Viable “*Haemophilus somnus*” Induces Myosin Light-Chain Kinase-Dependent Decrease in Brain Endothelial Cell Monolayer Resistance[∇]

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“*Haemophilus somnus*” causes thrombotic meningoencephalitis in cattle. Our laboratory has previously reported that *H. somnus* has the ability to adhere to, but not invade, bovine brain endothelial cells (BBEC) in vitro. The goal of this study was to determine if *H. somnus* alters brain endothelial cell monolayer integrity in vitro, in a manner that would be expected to contribute to inflammation of the central nervous system (CNS). Monolayer integrity was monitored by measuring transendothelial electrical resistance (TEER) and albumin flux. BBEC incubated with *H. somnus* underwent rapid cytoskeletal rearrangement, significant increases in albumin flux, and reductions in TEER. Decreased monolayer TEER was preceded by phosphorylation of the myosin regulatory light chain and was partially dependent on tumor necrosis factor alpha and myosin light-chain kinase but not interleukin-1 β . Neither heat-killed *H. somnus*, formalin-fixed *H. somnus*, nor purified lipooligosaccharide altered monolayer integrity within a 2-h incubation period, whereas conditioned medium from *H. somnus*-treated BBEC caused a modest reduction in TEER. The data from this study support the hypothesis that viable *H. somnus* alters integrity of the blood-brain barrier by promoting contraction of BBEC and increasing paracellular permeability of the CNS vasculature.

Thrombotic meningoencephalitis (TME) is a devastating neurological disease characterized by fibrinopurulent meningitis with hemorrhage, abscess formation, and thrombotic vasculitis throughout the central nervous system (CNS) (4, 33, 39). TME was the first reported clinical manifestation associated with “*Haemophilus somnus*” infection in cattle (33). Chronic lesions in animals that have undergone antibiotic therapy for TME include white matter degeneration, axonal spheroids, and focal microgliosis (80). Although *H. somnus* has been cultured from the brains of infected animals and microcolonies of the bacteria have been identified histologically in small blood vessels within necrotic foci in the CNS (60, 68), the pathogenesis of TME is not well defined.

Bacterial meningitis and CNS infection typically occur following septicemia and require a threshold level of bacteremia (40, 41). *H. somnus* meningitis, which has been reported to occur in association with septicemia, is rather unusual in its clinical presentation. The multifocal hemorrhages throughout the CNSs of affected animals are virtually pathognomonic for *H. somnus* infection (1, 38). However, it is unknown whether *H. somnus* has the ability to breach the endothelial cells of the blood-brain barrier or whether the neurological symptoms associated with TME are entirely the result of thrombotic occlusions in the CNS vasculature and resulting ischemia. Miller et al. (47) suggested that bacterial entry into the CNS might occur via veins that originate in the reproductive tract, where *H.*

somnus is frequently harbored, and course along the spinal cord.

Our laboratory has previously reported that *H. somnus* adheres to, but does not invade, bovine brain endothelial cells (BBEC) (8). We have also demonstrated proinflammatory and procoagulative responses in *H. somnus*-treated BBEC (7a) that could contribute to the destruction of the blood-brain barrier and dissemination of *H. somnus*. The apparent lack of cellular invasion by *H. somnus*, coupled with the profound dysregulation of the coagulation system associated with TME, suggests that interactions between *H. somnus* and endothelial cells of the blood-brain barrier might contribute to the pathology associated with TME. Thompson and Little (67) reported formation of inter-endothelial cell gaps in carotid artery explants treated with *H. somnus* and proposed that the resulting exposure of underlying basement membrane could be a trigger for the thrombus formation associated with TME. If endothelial cells of the blood-brain barrier respond to *H. somnus* in a similar manner, the resulting inter-endothelial cell gaps would result in increased paracellular permeability, edema, and exposure of the prothrombotic extracellular matrix. These changes could promote in situ thrombus formation and opening of a portal for inflammatory mediators, and possibly intact *H. somnus*, to enter the CNS.

Vascular barrier function is governed by the continuity of the endothelium, which in turn is regulated by the balance between contractile forces in individual endothelial cells and the tethering forces of intercellular junctions (18, 28, 44). In the blood-brain barrier, the intercellular junctions are composed of adherens junctions (57), tight junctions (42, 74, 76), and possibly gap junctions (48). The high transendothelial electrical resistance (TEER) and low paracellular permeability of the blood-brain barrier are attributed primarily to tight

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junctions (53), which are composed of integral transmembrane proteins and adaptor proteins that link the junctional complexes with the actin cytoskeleton (27, 76). Increased paracellular permeability would result from an imbalance between the adhesiveness of the inter-endothelial junctional complexes, particularly tight junctions, and cellular contraction.

Occludin is considered to be, and was used in this study as, a marker of tight junctions (74). Occludin is a transmembrane protein that was identified and initially characterized in the tight junctions of chicken intestinal epithelial and liver cells (25). Occludin is highly expressed in a continuous pattern along the cellular margins of the cerebral endothelium (34, 43) but is found at very low levels in endothelia outside the blood-brain and blood-testes barriers (25, 26, 36, 74). Decreased expression of occludin has been demonstrated, in association with decreased function of the blood-brain barrier, in numerous disease states (2, 11, 13, 16, 45).

Contractile forces in endothelial cells can be initiated by phosphorylation of the 20-kDa regulatory myosin light chain (rMLC) (28) and subsequent activity of myosin ATPase (66). The phosphorylation status of rMLC is determined by the net activity of kinases that directly phosphorylate rMLC (predominantly the Ca^{2+} /calmodulin-dependent MLC kinase [MLCK] [29] and also the Rho-associated protein kinase [12]) and removal of the phosphate groups by myosin light-chain phosphatase (MYPT).

In the present study, we investigated the ability of *H. somnus* to alter brain endothelial cell monolayer permeability in vitro. We provide evidence that viable *H. somnus* is capable of triggering cell contraction via MLCK, resulting in increased paracellular permeability.

MATERIALS AND METHODS

Chemicals and media. RPMI, Hanks' balanced salt solution, and trypsin were purchased from Cellgro (Kansas City, MO). 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) was purchased from BioMOL (Plymouth Meeting, PA). Soluble tumor necrosis factor receptor (sTNFR) and interleukin-1 (IL-1) receptor antagonist were generously provided by Amgen (Thousand Oaks, CA). Brain heart infusion agar was purchased from Difco (BD Biosciences, Franklin Lakes, NJ). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Endothelial cells. The simian virus 40-transformed BBEC line used in this study was described previously (61). The cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and were passaged by brief enzymatic digestion using 0.1% trypsin EDTA. All experiments were performed on cells prior to passage 50.

Bacteria. *Haemophilus somnus* strain 649, kindly provided by Lynette Corbeil (University of California, San Diego), was initially isolated from a clinical case of bovine abortion and has been described previously (15). The bacteria were stored as stationary-phase cells at -70°C in brain heart infusion broth with 10% (vol/vol) glycerol. Prior to each experiment, an aliquot of bacteria was thawed and inoculated at a 1:100 dilution in brain heart infusion broth supplemented with 0.5% yeast extract and 0.1% thiamine monophosphate. The bacteria were then cultured without shaking for 16 h at 37°C and 5% CO_2 . Prior to inoculation, bacteria were pelleted and resuspended in RPMI with 10% FBS. The number of bacteria present in the inoculum used in each experiment was extrapolated from growth curves determined in our laboratory and was confirmed in each experiment by plating and enumeration of CFU on sheep blood agar plates.

TEER measurements. BBEC were seeded onto translucent polyethylene terephthalate (PET) cell culture inserts (8.0- μm average pore size; BD Falcon, San Jose, CA) and cultured with daily medium changes (1 ml of medium on bottom side of insert and 500 μl on top side of insert) for 5 days in RPMI with 10% FBS. The large-pore-size inserts were selected to allow for determination of bacterial paracellular transmigration across the monolayer. TEER was measured using a voltmeter and End-ohm six-chamber cup electrodes (World Precision Instru-

ments, Sarasota, FL). Initial TEER values for each monolayer were determined prior to the beginning of each experiment, and subsequent recordings obtained during the course of the experiment were all normalized to this initial reading. Only monolayers with baseline TEER levels of $\leq 120 \Omega$ ($40 \Omega \text{ cm}^2$) were included in experiments. BBEC were treated with *H. somnus* (25 bacterial cells per endothelial cell), purified lipooligosaccharide (LOS) (0.25 to 10 $\mu\text{g/ml}$), lipopolysaccharide (LPS) (from *Escherichia coli*; 0.25 to 10 $\mu\text{g/ml}$), TNF- α (100 to 500 ng/ml), or conditioned medium derived from BBEC treated with *H. somnus* or were left untreated as negative controls. The TNF- α and LOS concentrations used were similar to or somewhat exceeded those that have been previously reported to increase permeability of endothelial cell monolayers (10, 35, 46, 77).

Conditioned medium was prepared by filtration (0.2 μm) of cell culture medium collected from BBEC incubated with *H. somnus* (25 bacterial cells per endothelial cell) for 4 h. The filtered conditioned medium was streaked on blood agar plates to ensure that there was no carryover of live *H. somnus*. Inhibitors (sTNFR, IL-1 receptor antagonist, and ML-7) were added to the BBEC 30 min prior to the addition of *H. somnus*. Resistance measurements were then made every 30 min and recorded manually.

Paracellular leakage measurements. At the onset of an experiment, medium was exchanged on both sides of the insert and fluorescein isothiocyanate (FITC)-labeled albumin (Sigma, St. Louis, MO) was added to the top side of the insert (5 mg/ml). The top side of the insert contained a final volume of 0.5 ml (medium and albumin), and the bottom side contained 1 ml medium. An insert with no cells was included to determine the maximal amount of albumin leakage. At each time point, 50 μl of cell culture medium was removed from the well on the bottom side of the insert and placed into individual wells in a 96 well plate. The plate was wrapped in foil and placed at 4°C between collection times. Upon completion of the experiment, the fluorescence of the samples was determined using a DTX 880 plate reader (excitation, 485 nm; emission, 535 nm), and albumin leakage across the treated monolayers was calculated as a percentage of albumin leakage across an insert with no cells, using the following equation: percent leakage = fluorescent units (FLU) of medium on bottom side of treated monolayer/FLU of medium on bottom side of monolayer-free insert $\times 100$.

Cytochemical staining for polymerized actin. BBEC were seeded at a density of 100,000 cells per well in Lab-Tek II culture chambers (Nunc, Rochester, NY). The following morning, treated wells were inoculated with *H. somnus* (25 bacterial cells per endothelial cell) and incubated for 30 to 180 min. The monolayers were then washed three times with phosphate-buffered saline (PBS) and fixed for 15 min at 4°C in 4% paraformaldehyde. Following three washes with PBS, the cells were permeabilized in 100 μl 0.2% Triton X-100 in PBS for 5 min. A working solution of FITC-phalloidin (Invitrogen, Carlsbad, CA) was prepared by adding 25 μl of FITC-phalloidin stock solution (200 units per ml in methanol) to 1 ml PBS. This staining solution (100 μl) was added to each well, and the cells were incubated for 20 min at 22°C in the dark. The wells were then washed four times with PBS, and slides were mounted in Prolong Gold antifade reagent with DAPI (4',6'-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA). Images were acquired using a Nikon Eclipse 800 fluorescence microscope.

Western blotting. BBEC were treated with *H. somnus* (25 bacteria per endothelial cell) for 5 to 240 min. BBEC were then washed with PBS and lysed in the tissue culture plates at -80°C in 100 μl MPER buffer (a detergent-based lysis buffer from Pierce, Rockford, IL) supplemented with HALT protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL). Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL) and equal amounts of lysate proteins were run on 4 to 20% sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to 0.2- μm nitrocellulose membranes. The membranes were blocked in Starter Block (Pierce, Rockford, IL) for 15 min at 22°C and incubated with primary antibody diluted in 1% bovine serum albumin-PBS-Tween 20 (PBST) overnight at 4°C . The following primary antibodies were used: anti-phospho(Ser 19)-MLC (1:5,000 dilution; Abcam, Cambridge, MA), polyclonal antioccludin (1:1,000 dilution; Invitrogen, Carlsbad, CA), and anti-phospho(Thr 850)-MYPT (1:1,000 dilution; Upstate/Millipore, Charlottesville, VA). The membranes were washed three times with PBST, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 22°C , and washed three times in PBST. Pierce West Pico substrate solution was used to detect the bound horseradish peroxidase, and bands were visualized by exposure to film. Blots were stripped and reprobed for β -actin using an anti- β -actin antibody (Sigma, St. Louis, MO).

Membrane protein extraction. BBEC were grown to confluence in T-25 culture flasks and incubated for 1 or 3 h with *H. somnus* (25 bacterial cells per endothelial cell). Untreated cells served as negative controls. Following incubation, the endothelial cell membrane proteins were extracted using the Proteo-Extract native membrane extraction kit (Calbiochem, San Diego, CA) as per the

manufacturer's instructions. This extraction method utilizes a differential detergent extraction. The initial detergent extraction only mildly disrupts the cell membrane to allow for the release of cytoplasmic proteins, followed by a more stringent detergent extraction to dissociate membrane-associated proteins from the cell. Briefly, monolayers were washed four times with cold PBS and soluble proteins extracted by a 10-min incubation on ice in 2 ml extraction buffer I with gentle agitation. The soluble fractions were then concentrated using a 10,000-kDa Amicon filtration device (Millipore, Billerica, MA). The membrane-associated proteins were extracted at 4°C by 30 min incubation in 1 ml extraction buffer II with gentle agitation. The protein concentration in each fraction was determined by bicinchoninic acid assay (Pierce, Rockford, IL), and samples were analyzed by Western blotting.

Statistical analysis. Data are presented as the means $\pm$ standard errors of the means (SEM) from at least three independent experiments. Analysis of variance followed by a Tukey posttest for significance was performed using the Prism 3 GraphPad statistical software package.

RESULTS

Incubation of BBEC with *H. somnus* alters monolayer TEER and albumin leakage. *H. somnus* elicited a rapid response from BBEC, characterized by a decline in the TEER and an increase in albumin flux across the monolayer. As shown in Fig. 1A, the TEER values for *H. somnus*-treated BBEC began to decline from the starting value at 60 min and further declined after 120 and 150 min of incubation. TEER is a measurement of the resistance across the total surface area of the monolayer; both paracellular and transcellular (e.g., pore formation and ion flux) resistance levels are reflected in the measurement. To determine if paracellular resistance was affected by *H. somnus*, we compared the leakages of FITC-labeled albumin across *H. somnus*-treated and control monolayers. As shown in Fig. 1B, there was greater leakage of FITC-albumin across the *H. somnus*-treated monolayers than across control monolayers. The increase in FITC-albumin leakage across the monolayers, which is indicative of increased paracellular permeability, lagged slightly behind the drop in TEER. We were able to culture *H. somnus* from the distal side of the insert, indicating that the gaps were sufficient in size to allow passage of the intact organism. After longer incubations (approximately 6 h), the BBEC began to detach from the insert and lactate dehydrogenase release was detectable (data not shown). For this reason, we focused on earlier time points in this study.

Our laboratory has previously demonstrated that *H. somnus* and its LOS have the ability to induce apoptosis in bovine pulmonary artery cells (62). We therefore sought to determine if apoptotic cell death was responsible for the increased permeability of *H. somnus*-treated BBEC monolayers. In the present study, the *H. somnus* induced decline in TEER was not altered by addition of the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk) (data not shown). These data suggest that apoptosis is not a major contributor to the decrease in TEER.

Roles of TNF- α and IL-1 β in the BBEC response to *H. somnus*. We have also previously reported secretion of the vasoactive cytokines TNF- α and IL-1 β (unpublished data) from *H. somnus*-treated BBEC. Therefore, we investigated the role of these cytokines in the *H. somnus*-induced decline in TEER. Figure 2 shows an examination of the roles of soluble factors (*H. somnus* and BBEC derived) and bacterial adhesion in the response of BBEC to *H. somnus*. Conditioned medium collected from *H. somnus*-treated BBEC (4 h with 25 *H. somnus* per endothelial cell) caused a 15% decline in TEER

FIG. 1. *H. somnus* increases paracellular permeability of BBEC monolayers. BBEC cultured on PET transwell inserts were inoculated with *H. somnus* (HS) (25 bacterial cells per endothelial cell) at 37°C. Evan's blue-albumin was added to the proximal sides of the inserts to quantify the paracellular flux across the monolayer. The TEER of each monolayer was measured at the beginning of the experiment and every 30 min thereafter. (A) Data are expressed as the normalized TEER (TEER at that time point divided by the initial TEER for that insert). (B) Data are expressed as the percentage of albumin on the distal side of an insert with BBEC compared to the amount of albumin on the distal side of a control insert with no cells (percent leakage = FLU of medium on bottom side of treated monolayer/FLU of medium on bottom side of monolayer-free insert $\times$ 100). Data are the means $\pm$ SEM from three independent experiments. *, $P < 0.05$ compared to control.

when added to BBEC monolayers (Fig. 2A). This effect was seen only when the culture medium was fully replaced by the conditioned medium. As also displayed in Fig. 2A, removal of unbound *H. somnus*, by replacement of the medium on the top side of the insert 30 min after the onset of the experiment, fully prevented *H. somnus*-induced TEER changes. Addition of sTNFR protected BBEC monolayers against a decline in TEER during a 120-min incubation with *H. somnus* ($P < 0.05$). However, at 180 min, the protective effect of sTNFR no longer reached statistical significance (Fig. 2B). To our surprise, incubation with recombinant TNF- α (up to 500 ng/ml) for up to 4 h had no effect on TEER (Fig. 2C and data not shown). We have previously demonstrated that TNF- α activation of BBEC increases the adherence of *H. somnus* to BBEC (8). To test whether TNF- α could enhance the ability of *H. somnus* to induce permeability, we coincubated BBEC monolayers with *H. somnus* and TNF- α . The coincubation caused a reduction in TEER of the BBEC monolayer that was statistically greater than the decline caused by *H. somnus* alone (Fig. 2C). Therefore, we hypothesize that sTNFR acts by limiting TNF- α activation of BBEC, thereby reducing interaction between *H.*

FIG. 2. Role of soluble mediators and bacterial adhesion in *H. somnus*-induced BBEC permeability. (A) Conditioned medium (CM) from *H. somnus* (HS)-treated BBEC was prepared by filtration (0.2 μ m) of medium from BBEC incubated with *H. somnus* (25 bacterial cells per endothelial cell) for 4 h at 37°C. The CM was then added to fresh BBEC monolayers (100% medium exchange) and incubated for the indicated times, and TEER was measured. Some BBEC were incubated for 30 min with *H. somnus* and then washed three times with PBS (HS wash 30). BBEC incubated with *H. somnus* alone or left untreated were included as positive and negative controls, respectively. Data represent the means $\pm$ SEM from three independent experiments (*, $P < 0.05$ compared to control). (B) sTNFR (100 ng/ml) was added to BBEC 30 min prior to the addition of *H. somnus*. BBEC treated with *H. somnus* alone and untreated BBEC were included as positive and negative controls, respectively. Data are the means $\pm$ SEM from three independent experiments (*, $P < 0.05$ compared to each other and the negative control). (C) BBEC were incubated with TNF- α (100 ng/ml) alone or together with *H. somnus* (25 bacteria cells per endothelial cell). BBEC treated with *H. somnus* alone and untreated BBEC were positive and negative controls, respectively. Data are the means $\pm$ SEM from three independent experiments (*, $P < 0.05$ compared to the negative control and each other). (D) BBEC cultured on transwell inserts were treated for 1 h with 1 U/ml heparinase III at 37°C, washed, and placed in fresh medium before *H. somnus* (25 bacteria per endothelial cell) was added. Cells treated with heparinase alone and cells incubated in medium for the duration of the experiment (3 h) were included as controls. The final change in TEER was calculated based on the TEER recorded prior to the heparinase treatment. Data represent the means $\pm$ SEM from three independent experiments ($P > 0.05$ for *H. somnus* versus *H. somnus* with heparinase).

somnus and the BBEC, rather than by blocking a direct permeability-inducing effect of TNF- α . Heat-killed or formalin-fixed *H. somnus* and *H. somnus* culture filtrates had no detectable effect on monolayer permeability (data not shown).

We have previously demonstrated that *H. somnus* adherence to BBEC is reduced by heparinase III treatment (8). Therefore, we next tested the ability of *H. somnus* to alter TEER of heparinase-treated BBEC. Although the results did not reach statistical significance, heparinase treatment of BBEC caused a modest reduction in the ability of *H. somnus* to decrease TEER (Fig. 2D).

LPS has been shown by other investigators to cause a decline in the barrier function of endothelial cell monolayers (7, 35, 70). However, we found that BBEC monolayers treated with either *H. somnus* LOS or *E. coli* LPS (500 ng/ml to 10 μ g/ml) showed no decline in TEER over a 4-h incubation (data not shown). We also sought to determine if IL-1 β , another vasoactive cytokine, contributed to *H. somnus*-induced declines in

TEER. The addition of IL-1 receptor antagonist (100-fold excess based on determination of IL-1 β in culture medium by enzyme-linked immunosorbent assay) had no effect on TEER at any time point tested (data not shown). Furthermore, the addition of 100 ng/ml bovine IL-1 β had no effect on BBEC monolayer resistance in our hands. Together, these data suggest that bacterial adherence of live *H. somnus* and the subsequent production of soluble mediators (e.g., TNF- α) from BBEC act in concert to enhance BBEC monolayer permeability.

Actin rearrangement in BBEC incubated with *H. somnus*. We next sought to determine what cellular events were contributing to the endothelial cell response to *H. somnus*. Increases in polymerized actin (F-actin) have been shown to correlate temporally with the development of isometric tension in endothelial cells (30). We stained *H. somnus*-treated and control BBEC with phalloidin, a phalloxin that binds actin only in the polymerized form, to assess actin polymerization and distribution in response to incubation with *H. somnus*. As

FIG. 3. *H. somnus* causes F-actin rearrangements in BBEC. BBEC were cultured for 5 days on Lab-Tek culture chambers. *H. somnus* was added (25 bacterial cells per endothelial cell), and the BBEC were incubated at 37°C for 30 to 120 min. BBEC monolayers were fixed in 4% paraformaldehyde for 15 min at 4°C, washed three times with PBS, and stained for polymerized F-actin with FITC-phalloidin (green). DAPI counterstain (blue) was used to identify nuclei. Inter-endothelial cell gaps (IEG) are prominent after 60 min of incubation. Incubation with *H. somnus* resulted in an increase in stress fiber number and prominence. Photos are representative of three independent experiments. Magnification, $\times 600$.

shown in Fig. 3, BBEC incubated with *H. somnus* (25 bacterial cells per endothelial cell) exhibited rapid rearrangements of the actin cytoskeleton. Formation of inter-endothelial cell gaps and an increased number and prominence of stress fibers were evident after 60 min of incubation with *H. somnus*. These became more pronounced and the gaps became more widespread and prominent after 120 min. Similar morphological changes were observed, but were more difficult to stain and visualize by microscopy, when the cells were stained on the PET culture inserts.

Alteration in occludin in BBEC incubated with *H. somnus*.

Based on the above observations, we hypothesized that alterations in cell conformation and actin polymerization would lead to loss of tight junction proteins from the membrane. To address the effect of *H. somnus* on tight junction protein localization, we examined the expression and distribution of occludin in *H. somnus*-treated and control BBEC. Phosphorylation of occludin, which increases the molecular weight of the protein and results in an upward shift in gel migration, has been linked to its localization in tight junctions and decreased cell permeability (78). As shown in Fig. 4A, the level of phosphorylated occludin in total cell lysates decreased over the course of a 4-h incubation with *H. somnus*, as indicated by the relative decline in the portion of occludin migrating in the higher-molecular-weight fraction. Despite the apparent change in phosphorylation, occludin was identified only in the membrane-associated fraction of the cell lysates at 1 and 3 h of incubation with *H. somnus* (Fig. 4B). The majority of occludin identified in the membrane-associated cell fractions was of higher molecular weight and hence phosphorylated. Taken together, these data indicate that there is a decrease in total cellular phosphorylated occludin following *H. somnus* treatment but that the fraction of phosphorylated occludin associated with the membrane remains unchanged.

MLC phosphorylation in BBEC incubated with *H. somnus*.

The rapidity of the decline in TEER and the lack of occludin in the soluble cell fraction led us to hypothesize that active contraction of the actin-myosin cytoskeletal network was involved in mediating the BBEC response to *H. somnus*. Polymerized actin acts in concert with myosin to mediate cell contraction. Phosphorylation of the MLC alone is sufficient to cause endothelial cell contraction (79). Therefore, we hypothesized that *H. somnus* would induce BBEC contraction via phosphorylation of MLC. To address the role of MLC in *H.*

somnus-induced BBEC contraction, we analyzed MLC phosphorylation by Western blotting. Phosphorylation of MLC in BBEC increased after 60 min of incubation with *H. somnus* and remained elevated at 2 h (Fig. 5A). One of the primary kinases responsible for phosphorylation of MLC is the Ca^{2+} /calmodulin-sensitive MLCK. We used ML-7 to pharmacologically inhibit MLCK. The forces generated by MLC phosphorylation and contraction of the cytoskeleton could be countered by changes at cell-cell junctions. The lack of occludin redistribution in response to *H. somnus* suggested that this might be the case. Therefore, we tested the ability of ML-7 to protect against the decline in TEER in *H. somnus*-treated BBEC. As shown in Fig. 5B, ML-7 reduced by 25% the *H. somnus*-mediated decline in TEER. The ability of ML-7 to reduce MLC phosphorylation was confirmed by Western blotting (Fig. 5C). Addition of the Rho-associated protein kinase inhibitor (Y-27632; Calbiochem) at doses ranging from 1 to 10 μM did not alter the *H. somnus*-induced decline in TEER (data not shown).

FIG. 4. Occludin remains in the membrane fraction in *H. somnus*-treated BBEC, despite a decline in phosphorylation. (A) BBEC were incubated for 5 min to 3 h with *H. somnus* (25 bacterial cells per endothelial cell). The BBEC were lysed and equal protein quantities of the lysates (60 μg) probed for occludin by Western blotting. The arrow indicates the slower-migrating, phosphorylated occludin, which decreases in the total cell lysate over time. (B) BBEC were incubated for 1 or 3 h with *H. somnus* (25 bacterial cells per endothelial cell). Detergent-soluble (S) and insoluble BBEC membrane (M) fractions were isolated from *H. somnus*-treated (HS) and control (C) BBEC and probed for occludin by Western blotting. In this set of experiments, 6 μg of protein was loaded in each lane. Occludin was detected only in the membrane fractions in both *H. somnus*-treated and control BBEC. The phosphorylated occludin (higher-migrating band) is concentrated in the membrane fraction in both control and *H. somnus*-treated cells. Data are representative of three independent experiments.

FIG. 5. MLCK contributes to *H. somnus*-induced BBEC permeability. (A) BBEC were treated for 5 to 120 min with *H. somnus* (25 bacterial cells per endothelial cell) and lysates probed for phosphorylated MLC (p-MLC) by Western blotting. β -Actin was used as a control for protein loading. (B) BBEC were preincubated for 30 min with the MLCK inhibitor ML-7 (20 μ M) prior to their incubation with *H. somnus* (25 bacterial cells per endothelial cell). BBEC treated with *H. somnus*, and untreated BBEC were included as positive and negative controls, respectively. TEER values were normalized to the resistance of each monolayer at the outset of the experiment. Data are the means $\pm$ SEM from three independent experiments (*, $P < 0.05$ compared to control BBEC). (C) BBEC were pretreated with 20 μ M of the MLCK inhibitor ML-7 prior to incubation with *H. somnus* (hs+ML-7) for 120 min. BBEC lysates were then analyzed for p-MLC by Western blotting. Data are representative of three independent experiments. (D) BBEC were incubated for 30 to 180 min at 37°C with 25 *H. somnus* cells per endothelial cell, and cell lysates were probed for phosphorylated MYPT by Western blotting. β -Actin was used as a control for protein loading. Data are representative of three independent experiments.

Increased MLC phosphorylation could also result from inhibition of MYPT. MYPT is inhibited by phosphorylation of its myosin binding subunit (73). We therefore examined *H. somnus*-treated BBEC cell lysates for phosphorylated MYPT. As shown in Fig. 5D, BBEC levels of phosphorylated MYPT were modestly increased after 2 and 4 h of incubation with *H. somnus*.

DISCUSSION

In this study, we demonstrate that *H. somnus* causes a rapid decline in the ability of BBEC monolayers to maintain electrical resistance (TEER) and restrict paracellular leakage of albumin. The decline in TEER occurred prior to the increase in albumin leakage. There are multiple explanations for this lag. First, we cannot rule out the possibility that transcellular changes precede paracellular gap formation in our system. Second, the TEER measurement is more sensitive than the optical determination of albumin leakage. Third, albumin is a relatively large molecule, and we would expect a need for paracellular gap formation to be correspondingly large to allow passage of a molecule of albumin's size. Finally, the extracellular matrix may remain intact for a period of time following cell contraction, allowing for reductions in TEER prior to leakage of albumin.

Our laboratory has previously reported that *H. somnus* and its LOS can induce apoptosis in bovine pulmonary endothelial cells. However, in the present study, addition of the pan-caspase inhibitor z-VAD-fmk had no effect on the *H. somnus*-induced decline in TEER. We considered the possibility that our cell line may be hyporesponsive as a result of transformation or repeated transfer in culture. This does not appear to be the case, because levels of TNF- α secreted by *H. somnus*-

treated BBEC are indistinguishable from those secreted by primary bovine pulmonary endothelial cells treated with the same number of bacteria (data not shown). Furthermore, this BBEC line has been used extensively by others to investigate the pathogenesis of a number of bacterial pathogens, including *E. coli* (3, 50) and *Streptococcus pneumoniae* (82).

Perhaps the timing of events is a critical factor. Apoptosis of *H. somnus*-treated primary bovine pulmonary endothelial cells is minimal at the early time points investigated in this study. *H. somnus*-treated BBEC do undergo appreciable levels of apoptosis following longer periods of incubation (data not shown). The link between MLCK activity and apoptosis is controversial. Although some investigators have demonstrated that pharmacological inhibition of MLCK protects cells against apoptosis (14), others have shown reduced MLCK activity to promote cell death (22). Clearly, the mechanistic coupling of cytoskeletal alterations and apoptosis, in *H. somnus*-induced vasculitis and in other systems, requires further investigation.

We found that conditioned medium from *H. somnus*-treated monolayers modestly decreased TEER when added to fresh BBEC monolayers. TNF- α has been reported by others to increase permeability of BBEC monolayers (46). However, some investigators have reported no alterations in brain endothelial permeability in response to TNF- α . For example, Schnell et al. (56) reported that stereotactic injection of TNF- α into the striata of rats resulted in no alteration of blood-brain barrier integrity. Seynhaeve et al. (58) recently reported that high doses of TNF- α had no impact on tumor endothelial cells in vitro, while coadministration of TNF- α and interferon caused severe morphological changes and inter-endothelial cell gap formation. In our system, addition of TNF- α alone did not cause any change in TEER. However, addition of an

sTNFR reduced the *H. somnus*-induced decline in TEER, and incubation of BBEC with TNF- α in combination with *H. somnus* resulted in a decline in TEER which was greater than that when the BBEC were incubated with *H. somnus* alone.

We have previously reported that TNF- α treatment enhances *H. somnus* adherence to BBEC (8). Our data suggest that addition of sTNFR prevented TNF- α -mediated activation of BBEC, thereby decreasing the interaction between BBEC and *H. somnus* and consequently reducing the *H. somnus*-induced decline in TEER. The reduced response of heparinase-treated BBEC, which we previously demonstrated have a decreased ability to bind *H. somnus* (8), also supports a role for bacterial adhesion in *H. somnus*-induced reductions in TEER. We interpret our data as suggesting that bacterial contact with BBEC is important in the induction of cell contraction. The initial interaction between *H. somnus* and the BBEC, however, is not sufficient to cause the decline in TEER observed when BBEC were incubated for longer times with the bacteria. The effect was lost when unbound *H. somnus* and the culture medium were removed 30 min after the onset of the experiment. Release of TNF- α or other factors produced by the BBEC in response to adherent *H. somnus* could play a role in promoting further interaction between *H. somnus* and the BBEC and hence increasing adhesion-mediated events that trigger cell contraction. Similar to our observations, *Neisseria meningitidis* adhesion-deficient strains are unable to induce the expression of TNF- α in endothelial cells (65).

The lack of a TEER response to TNF- α alone could be due to the absence of a required additional signal. For example, in a recent study by Friedl et al. (24), human umbilical vein endothelial cells (HUVEC) treated with TNF- α for 90 to 270 min showed no increase in permeability. However, when the HUVEC were incubated with TNF- α in the presence of factor VIII-deficient plasma, monolayer permeability increased within minutes. In the same study, antibodies against tissue factor eliminated the response to factor VIII-deficient plasma, indicating a role for the extrinsic coagulation cascade. Perhaps our cell culture system lacks components, such as active clotting factors, required to facilitate an optimal permeability response to TNF- α . We have previously demonstrated an increase in tissue factor activity in *H. somnus*-treated BBEC (unpublished data). Perhaps, as in the previously described study, addition of plasma proteins would promote TNF- α -mediated permeability in BBEC.

Both in vivo (9) and in vitro (77) studies have demonstrated that IL-1 β -induced brain endothelial permeability occurs after incubation times that exceed those used in the present study. Our laboratory has previously demonstrated that signaling through the IL-1 β receptor diminishes *H. somnus* LOS-mediated apoptosis (63). Therefore, we have evidence to support the effectiveness of the receptor antagonist in a bovine system. In our hands, IL-1 β does not appear to play a role in promoting *H. somnus*-induced permeability of BBEC. Similar to the case for TNF- α , it has recently been demonstrated that additional signals, such as tissue factor activity, are often required to elicit permeability changes in response to IL-1 β (52). Perhaps our in vitro system lacked a component required for an optimal response to IL-1 β .

Our data suggest that a soluble bacterial factor was not principally responsible for the *H. somnus*-induced decline in

TEER of BBEC monolayers, because neither heat-killed *H. somnus* nor culture filtrates had an effect on TEER. Other *H. somnus*-mediated events have also been reported to require viable bacterial cells. Gomis et al. (31) reported that infection of bovine alveolar macrophages with live *H. somnus* decreased their ability to phagocytose *Staphylococcus aureus*, while infection with heat-killed organisms had no effect on phagocytic function. The major outer membrane protein of *H. somnus* is a heat-modifiable protein highly homologous to outer membrane protein A (OmpA) of *E. coli* K-12 (64). Endothelial cell adherence by *E. coli* is dependent on OmpA (17, 51), and cellular attachment of *Neisseria* to eukaryotic cells is also mediated by its outer membrane proteins (32). Perhaps the heat-modifiable outer membrane protein of *H. somnus* initiates the cytoskeletal changes observed in BBEC, and this activity is lost when the bacterial cells are heated.

We were surprised at the inability of LOS or LPS to increase BBEC permeability. LPS has been shown by other investigators to cause a decline in the barrier function of endothelial cell monolayers (7, 35, 70), and the lipid A portion of LPS and LOS is highly conserved. However, the inability of heat-killed *H. somnus* or culture filtrates to alter TEER of the BBEC monolayer also speaks against a role for LOS in *H. somnus*-induced BBEC contraction. In other experiments performed in our lab, BBEC did not secrete TNF- α after a 6-h incubation with LOS, while they secreted more than 800 pg/ml of TNF- α after incubation with live *H. somnus*. The levels of TNF- α produced were indistinguishable between BBEC and primary bovine pulmonary endothelial cells that were incubated with *H. somnus* (data not shown). Furthermore, in experiments investigating the permeability of primary bovine pulmonary microvascular endothelial cells, the LPS-induced decline in TEER did not occur prior to 12 h of treatment (data not shown). Previous studies investigating the ability of LPS to induce permeability changes in brain endothelial cell monolayers focused on later time points than those used in the present study (77), confounding a direct comparison with our results. Pulmonary endothelial cell studies that focused on early events demonstrated at least a 2-h lag time between LPS-induced actin changes and alterations in permeability (6).

We also considered the possibility that BBEC might lack critical signaling components needed to respond to LOS. Perhaps the nonresponsiveness to LOS could be the result of inadequate serum levels of CD14 or LPS binding protein (LBP) in our culture system. In a study performed by Bannerman and Goldblum (6), a 200,000-fold increase in LPS concentration was required to induce monolayer permeability in the absence of LBP and CD14. However, we detected Toll-like receptor 4 in BBEC cell lysates by Western blotting (data not shown) and LOS concentrations of as high as 10 μ g/ml had no deleterious effect on BBEC monolayer permeability. Furthermore, all experiments were performed in the presence of 10% FBS, which should provide LBP.

The compromised barrier function of *H. somnus*-treated BBEC monolayers correlated with rearrangement of the actin cytoskeleton and formation of inter-endothelial cell gaps. Paracellular gaps lead to a loss of vascular barrier function in a number of disease states. For example, in pneumolysin-induced vascular injury, development of lung edema in vivo correlated with increased paracellular permeability in toxin-

treated HUVEC monolayers in vitro (75). Likewise, the formation of inter-endothelial cell gaps preceded plasma leakage in a rat tracheal model of substance P-mediated vascular leakage (5). *H. somnus* caused the formation of inter-endothelial cell gaps in BBEC monolayers in vitro. The formation of these gaps typically results from an imbalance in the forces that regulate endothelial cell shape and cell-to-cell connectivity (44). If similar changes occur in vivo, the paracellular gaps could allow dissemination of *H. somnus* and leakage of systemic inflammatory mediators into the brain parenchyma and could contribute directly to the clinical and pathological findings associated with TME.

Activation of MLCK and phosphorylation of MLC contribute to decreased barrier function of intestinal and endothelial cells in a number of disease states. For example, increased intestinal epithelial cell permeability in response to infection with *Helicobacter pylori* (23) or enteropathogenic *Escherichia coli* (81) is MLCK dependent. Cytotoxic necrotizing factor 1, a cytotoxin of enteropathogenic *E. coli*, increases MLC phosphorylation by activating Rho GTPase, without inhibiting MYPT (20). In contrast, *Pasteurella multocida* toxin increases endothelial cell permeability by activation of Rho/Rho kinase and inhibition of MYPT (19). Rho/Rho kinase inhibition of MYPT also induces the formation of actin stress fibers and intercellular gaps in endothelial cells treated with mildly oxidized low-density lipoprotein (21). Inhibition of MLCK reduced phosphorylation of MLC and actin stress fiber formation in burn plasma-treated rat lung microvascular endothelial monolayers and attenuated burn-induced increases in permeability of mesenteric venules (37, 69). Although the role of MLCK in bacterially mediated endothelial cell contraction has not been well characterized, inhibition of myosin ATPase activity and MLCK was reported to significantly reduce *E. coli* invasion of brain microvascular endothelial cells in vitro (55).

In the present study, the *H. somnus*-induced increase in BBEC paracellular permeability correlated with phosphorylation of MLC and was partly dependent on MLCK. We were unable to completely eliminate phosphorylation of MLC by using concentrations of the inhibitor ML-7 that were in fivefold excess of the concentration that inhibits 50% of the MLCK activity. This observation might be explained in at least three ways. First, brain endothelial cells may have efflux pumps that are capable of reducing the effective intracellular concentration of ML-7. Second, an alternative kinase (e.g., a P21-activated kinase) may be responsible for a portion of MLC phosphorylation (55). Third, ML-7 may be less effective at inhibiting bovine than human MLCK. *H. somnus*-treated BBEC also had increased levels of phosphorylated (and hence inactive) MYPT, although this increase occurred later than phosphorylation of MLC. This finding is consistent with previous reports regarding the regulation of endothelial cell contraction. In those studies, MLC phosphorylation in thrombin-stimulated endothelial cells occurred within seconds, peaked at 1 to 2 min, and then was rapidly reversed (28). On the other hand, inhibition of phosphatase activity in endothelial cells results in increased phosphorylation of MLC and increased permeability, which are evident for at least 1 h (73). In *H. somnus*-induced endothelial monolayer permeability, the initial increase in MLC phosphorylation appears to be partly the result of MLCK activity. Perhaps inhibition of MYPT plays a

FIG. 6. Proposed model. *H. somnus* adherence to the BBEC promotes cell contraction and subsequent vascular permeability. We infer from these data and prior results from our lab that *H. somnus*-induced BBEC permeability is dependent on bacterial adhesion. We hypothesize that heparan-sulfate proteoglycans, previously shown to be involved in *H. somnus* adherence to BBEC, play an initiating role in the cascade of events that lead to cell contraction. Heparan-sulfate proteoglycans could act as signal transducers or facilitate the interaction between *H. somnus* and another, unidentified signaling component. MLC phosphorylation and MLCK activity play a role in *H. somnus*-induced permeability. Activation of MLCK is Ca^{2+} dependent. Therefore, we also hypothesize that Ca^{2+} elevations (via either pore formation or release of intracellular stores) also contribute to *H. somnus*-mediated permeability.

role in prolonging the contractile response. Due to the relative insensitivity of Western blotting, however, we do not know if very low levels of MYPT phosphorylation occur at earlier time points. If so, this could contribute to the portion of *H. somnus*-induced BBEC permeability that is ML-7 insensitive.

Activation of MLCK can cause increased epithelial cell permeability that is partially characterized by redistribution of occludin (59). While we were able to detect a decrease in phosphorylated occludin in total cell lysates, occludin was detectable only in membrane-associated fractions in *H. somnus*, which was likely a result of the lesser amount of protein that was examined in the membrane fractions. More importantly, there was no detectable alteration in the relative component of occludin that remained in the membrane following exposure to *H. somnus*. These data might indicate that occludin localization is regulated differently in brain endothelial cells than in epithelial cells. We also focused on the early events contributing to BBEC permeability. Longer incubations of BBEC with *H. somnus* and a prolonged contractile response may have a more obvious effect on occludin distribution. Phosphorylation of occludin often correlates with decreased permeability in both epithelial and endothelial cells (54, 78). Perhaps the decrease in phosphorylated occludin that we observed in *H. somnus*-treated BBEC contributed to monolayer permeability without causing a detectable redistribution of occludin within the cells.

In summary, this study shows that the presence of viable *H. somnus* increases BBEC monolayer permeability in part by triggering contraction of endothelial cells. Although $\text{TNF-}\alpha$ and other BBEC-derived soluble mediators appear to play some contributory role, our data support a key role for bacterial adherence in the process. These observations may provide new insights into

how *H. somnus* causes meningoencephalitis without directly invading brain endothelial cells. We have previously reported a lack of brain endothelial cell invasion by *H. somnus* in vitro (8), which is rather unusual for a meningeal pathogen. In contrast, encapsulated strains of *Neisseria meningitidis* are capable of invading human brain endothelial cells (49), and both *Streptococcus suis* serotype 2 (71) and *Haemophilus parasuis* (72) invade porcine brain microvascular endothelial cells. The ability of *H. somnus* to elicit rapid cytoskeletal rearrangement, resulting in the formation of large intercellular gaps, could compensate for the lack of direct endothelial cell invasion. Viewed in the context of TME, inter-endothelial cell gaps and increased paracellular permeability could open a portal for cytokines, reactive oxygen species, activated leukocytes, and other components of the inflammatory response to enter the CNS and cause neural pathology. *H. somnus* may be unique in its ability to induce such a devastating neurological disease without invading the brain endothelial cell. In Fig. 6 we propose a model that illustrates a potential series of events that could contribute to the CNS pathology associated with TME. Because this is the first study to identify a bacterial pathogen of the CNS that induces endothelial cell contraction, we do not know whether other bacteria also use this mechanism to promote CNS inflammation without cellular invasion.

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