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Viability of microvascular endothelial cells to direct exposure of formalin, λ-carrageenan, and complete Freund's adjuvant

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Abstract

We investigated three inflammatory agents to establish if these substances elicit a direct effect on the functional and structural integrity of the blood–brain barrier. Cellular cytotoxicity and paracellular permeability were assessed in vitro using primary bovine brain microvascular endothelial cells exposed to formalin, λ-carrageenan, or complete Freund's adjuvant for 1, 3, or 72 h, respectively. Results showed that only the highest concentration (0.025%) of formalin produced a decrease in cell viability (~ 34%) and a significant increase in cell permeability to [^{14}C]sucrose at 120 min (~ 137%). Brain perfusion using female Sprague–Dawley rats showed no difference in paracellular permeability to [^{14}C]sucrose for any inflammatory agent. Western blot analyses were performed on isolated rat brain microvessels to assess the structural integrity of blood–brain barrier tight junctions. Results indicate that expression of zonula occludens-1, occludin, claudin-1, and actin remain unchanged following intravenous exposure to inflammatory agents. This study confirms that changes seen at the blood–brain barrier following a peripheral inflammation are due to physiological responses to the given inflammatory agent and not to any direct interaction between the inflammatory agent and the brain microvasculature.

Keywords: Inflammation; Tight junctions; Pain; Occludin; Zonula occludens-1; Blood–brain barrier

1. Introduction

Research involving peripheral inflammation has been used for several decades to assess pain neurobiology and its contributions have created a number of well-characterized pain models to evaluate the profile of nociceptive, behavioral, and physiological components of noxious stimuli (Lidow et al., 2001; Porro and Cavazzuti, 1993; Brown et al., 1968; Winter et al., 1962).

To date, most research using pain models has focused on specific parameters of pain (such as nociception, immune response, neurotransmitter release, etc.) or the efficacy of anti-inflammatory drugs (Gilbert and Frankin, 2001; Lidow et al., 2001; Saleh et al., 1996). Recently, we evaluated three well-characterized inflammatory pain models (formalin, λ-carrageenan, and complete Freund's adjuvant) to determine their effects on cerebral microvascular beds. These inflammatory pain models have different onsets of action, duration times, and physiological responses at the site of action. Our current findings show that all three inflammatory agents affected blood–brain barrier permeability (Huber et al., 2001a). The structural and functional changes seen during systemic inflammatory pain have thus far been credited to the pathophysiological response of the inflammatory agents through a combination of immune, hormonal, and neuronal responses. However, a primary concern exists as to whether the inflammatory agents can elicit a direct effect on the blood–brain barrier by absorbing into the systemic circulation from the site of injury and subsequently affect cerebral microvascular endothelial cells.

Using primary cultured bovine brain microvessel endothelial cells, we assessed the cellular viability and paracellular permeability of a confluent monolayer in the presence of each of the three inflammatory agents. The high concentration of each agent was calculated as the amount present if the entire bolus given via a subcutaneous injection into the paw were absorbed into the peripheral circulation at

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once. Lower concentrations of each substance were assessed to determine if any effects would be seen at levels several fold less than maximal, which would be more indicative of absorption from the site of injection. Furthermore, we assessed the functional and structural integrity of an intact blood–brain barrier using an in situ brain perfusion and Western blot analyses of isolated cerebral microvessels following an i.v. bolus of an inflammatory agent at a concentration equivalent to the amount injected subcutaneously into the hind paw. The results of this investigation clearly show that the functional and structural alterations seen at the blood–brain barrier following a peripheral inflammatory insult are due to physiological actions in response to the inflammatory pain and not due to any direct interaction between the blood–brain barrier microvasculature and the inflammatory-inducing agent.

2. Materials and methods

2.1. Radioisotopes/antibodies/chemicals

[^{14}C]Sucrose was obtained from ICN Pharmaceuticals (specific activity: 492 mCi/mmol, >99.5% purity; Irvine, CA). Primary antibodies (anti-zonula occludens-1, anti-occludin, anti-claudin 1) were obtained from Zymed (San Francisco, CA). Conjugated anti-rabbit immunoglobulin G and anti-mouse immunoglobulin G-horseradish peroxidase were purchased from Amersham Life Science Products (Springfield, IL). Anti-actin and all other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

2.2. Animals/treatments

Female Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–300 g were housed under standard 12 h light/dark conditions and received food ad libitum. All protocols involving animals were approved by the University of Arizona Institutional Animal Care and Use Committee and abide by NIH guidelines. Rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.) and subsequently injected (100 μl; i.v.) with inflammatory agent (5% formalin, 3% λ-carrageenan, or 50% complete Freund's adjuvant) into the tail vein and placed into two groups. Concentrations of inflammatory agents were equivalent to the amount commonly injected into the hind paw when conducting nociceptive testing. The first group of animals underwent a 20-min in situ brain perfusion at 1, 3, or 72 h post-injection, respectively. These time points represent commonly used time points for nociceptive testing of each agent (Wei et al., 1999; Nozaki-Tagaguchi and Yamamoto, 1998; Winter et al., 1962). Animals from the second group were anesthetized with sodium pentobarbital and the brains were harvested at 1, 3, or 72 h for Western blot analyses. Control animals were injected (100 μl, i.v.) with 0.9% saline into the tail vein. Naïve controls showed no significant difference in BBB alterations compared to the saline-treated controls and were therefore not included in this study.

2.3. Bovine brain microvessel endothelial cell isolation

Fresh bovine brains were obtained from the University of Arizona Animal Sciences Meat Laboratory. Bovine brain microvessel endothelial cells were isolated from cerebral cortex gray matter and cryopreserved, as previously described (Audus and Borchardt, 1987). Isolated cells were seeded at a cell density of 50,000 cells/cm onto rat tail collagen and fibronectin coated Transwells® containing a 25 mm polyester membrane insert with a 0.4 μm pore size or 12-well tissue culture plates (Costar Nucleopore, Cambridge, MA). Bovine brain microvessel endothelial cells were grown to confluent monolayers (10–12 days) prior to being used for cell viability or transendothelial cell permeability studies. Endothelial cells used for these studies were primary cultured cells, which have been shown to maintain excellent blood–brain barrier characteristics as well as a good in vivo correlation (i.e., 0.75) (Weber et al., 1993; Brownson et al., 1994; Abbruscato et al., 1996, 1997).

2.4. Endothelial cell viability assay

Viability of bovine brain microvessel endothelial cells following exposure to formalin, λ-carrageenan, or complete Freund's adjuvant was assessed using the MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) cytotoxicity assay (Hansen et al., 1989). Confluent bovine brain microvessel endothelial cell monolayers were exposed to formalin (0.025% and 0.0025%), λ-carrageenan (0.015% and 0.0015%), or complete Freund's adjuvant (0.25% and 0.025%) for (1, 3, and 72 h, respectively). Cell culture medium was removed and cells were rinsed with phosphate buffered saline (PBS) and incubated with 200 μl of MTT (5 mg/ml) for 2 h at 37 °C. Excess MTT was removed and cells rinsed with PBS prior to solubilizing with 200 μl of a 50:50 mixture of dimethyl formamide and 20% (w/v) sodium lauryl sulfate (pH 4.7). Absorbance readings were taken at 550 nm using a Lab systems Multiskan RC™ microplate reader (Fisher, Tustin, CA). Cell viability was expressed as a percent of control bovine brain microvessel endothelial cells (untreated).

2.5. Bovine brain microvessel endothelial cell monolayer permeability to [^{14}C]sucrose

Passage of [^{14}C]sucrose across bovine brain microvessel endothelial cell monolayers were used to assess paracellular permeability changes induced by three inflammatory pain models, formalin (0.025%, 0.0025%, 0.00025%), λ-carrageenan (0.015%, 0.0015%, 0.00015%), or complete Freund's adjuvant (0.25%, 0.025%, 0.0025%) for 1, 3, or 72 h, respectively. Following treatment, confluent bovine brain microvessel endothelial cell monolayers were incu-

bated with assay buffer, consisting of (122 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose and 0.4 mM K_2HPO_4) for 30 min at 37 °C. Paracellular permeability across bovine brain microvessel endothelial cell monolayers was determined by adding [^{14}C]sucrose (0.5 μCi) to the luminal side (upper compartment of Transwell®) and samples (50 μl) were removed from the abluminal side (lower chamber of Transwell®) at 0 and 120 min and replaced with fresh assay buffer. Concentrations of [^{14}C]sucrose applied to the luminal side were determined by removing samples (50 μl) at time zero. Amount of radioactivity in the samples from permeability studies was determined using a Beckman LS5000 TD beta-counter (Fullerton, CA). Permeability coefficients (PC) for [^{14}C]sucrose were expressed as previously described (Deli et al., 1995) where V = volume in receiver chamber (1.5 cm^3), SA = surface area of cell monolayer (1 cm^2), C_d = concentration of marker in donor chamber at time zero, and C_r = concentration of marker in receiver at time (T).

$$\mathrm{PC(cm/min)} = \frac{V}{\mathrm{SA}^* C_d}\frac{C_r}{T}$$

2.6. In situ brain perfusion

Following treatment with inflammatory agents, rats were anesthetized, as above, and heparinized (10,000 U/kg). Body temperature was maintained using a heating pad. The right common carotid artery was exposed and cannulated with silicone tubing connected to a perfusion circuit. Perfusate consisted of a modified mammalian Ringer's solution (117 mM NaCl, 4.7 mM KCl, 0.8 mM $MgSO_4$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 10 mM D-glucose, dextran [MW 70,000; 10 g/l]; bovine serum albumin-type V, 1 g/l, pH 7.4) (Preston et al., 1995). Addition of Evan's blue (55 mg/l) to the Ringer's solution provided a control for blood–brain barrier integrity. Perfusate was aerated with 95% O_2/5% CO_2, and warmed to 37 °C. The ipsilateral vein was sectioned to allow drainage. Once the desired perfusion pressure and flow-rate were achieved (85–95 mm Hg; 3.1 ml/min, respectively), the contralateral carotid artery was cannulated and perfused as described above. Radiolabeled sucrose was infused into the inflow of the perfusate using a slow-drive syringe pump (0.5 ml/min/hemisphere; model 22: Harvard Apparatus, South Natick, MA) for 20 min. The animal was decapitated and brain was removed. Choroid plexi and meninges were excised and cerebral hemispheres sectioned and homogenized. Perfusate containing the radiolabeled marker was collected from each carotid cannula at the termination of the perfusion to serve as a reference.

Cerebral hemispheres (~ 500 mg) and 100 μl of perfusate were prepared for liquid scintillation counting by adding 1 ml of tissue solubilizer (TS-2; Research Products International, Mount Pleasant, IL). After 2 days of solubilization, 100 μl of 30% glacial acetic acid was added to

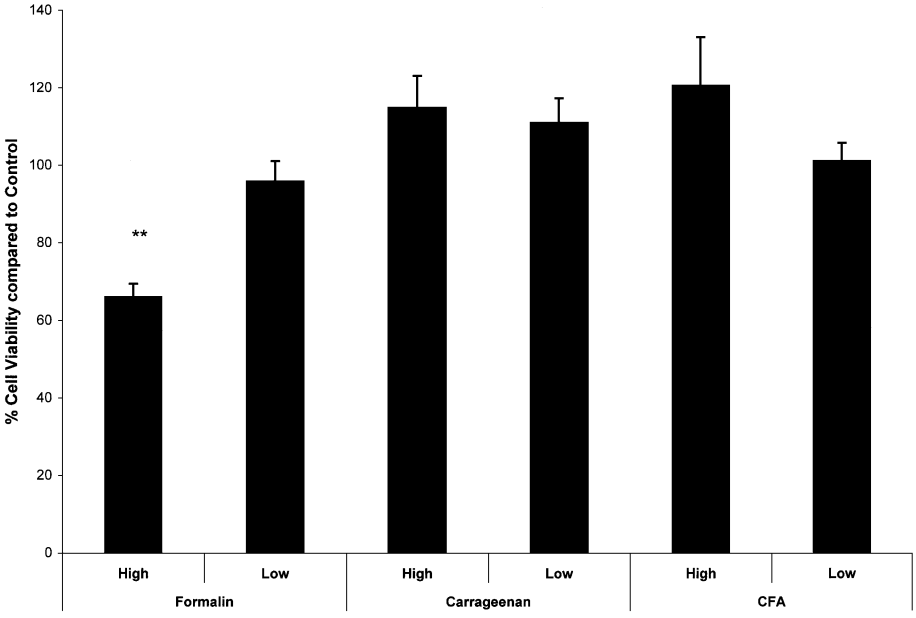

Fig. 1. MTT cell viability study conducted on bovine brain microvessel endothelial cell monolayers (primary culture, 10–12 days confluent) following incubation in media conditioned with an inflammatory agent (formalin, λ-carrageenan, or complete Freund's adjuvant). High (0.025%, 0.015%, and 0.25%, respectively) and low (0.0025%, 0.0015%, and 0.025%, respectively) concentrations of each inflammatory agent were added to the media for a preset period of time (formalin–1 h, λ-carrageenan–3 h, and complete Freund's adjuvant—72 h). The high concentration of each inflammatory agent was designated as the amount present if the entire amount given via a subcutaneous injection into the paw were absorbed into the peripheral circulation at once, while the lower concentration was used to determine if any effects may be seen at levels several fold less than maximal. Each bar represents mean ± S.E.M. (n = 6). Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc test. ** Indicates (P < 0.01) significance between treatments as compared to control. No significant interactions were observed.

eliminate chemiluminescence. Four milliliters of Budget Solve Liquid Scintillation Cocktail (Research Products International) were added and samples measured for radioactive counts (model LS 5000 TD Counter; Beckman Instruments).

2.7. Capillary depletion

Measurement of the vascular component to total brain uptake was performed using capillary depletion (Triguero et al., 1990). After a 20 min in situ brain perfusion, the brain was removed and the choroid plexi and meninges excised. Homogenization procedures were performed within 2 min of sacrificing the animal. Brain tissue (50 mg, wet weight) was homogenized (Polytron homogenizer; Brinkman Instruments, Westbury, NY) in 1.5 ml of capillary depletion buffer [10 mM 4-(2-hydroxyethyl)-piperaxineethane sulfonic acid; 141 mM NaCl; 4 mM KCl; 2.8 mM $CaCl_2$; 1 mM $MgSO_4$; 1 mM NaH_2PO_4; 10 mM, D-glucose; pH 7.4] and kept on ice. Two milliliters of ice-cold 26% clinical grade dextran was added and homogenization repeated. Aliquots of homogenate were centrifuged at $5400 \times g$ for 15 min. Capillary-depleted supernatant was separated from the vascular pellet. Homogenate, supernatant, and pellet were taken for radioactive counting. The amount of [^{14}C]sucrose in the brain homogenate, supernatant, and pellet was expressed as the percentage ratio of tissue (C_{Tissue} disintegrations/min/g of disintegrations/min/ml) to perfusate activities ($C_{Perfusate}$ disintegrations/min/ml) and expressed as R_{Brain}%.

$$R_{Brain}\% = (C_{Brain}/C_{Perfusate}) \times 100$$

2.8. Microvessel isolation

At each time point, following inflammatory insult, the rats were anesthetized with sodium pentobarbital (60 mg/kg), decapitated, and their brains removed. Meninges and choroid plexi were excised and the cerebral hemispheres were homogenized in 4 ml of microvessel isolation buffer (103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, 2.5 mM $NaHCO_3$, 10 mM D-glucose, 1 mM sodium pyruvate, dextran [MW 64,000; 10 g/l]; pH 7.4) with a protease inhibitor cocktail (0.2 mM phenylmethylsulfonyl fluoride; 1 mM benzamide; 1 mM $NaVO_4$; 10 mM NaF; 10 mM sodium pyrophosphate; and 10 μg/ml of aprotinin and leupeptin). Four milliliters of 26% dextran were added and the homogenates vortexed. Homogenates were centrifuged at $5600 \times g$ for 10 min and the supernatant aspirated. Pellets were resuspended in 10 ml of microvessel isolation buffer and passed through a 100-μm filter (Falcon, Becton–Dickinson; Franklin, NJ). The filtered homogenates were centrifuged at $3000 \times g$. Protein was extracted from the pellets

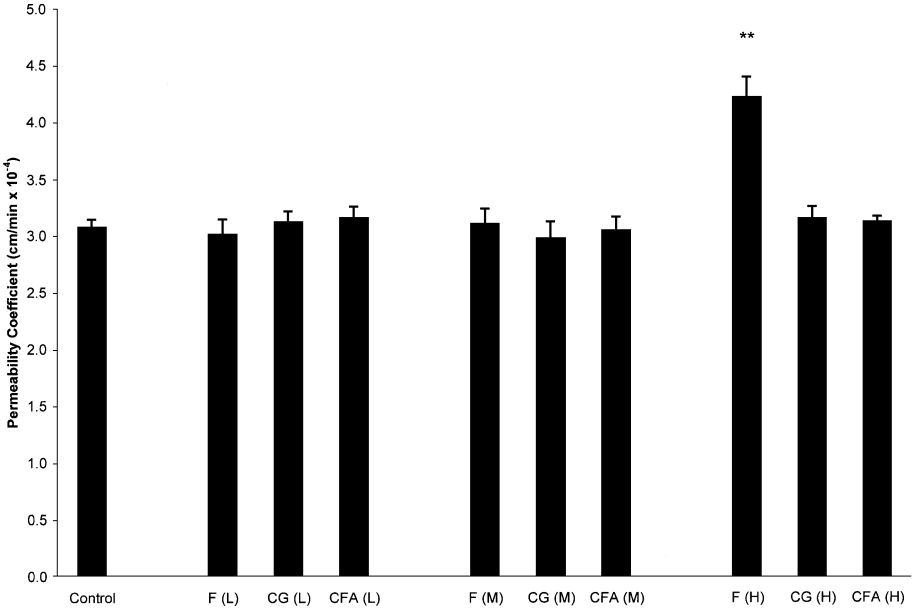

Fig. 2. In vitro assessment of permeability across bovine brain microvessel endothelial cell monolayers (primary culture, 10–12 days) using [^{14}C]sucrose following incubation in media conditioned with an inflammatory agent (formalin, λ-carrageenan [CG], or complete Freund's adjuvant [CFA]) at 120 min. Low (L) (0.00025%, 0.00015%, and 0.0025%, respectively), medium (M) (0.0025%, 0.0015%, and 0.025%, respectively) and high (H) (0.025%, 0.015%, and 0.25%, respectively) concentrations of each inflammatory agent were added to the media for a preset period of time (formalin—1 h, λ-carrageenan—3 h, and complete Freund's adjuvant—72 h). The highest concentration of each inflammatory agent was designated as the amount present if the entire amount given via a subcutaneous injection into the paw were absorbed into the peripheral circulation at once, while the lower concentrations were used to assess bovine brain microvessel endothelial cell permeability at levels several fold less than maximal. Each bar represents mean ± S.E.M. ($n = 4$). Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc test. No significant interactions were observed. ** Indicates ($P < 0.01$) significance between treatments as compared to control.

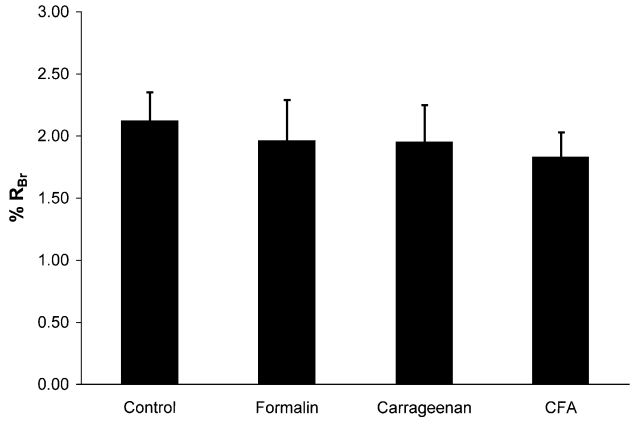

Fig. 3. [^{14}C]Sucrose blood–brain barrier permeability after i.v. injection of inflammatory agent (formalin, λ-carrageenan, or complete Freund's adjuvant) into the tail vein. Concentrations of inflammatory agents are equivalent to the amount commonly injected into the hind paw when conducting nociceptive testing. After a preset period of time (formalin—1 h, λ-carrageenan—3 h, and complete Freund's adjuvant—72 h), a 20 min in situ brain perfusion was performed. R_{Br}%, ratio of radioactivity found in the brain compared with the radioactivity found in the perfusate media. Each bar represents mean ± S.E.M. ($n=4$).

using 6 M urea lysis buffer (6 M urea; 0.1% Triton X − 100; 10 mM Tris, pH 8.0; 1 mM dithiothreitol; 5 mM $MgCl_2$; 5 mM EGTA; 150 mM NaCl) with the protease inhibitor cocktail. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL).

2.9. Tight junctional protein analyses

Isolated microvessel homogenates were analyzed for expression of occludin, zonula occludens-1, claudin-1, and actin using Western blot. Microvessel samples (20 μg) were resolved on a 4–12% Tris–glycine gel (Novex, San Diego, CA) for 90 min at 125 V and transferred to a polyvinylidene difluoride membrane for 40 min at 240 mA. Gel-Stain Blue (Pierce) was used to insure proper protein loading. Polyvinylidene difluoride membranes were blocked in Tris-buffered saline (141 mM NaCl, 10 mM Tris-base, 0.1% Tween-20) with 5% non-fat milk for 4 h. Blots were incubated in primary antibody at room temperature for 2 h, rinsed in Tris-buffered saline with 5% non-fat milk for 1 h, and incubated with secondary antibody at room temperature for 30 min. Blots were developed using enhanced chemiluminescence (ECL+; Amersham Life Science Products) and analyzed using Scion Image (Scion, Frederick, MD).

3. Results

3.1. MTT cell viability

Bovine brain microvessel endothelial cells were exposed to formalin (1 h), λ-carrageenan (3 h), or complete Freund's adjuvant (72 h) and cell viability was assessed using an MTT assay (Fig. 1). Cell viability was significantly ($P<0.01$) decreased at the high concentration of formalin (0.025%) resulting in a 34% decrease in cell viability. No significant difference occurred between any other treatment group when compared to the control group ($P\geq 0.05$).

3.2. In vitro bovine brain microvessel endothelial cell permeability of [^{14}C]sucrose

Effects of exposure to inflammatory agents (formalin, λ-carrageenan, or complete Freund's adjuvant) for a preset incubation period (1, 3, and 72 h, respectively) were studied using bovine brain microvessel endothelial cell permeability to [^{14}C]sucrose. Fig. 2 shows the calculated permeability coefficients for each agent at various doses. Results indicate the high concentration (0.025%) of formalin significantly ($P<0.01$) increased bovine brain microvessel endothelial cell monolayer permeability to [^{14}C]sucrose at 120 min with a permeability coefficient of 4.22 ± 0.18 as compared to 3.08 ± 0.07 for the control.

3.3. In situ brain perfusion

Effects of inflammatory agent (formalin, λ-carrageenan, or complete Freund's adjuvant) exposure for a preset incubation period (1, 3, or 72 h, respectively) on basal permeability across an in vivo blood–brain barrier were assessed using in situ perfusion of the brain with [^{14}C]sucrose, a membrane impermeant marker. Visual inspection of the brain immediately following in situ perfusion showed no influx of Evan's blue albumin into the brain parenchyma.

Fig. 3 shows R_{Br}% for the treated and control groups. Control R_{Br}% value of 2.12 ± 0.23, representative of vascular space volume, was converted to a vascular space of 21.2 μl/g brain tissue. Results demonstrate no significant difference in amount of sucrose associated with the brain (formalin—92.6 ± 10.9, λ-carrageenan—92.2 ± 10.2, complete Freund's adjuvant—86.2 ± 6.8% compared to control, respectively). Capillary depletion data following the 20 min in situ brain perfusions showed the amount of [^{14}C]sucrose trapped in the pellet was not significantly different from control (Table 1). Furthermore, the study revealed the

Table 1
Capillary depletion studies after a 20 min in situ brain perfusion

	Pellet	Supernatant	Homogenate
Control	0.34 ± 0.12[a]	1.84 ± 0.41	1.87 ± 0.53
Formalin	0.25 ± 0.16[a]	1.61 ± 0.16	1.57 ± 0.30
λ-Carrageenan	0.31 ± 0.09[a]	1.45 ± 0.06	1.50 ± 0.17
Complete Freund's adjuvant	0.11 ± 0.04[a]	1.36 ± 0.16	1.40 ± 0.14

Values are mean ± S.E.M.; $n=3$. Data are the percent values after a 20 min in situ brain perfusion with [^{14}C]sucrose. Statistical significance was determined using two-way ANOVA. No significant difference was determined between treatment groups and no significant interactions were observed ($P\geq0.05$).

[a] $P<0.01$, significant difference from homogenate within a treatment group.

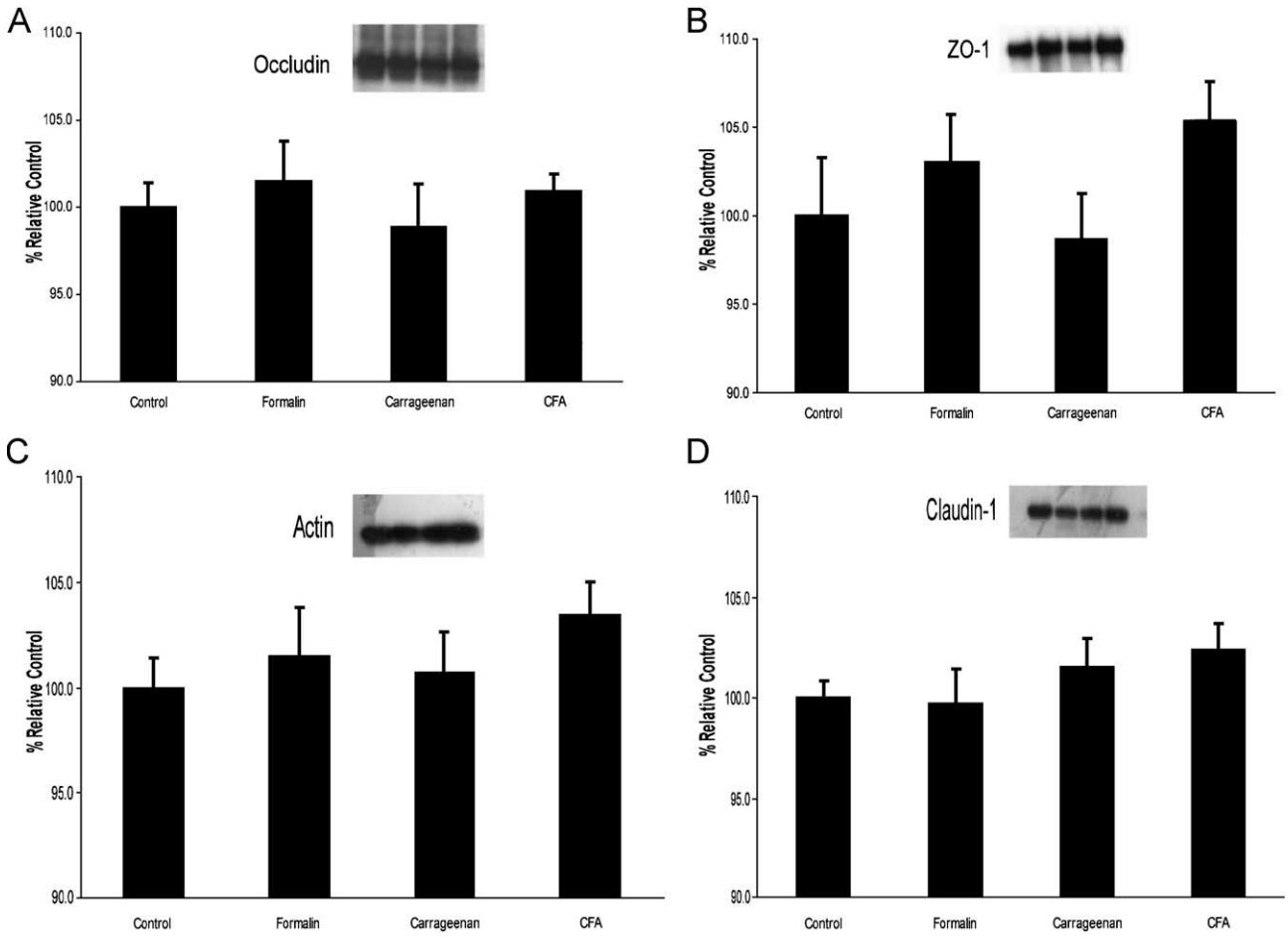

Fig. 4. Western blot analyses of isolated microvessels indicate that expressions of tight junctional proteins are not altered by i.v. injection of an inflammatory agent, formalin (λ-carrageenan, or complete Freund's adjuvant) into the tail vein. (A) occludin, (68 kDa); (B) zonula occludens-1, (220 kDa); (C) actin, (42 kDa) (D) claudin-1, (46 kDa). Insets: representative Western blots. Lane 1—control, lane 2—formalin, lane 3—λ-carrageenan, lane 4—complete Freund's adjuvant. Each bar represents mean ± S.E.M. ($n = 3$).

percentage amount of [^{14}C]sucrose associated with actual entry into the brain parenchyma was not statistically different from the homogenate (Table 1).

3.4. Tight junctional protein analyses

Western blot analyses indicated no significant change in expression of the tight junctional proteins (occludin, zonula occludens-1, and claudin-1) and the cytoskeletal protein (actin) following i.v. injection of inflammatory agents (formalin, λ-carrageenan, and complete Freund's adjuvant) for a preset period (1, 3, and 72 h, respectively). Fig. 4A–D shows the relative difference (%) from control of optical density for each protein.

4. Discussion

In this study, we investigated three inflammatory agents to establish if these agents elicited a direct effect on the functional and structural integrity of the blood–brain barrier. Using in vitro and in situ techniques, we were able to evaluate the effect of these inflammatory agents on cell viability, permeability, and tight junctional protein expression. In previous studies, we have shown that formalin, λ-carrageenan, and complete Freund's adjuvant, when administered subcutaneously into the hind paw, induced a time-dependent increase in blood–brain barrier permeability with a concomitant alteration in tight junctional and cytoskeletal protein expression (Huber et al., 2001a). Furthermore, we showed that hind paw injection of λ-carrageenan produced a biphasic increase in blood–brain barrier permeability, occurring from 1 to 6 h and then again at 48 h (Huber et al., 2002). However, to confirm that the observed changes were elicited by the inflammatory pain and not directly due to the inflammatory agents entering the systemic circulation and affecting the cerebral microvasculature of the blood–brain barrier, these experiments were conducted.

MTT cytotoxicity assays were performed to determine the viability of bovine brain microvessel endothelial cells exposed to the inflammatory agents at concentrations equivalent to that potentially in the systemic circulation following

injection into the hind paw, if it were to entirely enter at the same time. Results indicate that only the highest concentration of formalin (0.025%) affected bovine brain microvessel endothelial cell viability (~ 34% cytotoxic) (Fig. 1). Subsequently, we investigated the effect of these same agents on bovine brain microvessel endothelial cell permeability. The results again showed that only the highest concentration of formalin affected the permeability coefficient of bovine brain microvessel endothelial cell monolayers (Fig. 2), which is directly related to the decreased cell viability seen at this same concentration. Therefore, bovine brain microvessel endothelial cells appear to be viable and functional in media containing the inflammatory agents at concentrations much higher than would be found in the systemic circulation.

We next assessed the basal permeability across an intact blood–brain barrier following an i.v. injection of formalin, λ-carrageenan, or complete Freund's adjuvant into the tail vein. After a preset period of time (formalin—1 h, λ-carrageenan—3 h, and complete Freund's adjuvant—72 h), a 20 min in situ brain perfusion was performed. The control R_{Br}% value of 2.12 ± 0.23 was converted to a vascular space of 21.2 μl/g brain tissue, which is similar to our previous studies (Huber et al., 2001a, 2002) and to other studies using vascular space markers (Blasberg et al., 1983; Williams et al., 1996). No significant difference was noted in the amount of sucrose associated with the brain in any of the treated groups compared to control, indicating no change in blood–brain barrier permeability after a direct i.v. bolus.

Finally, we investigated the structural integrity of the tight junctional complex between blood–brain barrier endothelial cells using Western blot analyses. Tight junctions form a rate-limiting barrier between the extracellular fluid surrounding brain parenchyma and the systemic circulation. Tight junctions are primarily comprised of two transmembrane proteins, claudins and occludin, which attach one endothelial cell to another, and several accessory proteins, including zonula occludens-1, which form a scaffold to attach the tight junction complex to the cytoskeleton (actin). As has previously been discussed (Huber et al., 2001a,b), changes in these proteins indicate a significant alteration in blood–brain barrier structural integrity that can be correlated with functional changes. Therefore, we have focused our attention on these four proteins. In a previous study, we show a clear, time-dependent change in occludin, zonula occludens-1, and actin expression at peak inflammation after the inflammatory agent was injected subcutaneously into the hind paw. In these studies, Western blot analyses show no significant difference in expression of any of the chosen proteins following i.v. injection of the inflammatory agent, indicating that the effects of inflammatory pain on the blood–brain barrier are mediated by the inflammatory response and its effector molecules rather than the inflammatory agent itself.

In summary, we have clearly shown that the effects on blood–brain barrier functional and structural integrity seen in our previous studies are not due to a direct chemical interaction between the inflammatory agent and the cerebral endothelial cells of the BBB. Furthermore, this study shows that, at the concentrations used to elicit peripheral inflammation (either i.p. or s.c.), none of the inflammatory agents investigated are systemically toxic, which was a great concern especially for the formalin model (Pandey et al., 2000). This study helps to confirm that the changes seen at the blood–brain barrier following inflammation are due to the physiological response to the given inflammatory agent. By using three different inflammatory pain models, we are able to explore many different facets of the pain response (onset, time course, neuronal/immune contributions, etc.) and insure that any novel findings are not solely an artifact of a single pain model. Finally, this study strengthens these inflammatory pain models as experimental tools for blood–brain barrier assessment during pain.

Acknowledgements

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