvascular endothelial cell monolayers. *Pharm. Res.* **16**, 1360-1365.

Mark, K. S., Trickler, W. J. and Miller, D. W. (2001). Tumor necrosis factor-alpha induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther.* **297**, 1051-1058.

Mark, K. S. and Davis, T. P. (2002). Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. *Am. J. Physiol. Heart Circ. Physiol.* **282**, H1485-H1494.

Mitic, L. L., Schneeberger, E. E., Fanning, A. S. and Anderson, J. M. (1999). Connexin-occludin chimeras containing the ZO-binding domain of

occludin localize at MDCK tight junctions and NRK cell contacts. *J. Cell Biol.* **146**, 683-693.

Nag, S., Takahashi, J. L. and Kilty, D. W. (1997). Role of vascular endothelial growth factor in blood-brain barrier breakdown and angiogenesis in brain trauma. *J. Neuropathol. Exp. Neurol.* **56**, 912-921.

Okumura, N., Takimoto, K., Okada, M. and Nakagawa, H. (1989). C6 glioma cells produce basic fibroblast growth factor that can stimulate their own proliferation. *J. Biochem. (Tokyo)* **106**, 904-909.

Plate, K. H., Breier, G., Millauer, B., Ullrich, A. and Risau, W. (1993). Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res* **53**, 5822-5827.

Pohlman, T. H. and Harlan, J. M. (2000). Adaptive responses of the endothelium to stress. *J. Surg. Res.* **89**, 85-119.

Raub, T. J., Kuentzel, S. L. and Sawada, G. A. (1992). Permeability of bovine brain microvessel endothelial cells in vitro: barrier tightening by a factor released from astroglioma cells. *Exp. Cell Res.* **199**, 330-340.

Reese, T. S. and Karnovsky, M. J. (1967). Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**, 207-217.

Sasaki, H., Ray, P. S., Zhu, L., Galang, N. and Maulik, N. (2000). Oxidative stress due to hypoxia/reoxygenation induces angiogenic factor VEGF in adult rat myocardium: possible role of NFkappaB. *Toxicology* **155**, 27-35.

Selzman, C. H., Shames, B. D., McIntyre, R. C., Jr, Banerjee, A. and Harken, A. H. (1999). The NFkappaB inhibitory peptide, IkappaBalpha, prevents human vascular smooth muscle proliferation. *Ann. Thorac. Surg.* **67**, 1227-1231.

Sobue, K., Yamamoto, N., Yoneda, K., Hodgson, M. E., Yamashiro, K., Tsuruoka, N., Tsuda, T., Katsuya, H., Miura, Y., Asai, K. and Kato, T. (1999). Induction of blood-brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. *Neurosci. Res.* **35**, 155-164.

Takakura, Y., Audus, K. L. and Borchardt, R. T. (1991). Blood-brain barrier: transport studies in isolated brain capillaries and in cultured brain endothelial cells. *Adv. Pharmacol.* **22**, 137-165.

Tsukita, S. and Furuse, M. (1999). Occludin and claudins in tight-junction strands: leading or supporting players? *Trends Cell Biol.* **9**, 268-273.

Weber, S. J., Abbruscato, T. J., Brownson, E. A., Lipkowski, A. W., Polt, R., Misicka, A., Haaseth, R. C., Bartosz, H., Hruby, V. J. and Davis, T. P. (1993). Assessment of an in vitro blood-brain barrier model using several [Met5]enkephalin opioid analogs. *J. Pharmacol. Exp. Ther.* **266**, 1649-1655.

Wolburg, H., Neuhaus, J., Kniesel, U., Krauss, B., Schmid, E. M., Ocalan, M., Farrell, C. and Risau, W. (1994). Modulation of tight junction structure in blood-brain barrier endothelial cells. Effects of tissue culture, second messengers and cocultured astrocytes. *J. Cell Sci.* **107**, 1347-1357.