

Diclofenac Attenuates the Regional Effect of λ -Carrageenan on Blood-Brain Barrier Function and Cytoarchitecture

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Received December 18, 2007; accepted February 25, 2008

ABSTRACT

The microenvironment of the brain requires tight regulation for proper neuronal function. Protecting the central nervous system (CNS) from the varying concentrations of ions, proteins, and toxins in the periphery is the dynamically regulated blood-brain barrier (BBB). Recent studies have demonstrated significant modulation of the BBB in a number of diseases and physiological states, including pain. This study expands on previous explorations of acute and chronic pain-induced effects on the function and molecular cytoarchitecture of the barrier. It describes the role of cyclooxygenase (COX) up-regulation by blocking with diclofenac (30 mg/kg, i.p.), and it examines the variation in BBB regulation through various brain regions. Edema and hyperalgesia were induced by λ -carrageenan and attenuated by the additional administration of diclofenac. Examination of unidirectional [14 C]sucrose permeability with multitime in situ perfusion studies demonstrated

that λ -carrageenan significantly increased cerebral permeability and decreased brainstem permeability. There were no significant changes in any of the other brain regions examined. These permeability changes correlated with up- and down-regulation of the tight junction (TJ) protein claudin-5 in the cerebrum and brainstem, respectively. Diclofenac administration attenuated the cerebral permeability uptake as well as the claudin-5 up-regulation. In addition, diclofenac reversed the lowered permeability in the brainstem, but it did not attenuate TJ protein expression. These studies demonstrate the complex regulation of the BBB occurring during inflammatory pain and the role of COX in this process. An understanding of BBB regulation during pain states is critically important for pharmacotherapy, and it holds great promise for new therapies to treat central nervous system pathologies.

The central nervous system (CNS) is one of the most vital and delicate systems of the human body, requiring tight regulation. Ion and nutrient concentrations within the extracellular and interstitial fluid of the brain are precisely controlled. Independence from the peripheral circulation is essential for such control, protecting the CNS from fluctuation in ion concentrations, toxins, and the immune system. This environmental maintenance is carried out by the blood-CNS barrier, consisting of the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (Sharma et al., 2004). The BBB, situated at the level of endothelial tight junctions of cerebral microvessels (Reese and Karnovsky, 1967; Brightman et al., 1970), is critical for regulating and maintaining the brain microenvironment and, thus, proper neuronal function.

The basis of the blood-brain barrier is the cerebral capillary endothelium with a continuous and large surface area that selectively allows passage into the CNS. The BBB endothelial cells are characterized by a lack of fenestrations, decreased pinocytosis, and the presence of tight junction (TJ) proteins, multiple transport systems, and enzymatic detoxification enzymes (Fenstermacher et al., 1988; Kniessel and Wolburg, 2000; Löscher and Potschka, 2005). The high transmembrane electrical resistance and low permeability, characteristic of the cerebral microvasculature, are conferred primarily by the TJ (Butt, 1995). Within the CNS, they govern the paracellular route of entry into the brain, spanning the apical region of the paracellular cleft and tightly linking neighboring endothelial cells together. The primary seal of the TJs is a complex interaction of numerous transmembrane, accessory, and cytoskeletal proteins—the transmembrane proteins occludin and the claudins, with the accessory protein zonula occludens-1 (ZO-1) interacting with their C termini, are linked to the actin cytoskeleton (Farshori and Kachar, 1999; Mitic et al., 2000). These junctions are dy-

This work was supported by National Institutes of Health Grants NS 42652 and NS 39592 (to T.P.D.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.107.135632.

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; TJ, tight junction; ZO-1, zonula occludens-1; COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; PGE₂, prostaglandin E₂; GFAP, glial fibrillary acidic protein; GLUT-1, glucose transporter 1; IR, infrared; NVU, neurovascular unit; AUC, area under the curve; OD, optical density; IL, interleukin; TNF, tumor necrosis factor; S/W, saline/water; S/D, saline diclofenac; C/W, carrageenan/water; C/D, carrageenan/diclofenac.

namic structures modulated by physiological and pathological conditions (Hawkins and Davis, 2005; Cerejido et al., 2007). Changes in expression and intracellular localization of these TJ proteins are associated with alterations in paracellular transport (Balda et al., 2000; Coyne et al., 2003; Brooks et al., 2005, 2006).

BBB failure is a critical event in the development and progression of numerous diseases affecting the CNS. Increased permeability across the BBB, as a result of decreased function, is an effect of disease, as with ischemic stroke or traumatic brain injury (Ilzecka, 1996; Morganti-Kossmann et al., 2001), whereas in other cases the permeability change is causative, as with multiple sclerosis (DeVries, 2004). In other cases, the role of BBB breakdown is unclear, as with Alzheimer's disease currently being studied (Wardlaw et al., 2003). Recent work has shown BBB perturbation with various pain stimuli, including changes in transcellular and paracellular transport (Huber et al., 2001, 2002a; Hau et al., 2004; Brooks et al., 2005, 2006; Seelbach et al., 2007).

Studies with various inducers of inflammatory pain have highlighted similar regulation of BBB molecular and functional integrity. Both the acute λ -carrageenan and the chronic complete Freund's adjuvant model induced a biphasic change in transmembrane TJ proteins and permeability of [14 C]sucrose at the BBB (Huber et al., 2002a; Brooks et al., 2006). The λ -carrageenan model develops inflammatory pain in a significantly shorter timeline than complete Freund's adjuvant. Therefore, pharmacological intervention of the potential mechanisms mediating alterations of the BBB was possible without concerns of constant dosing and the clearance and half-life of drugs. Thus, to examine the mechanisms leading to BBB alterations, the current studies focused on the acute, λ -carrageenan model of inflammatory pain that molecularly and functionally change the barrier (Huber et al., 2001, 2002a; Hau et al., 2004; Seelbach et al., 2007). There are multiple signals and pathways involved in λ -carrageenan-induced inflammatory pain, including the serotonin system, nitric oxide, various inflammatory and pain mediators, and the cyclooxygenase (COX) pathway.

The COX system, including COX-1 and -2, is responsible for converting arachidonic acid into prostaglandins. COX-1 is constitutively expressed throughout the body, whereas COX-2 is an inducible enzyme. Screening of novel nonsteroidal anti-inflammatory drugs (NSAIDs), which nonspecifically inhibit the COX pathway of inflammation, is often performed using the λ -carrageenan model of pain (Rao et al., 2006). It is clear that the COX system is intimately involved in λ -carrageenan-induced inflammatory pain, with evidence of both peripheral and central effects (Dirig et al., 1998). λ -Carrageenan has been demonstrated to induce COX-2 activity, and subsequently prostaglandin E₂ (PGE₂), at the site of injection (i.e., the hindpaw) and other organ systems, including the CNS (Dirig et al., 1998; Nantel et al., 1999; Ibuki et al., 2003; Guay et al., 2004).

At this time, we expand on our previous studies with inflammatory pain by detailing functional and molecular changes in the BBB (Huber et al., 2001, 2002a; Hau et al., 2004; Brooks et al., 2005, 2006; Seelbach et al., 2007). Using diclofenac, a classic NSAID with 10-fold preference for COX-2 inhibition, the role of the COX system in these changes was examined. We focused our studies 3 h post- λ -carrageenan injection, which demonstrated the greatest change in BBB function (Huber et al., 2002a). Other works have described pathological insults altering BBB characteristics in specific brain regions (Huber et al.,

2006a,b). Therefore, to explore the regional regulation of BBB perturbations and the role of COX in these regions, these studies expanded previous studies of the cerebral BBB to seven distinct brain regions: the hypothalamus, cerebellum, midbrain, cerebrum, hypothalamus, thalamus, and brainstem. Elucidation of the particular mechanisms of inflammatory pain that lead to BBB disruption promises to uncover novel therapeutic targets for the treatment of pain states.

Materials and Methods

Radioisotopes, Antibodies, and Chemicals. [14 C]Sucrose with a specific activity of 492 mCi/mmol and >99.54% purity was purchased from Valeant Pharmaceuticals (Costa Mesa, CA). Primary antibodies for the TJ proteins ZO-1 (mouse), occludin (rabbit), and claudin-5 (mouse) were obtained from Zymed Laboratories (San Francisco, CA); the marker proteins glial fibrillary acidic protein (GFAP) (mouse) and α smooth muscle actin (mouse) were obtained from Sigma-Aldrich (St. Louis, MO); and glucose transporter 1 (GLUT-1) (rabbit) was obtained from EMD Chemicals (San Diego, CA). Conjugated anti-mouse and anti-rabbit IgG horseradish peroxidase were purchased from Amersham Life Science Products (Springfield, IL). λ -Carrageenan, diclofenac, and all other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO).

Animals and Treatments. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Arizona, and they abide by the Institute of Laboratory Animal Resources (1996) for the proper treatment of animals. Female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 300 g were housed under standard 12-h light/dark conditions, and they received food and water ad libitum. Preinjection paw volume and thermal sensitivity were measured as described below. Rats received injections with either 150 μ l of λ -carrageenan (3% w/v dissolved in 0.9% saline) or vehicle control in the plantar surface of the right hindpaw. After 15 min, an i.p. injection of either diclofenac (30 mg/kg dissolved in distilled water) or vehicle control was administered. Thus, there were four experimental groups, as detailed in Table 1. After 3 h, postinjection animals were assessed for thermal sensitivity, and final paw volume was measured immediately before anesthetization with pentobarbital sodium (64.7 mg/kg i.p.) for in situ brain perfusion of microvessel isolation. To determine localized changes in BBB paracellular permeability and molecular architecture, seven brain regions were dissected in the following order: hypothalamus, cerebellum, midbrain, cerebrum, hippocampus, brainstem, and thalamus.

Edema and Thermal Sensitivity. Paw volume was measured by the displacement of electrolyte solution in a plethysmometer (model 7141; Ugo Basile, Comerio VA, Italy) at the time points described above. Sensitivity to a thermal stimulus, where a decrease is indicative of thermal hyperalgesia, was measured as paw removal latency upon application of infrared (IR) heat. The IR heat source heats linearly from room temperature (23.5°C) to 37°C over 15 s. Rats were first habituated to the individual boxes elevated on a glass table for at least 20 min before exposing the hindpaws to the mobile IR source. At least 1 min was allowed before repeat measurements of the same hindpaw, and the IR source was allowed to cool to room temperature between uses. Paw withdrawal latencies were defined

TABLE 1
Experimental groups

	Paw Injection	Intraperitoneal Injection
S/W	Saline	Water
S/D	Saline	Diclofenac
C/W	λ -Carrageenan	Water
C/D	λ -Carrageenan	Diclofenac

as the time (in seconds) taken for the rat to remove its hindpaw from the IR source.

In Situ Brain Perfusion. In situ brain perfusion was performed after previous methodology (Zlokovic et al., 1985, 1989; Zlokovic, 1995; Deane et al., 2004). In brief, animals were anesthetized with pentobarbital sodium (64.7 mg/kg i.p.) and heparinized by injection (10,000 U/kg i.p.). A ventral midline incision was made at the neck, and the common carotid arteries were exposed and cannulated with silicone tubing. Once the perfusion began, the jugular veins were cut to relieve pressure. The perfusion medium was a simple Ringer solution (117 mM BaCl, 4.7 mM KCl, 0.8 mM MgSO₄, 24.8 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 10 mM D-glucose, 39 g/l dextran, and 10 g/l bovine serum albumin, pH 7.4) containing Evans blue-labeled albumin. The solution was oxygenated with 95% O₂/5% CO₂ gas and passed through a heating coil (37°C) and a bubble trap via a peristaltic pump. When 100 mm Hg perfusion pressure and a flow rate of 3.1 ml/min were reached, [¹⁴C]sucrose (0.5 mCi/ml in Ringer solution) was infused at a rate of 0.5 ml/min into each carotid artery. Animals were perfused for 5, 10 or 20 min, after which they were decapitated and their brains were harvested. Samples of the radioactive perfusate were collected from each cannula as a reference. The choroid plexis and meninges were removed during the dissection of the brain into the aforementioned seven regions. The regions were then sectioned and homogenized before preparation for liquid scintillation counting by incubating with 1 ml of TS-2 tissue solubilizer (Research Products International, Mt. Prospect, IL) for 2 days. Radiation was counted after the addition of 100 ml of 30% acetic acid and 2.5 ml of OptiPhase SuperMix liquid scintillation cocktail (PerkinElmer, Waltham, MA). Results are reported as the ratio of radioactivity in the individual brain regions to the radioactivity in the reference perfusate (%R_{br}, measured in milligram per gram):

$$\%R_{br} \text{ (ml/g)} = (C_{\text{brain}} \text{ (dpm/g)} / C_{\text{perfusate}} \text{ (dpm/ml)}) * 100 \quad (1)$$

A least-squares regression of %R_{br} versus perfusion time (*t*, measured in minutes) was performed for each animal group, where *V_D* is the calculated initial volume of distribution of [¹⁴C]sucrose and *K_{in}* is the unidirectional transfer coefficient:

$$\%R_{br} (t) = V_D + K_{in} t \quad (2)$$

Microvessel Isolation. While under pentobarbital sodium anesthesia, rats were decapitated and their brains were removed. Meninges and choroids plexus were removed as the brain was dissected into the seven brain regions as detailed above. Regions from three animals were grouped to obtain sufficient protein. The pooled regions were then homogenized in 3 to 7 ml of microvessel isolation buffer that contained the following: 103 mM NaCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 15 mM HEPES, 2.5 mM NaHCO₃, 10 mM D-glucose, and 1 mM sodium pyruvate with 10 g/l dextran (64-kDa molecular mass), pH 7.4, and a protease inhibitor cocktail containing 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 1 mM NaVO₄, 10 mM NaF, 10 mM sodium pyrophosphate, and 10 μg/ml aprotinin and leupeptin. An aliquot was taken from homogenates for protein analysis. Four to 8 ml of 26% dextran was added to each homogenate, and the mixture was vortexed and centrifuged at 5600 *g* for 10 min. The supernatant was removed, and pellets were resuspended in 5 to 10 ml of microisolation buffer and passed through a 70-μm filter (Falcon; BD Biosciences, San Jose, CA). The filtered homogenates were centrifuged at 3000*g* for 10 min at 4°C. This isolation results in a highly enriched preparation of microvessels. Protein was isolated from whole-brain homogenates and microvessel preparations from each pooled brain region pellets with 6 M urea lysis buffer (6 M urea, 0.1% Triton X-100, 10 mM Tris, pH 8.0, 1 mM dithiothreitol, 5 mM MgCl₂, 5 mM EGTA, and 150 mM NaCl) with the protein inhibitor cocktail. Protein concentrations were determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL); the protein samples were stored at -40°C until use.

Protein Expression and Analysis. Isolated proteins from whole brains and isolated microvessels were analyzed for the expression of the neurovascular unit (NVU) protein markers GFAP, α smooth muscle actin, and GLUT-1, as well as the TJ proteins ZO-1, occludin, and claudin-5, confirming the enrichment of microvessels from the isolation procedure described above. Moreover, the microvessels isolated from the pooled brain regions were analyzed for the expression of the transmembrane TJ proteins occludin and claudin-5. A total of 20 μg of microvascular protein isolated was electrophoresed on a 4 to 20% Tris-HCl criterion gel (Bio-Rad, Hercules, CA) at 200 V for 1 h. The gel was then transferred to a nitrocellulose membrane (Bio-Rad) at 240 mAmps for 2 h at 4°C. Gel-Code Blue (Pierce) was used to stain the gels and ensure proper protein loading. The membranes were incubated with TBST blocking buffer (20 mM Tris base, 137 mM NaCl, 2 M HCl, 0.1% Tween 20, pH 7.6) with 5% nonfat milk for 1 h at room temperature. Blots were then incubated overnight at 4°C with primary antibody (claudin-5, 1:500; occludin, 1:1000), washed with TBST with 5% nonfat milk at room temperature for 1 h, and incubated with secondary antibody (1:1500 anti-mouse or 1:2000 anti-rabbit) for 45 min at room temperature. Blots were developed using enhanced chemiluminescence (ECL+; Amersham Life Science Products, Buckinghamshire, UK) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis. For the in situ brain-perfusion experiments, statistical comparisons of the regression coefficients *K_{in}* and *V_D* were performed as described previously (Brooks et al., 2005) in accordance with the methods of Glantz (2002). Analyses of all the data were performed using a one-way analysis of variance followed by a post hoc Student Newman-Keuls test.

Results

Role of COX-2 in the Onset of Hyperalgesia and Edema. The hyperalgesic response to λ-carrageenan is localized, rapid, and robust. Only the paw receiving injections, and not the contralateral paw, demonstrate a decrease in paw removal latency from the baseline time of 10.4 ± 0.3 s. There is a maximal decrease in paw removal latency to 2.4 ± 0.4 s within 15 min of λ-carrageenan administration (Fig. 1, A and B). Although previous studies demonstrated that 30 mg/kg diclofenac (i.p.) reversed λ-carrageenan-induced hyperalgesia (Ibuki et al., 2003), the present study endeavored to find the lowest effective dose of diclofenac to reverse the hyperalgesia and modulate the edema formation while not affecting the viability of the rat.

A range of diclofenac doses from 2 to 30 mg/kg (i.p.) were examined post-λ-carrageenan injection; paw removal latency was measured at a series of time points from 0 to 180 min in both the paw receiving injections and the contralateral paw, from which the area under the curve (AUC) was calculated (Fig. 1, A–C). λ-Carrageenan significantly decreased the hyperalgesic AUC from 1766.0 ± 73.7 to 398.3 ± 22.6 (*p* < 0.0001). Although 10 mg/kg was able to fully attenuate the λ-carrageenan-induced change in AUC from 398.3 ± 22.6 to 1504.5 ± 207.3, post-λ-carrageenan injection paw removal latency was significantly decreased from 9.1 ± 2.9 s at 150 min to 5.5 ± 2.4 s at 180 min (*p* < 0.05). In comparison, 30 mg/kg also reversed the decreased AUC from 398.3 ± 22.6 to 1621.7 ± 108.6, and the paw removal latency remained fully attenuated through 180 min (Fig. 1, B and C).

Modulation of edema onset at 180 min was examined with the same range of diclofenac doses (0–30 mg/kg i.p.) (Fig. 1D). Consistent with previous studies and reports, λ-carrageenan alone significantly increased the paw volume from 1.2 ± 0.0 to 2.2 ± 0.0 ml (*p* < 0.01). Diclofenac showed a

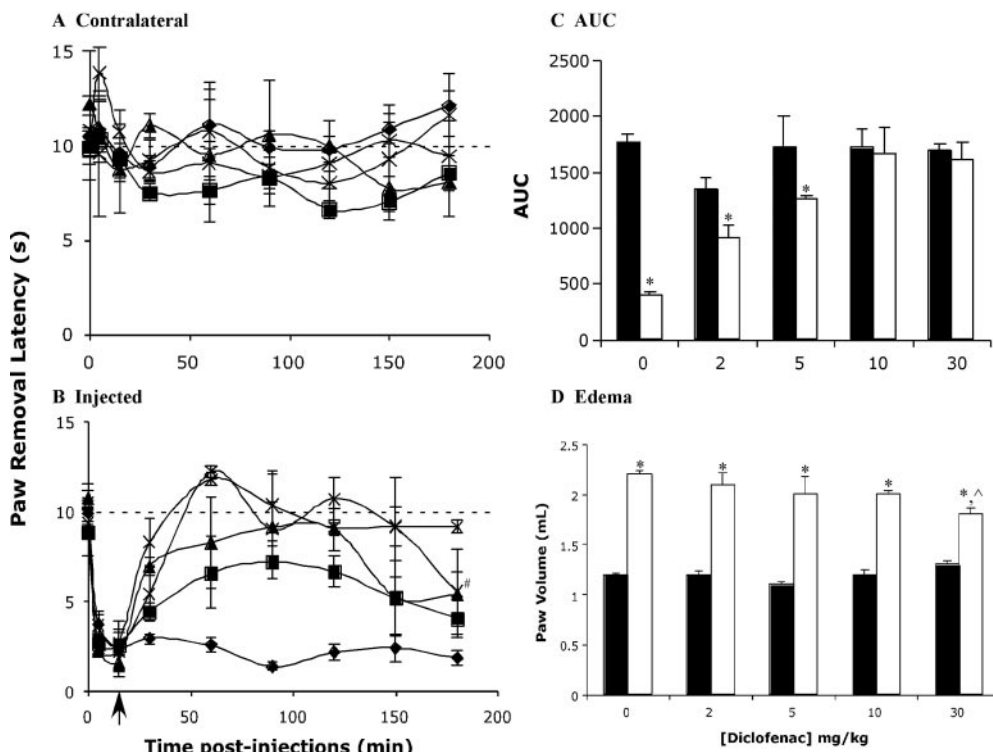


Fig. 1. Dose-dependent effects of diclofenac on hyperalgesia and edema. Paw removal latency from an infrared heat source was determined in the contralateral (A) and injected paws of λ -carrageenan-treated animals (B) at 0 (◆), 2 (■), 5 (▲), 10 (X), and 30 (*) mg/kg diclofenac (i.p.). C, the AUC of paw removal latency over time (0–180 min) was calculated for each dose of diclofenac in the contralateral paw (filled bars) and the paw receiving injections (open bars). D, the effect of each dose of diclofenac on paw volume and edema formation was also measured. Experiments represent $n \geq 3$ animals; *, $p < 0.05$, compared to the corresponding contralateral paw; #, $p < 0.05$, compared to all earlier time points within same treatment dose; ^, $p < 0.05$, compared to other doses of diclofenac.

dose-dependent decrease in edema formation, with maximal attenuation to 1.8 ± 0.1 ml with 30 mg/kg. Based on the consistent attenuation of both the hyperalgesia and edema onset, 30 mg/kg diclofenac was chosen for all subsequent experiments.

This dose, administration of its corresponding vehicle, and all relevant experimental groups were fully characterized in terms of hyperalgesia and edema formation (Fig. 2). Injection of saline into the right hindpaw did not decrease paw removal latency from baseline; in addition, diclofenac did not affect the latency in this group, compared to the water-vehicle-matched group. λ -Carrageenan rapidly decreased the paw removal latency, demonstrating the onset of hyperalgesia. Water administration (i.p.) did not alter this response, whereas diclofenac significantly attenuated the paw removal latency (Fig. 2B). Paw size was consistent between all animals receiving saline-paw injections. λ -Carrageenan significantly increased paw volume after both subsequent administration (i.p.) of water ($p < 0.0001$) or diclofenac ($p < 0.01$). However, diclofenac (30 mg/kg) significantly decreased the volume of the paw within the groups receiving λ -carrageenan-paw injections ($p < 0.01$) (Fig. 2B).

λ -Carrageenan Alters Paracellular Permeability Regionally. Paracellular permeability was examined with multitime uptake studies of [14 C]sucrose in seven regions of the brain. The rates of sucrose uptake were calculated from multitime data, and basal uptake rates ranged from 0.2628 to 1.347 μ l/g/min (Fig. 3; Table 2). λ -Carrageenan (3 h) significantly altered the rates of uptake in the cerebrum and brainstem only. Within the cerebrum, uptake was increased from 0.2628 ± 0.2026 to 0.8862 ± 0.0396 μ l/g/min ($p < 0.01$). In contrast, unidirectional transport of sucrose into the brainstem was decreased from 1.194 ± 0.3959 to 0.0155 ± 0.400 μ l/g/min ($p < 0.05$) (Fig. 4, D and G, respectively).

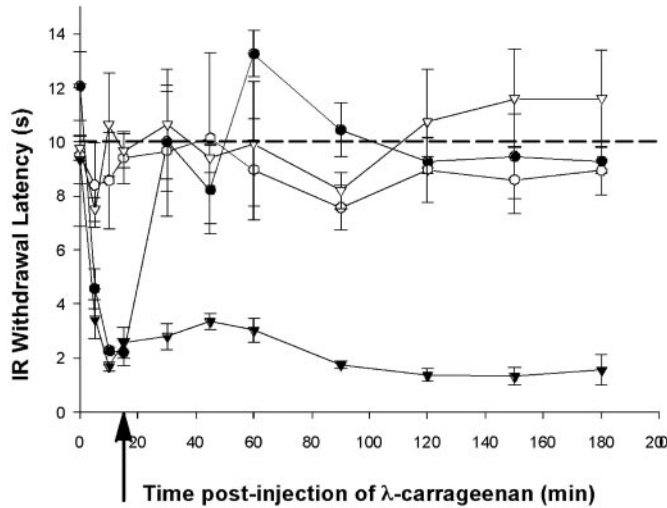
The role of COX up-regulation by λ -carrageenan in sucrose

permeability changes was examined with diclofenac administration (i.p.) 15-min postinjection into the paw. There was no significant change caused by diclofenac alone, as determined by the saline/water (S/W) and saline/diclofenac (S/D) groups (Fig. 4; Table 2). Whereas administration of diclofenac after λ -carrageenan (C/D group) moderately attenuated the increased unidirectional sucrose transport into the cerebrum [decreased from 0.8862 ± 0.0396 to 0.5143 ± 0.2503 μ l/g/min, carrageenan/water (C/W) to carrageenan/diclofenac (C/D), respectively], this was not a significant change ($p > 0.1$). However, in the brainstem diclofenac (C/D), compared to vehicle (C/W) control, administration after λ -carrageenan significantly increased the rate of sucrose uptake from 0.0155 ± 0.400 to 1.223 ± 0.2897 μ l/g/min ($p < 0.05$), fully reversing the lowered transport caused by λ -carrageenan alone (Fig. 4; Table 2).

Changes in Transmembrane TJ Proteins. The TJs control the paracellular permeability through the BBB, primarily via the transmembrane proteins occludin and the claudins, including claudin-5. The microvasculature isolated from the various brain regions, compared to whole-brain lysate, was enriched for the endothelial cell marker GLUT-1 as well as the integral TJ proteins ZO-1, occludin, and claudin-5. At the same time, the glial marker GFAP and the pericyte marker α smooth muscle actin was decreased (Fig. 5).

Within the seven examined brain regions, and across the four experimental groups, occludin expression seemed fairly stable and static. Whereas there was a moderate decrease in expression with λ -carrageenan injection within the cerebrum (C/W), semiquantitative comparison of optical density (OD) did not reach significance (Fig. 6). In comparison, claudin-5 was dynamically regulated throughout these same brain regions. Expression seemed up-regulated in the cerebellum and the hippocampus, and it decreased in the midbrain and thal-

A Hyperalgesia



B Edema

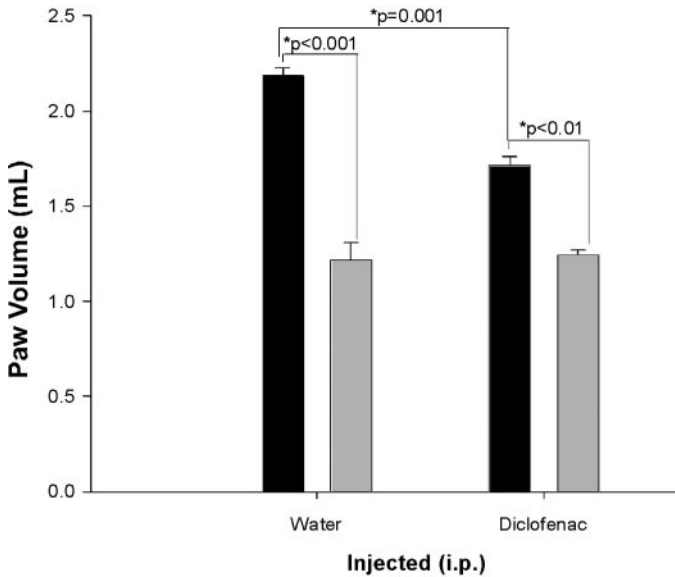


Fig. 2. Effect of 30 mg/kg diclofenac on hyperalgesia and edema in all experimental groups. Hyperalgesia (A) and edema (B) formation were examined in the S/W (∇), S/D (\circ), C/W (\blacktriangledown), and C/D (\bullet) groups. Arrow indicates i.p. injection time. All paw injections lead to an immediate (5 min) decrease in withdrawal latency, which rebounded to baseline (dashed line) by 10 min in the saline groups and maximally decreased by 15 min in the λ -carrageenan groups. Injection of diclofenac did not affect withdrawal latency in the S/D, compared to the S/W, group, whereas it reversed the hyperalgesic effect in the C/D, compared to the C/W, group. λ -Carrageenan caused significant edema formation under all conditions, but it is significantly ($p < 0.01$) attenuated with diclofenac administration. All data points are representative of $n \geq 9$ animals.

amus, after λ -carrageenan injection (C/W). However, semi-quantitative statistical analysis of the ODs failed to reach significance. λ -Carrageenan injection strongly up-regulated claudin-5 in the cerebrum (2-fold, as determined by OD; $p < 0.01$). This increase was attenuated and reversed with diclofenac administration (C/D versus C/W; $p < 0.05$). There was also a significant decrease in claudin-5 expression within the brainstem (C/W versus S/W; $p < 0.05$), which was not effected by diclofenac (C/D) (Fig. 7).

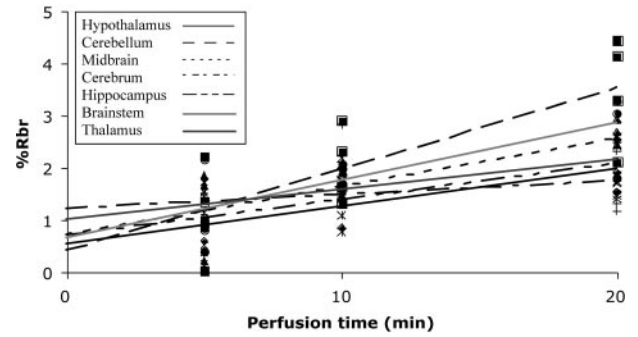


Fig. 3. Regional variation in multitime uptake of [14 C]sucrose. [14 C]Sucrose was used as a marker of paracellular permeability in the hypothalamus (solid line), cerebellum (long dash), midbrain (short dash), cerebrum (1 long:1 short variable dash), hippocampus (1 long:2 short variable dash), brainstem (thick gray line), and thalamus (dark thick line). $n = 4$ control rats/time point.

Discussion

In the current study, we demonstrated that the effect of inflammatory pain on BBB function was not global (i.e., across all regions of the brain studied). Permeability to [14 C]sucrose was increased in the cerebrum, decreased in the brainstem, and unaltered in five other regions examined. Examination of the transmembrane TJs that are integral to BBB maintenance revealed dynamic regulation of claudin-5. Claudin-5 was significantly increased in the cerebrum, correlating with an increase in paracellular permeability, and decreased in the brainstem where it correlated with decreased paracellular permeability. Furthermore, this study explored the role of the COX system by using diclofenac. Dose-dependent studies showed that 30 mg/kg diclofenac (i.p.) rapidly and significantly attenuates the onset of both edema and hyperalgesia due to injection of λ -carrageenan in the rat hindpaw. This dose attenuated the changes in cerebral BBB function and fully reversed changes in brainstem paracellular permeability. Moreover, diclofenac affects the dysregulation of claudin-5 in the cerebrum, corresponding with its attenuation of BBB function (i.e., permeability).

The TJ is a complex structure comprised of integral transmembrane proteins linked to the actin cytoskeleton. Alterations in the transmembrane proteins occludin and claudin-5 have previously correlated with alterations in BBB function (Huber et al., 2001, 2002a; Brooks et al., 2005, 2006). In the present study, there was a trend toward a decrease in occludin expression within the cerebral microvasculature (C/W versus S/W), but analysis of the OD did not reveal statistical significance. This finding does not correlate with previous descriptions of a significant decrease in occludin under similar conditions (Huber et al., 2001, 2002a) and may be due to differences in brain regions studied or antibody specificity. Although the initial λ -carrageenan studies did not examine claudin-5 regulation, the protein up-regulation within the cerebral microvasculature demonstrated in the current study agrees with molecular alterations seen with other models of inflammatory pain (Huber et al., 2001, 2002a; Brooks et al., 2005, 2006).

The complex and dynamic regulation of claudin-5 seems to be a common feature of BBB dysregulation after peripheral inflammatory pain (Brooks et al., 2005, 2006). Increased cerebral expression correlates with greater paracellular permeability, and the diclofenac-induced reversal

TABLE 2

Unidirectional permeability coefficients

The K_{in} as calculated from the multitime uptake [14 C]sucrose studies.

	S/W	S/D	C/W	C/D
Hypothalamus	0.7134 ± 0.2996	0.4952 ± 0.4603	1.069 ± 0.919	0.9521 ± 0.3608
Cerebellum	1.347 ± 0.1655	1.107 ± 0.7367	1.148 ± 0.8563	1.294 ± 0.4291
Midbrain	0.9246 ± 0.02543	0.5754 ± 0.3	0.8699 ± 0.3644	0.5754 ± 0.3
Cerebrum	0.2628 ± 0.2026	0.3071 ± 0.6325	0.8862 ± 0.03964*	0.5143 ± 0.2503
Hippocampus	0.7735 ± 0.1783	0.7418 ± 0.2975	0.6963 ± 0.1115	0.4384 ± 0.7356
Brainstem	1.194 ± 0.3959	0.803 ± 0.6755	0.01548 ± 0.4003#	1.223 ± 0.2897
Thalamus	0.5189 ± 0.4353	0.4481 ± 0.6865	0.1106 ± 0.739	0.5599 ± 0.4578

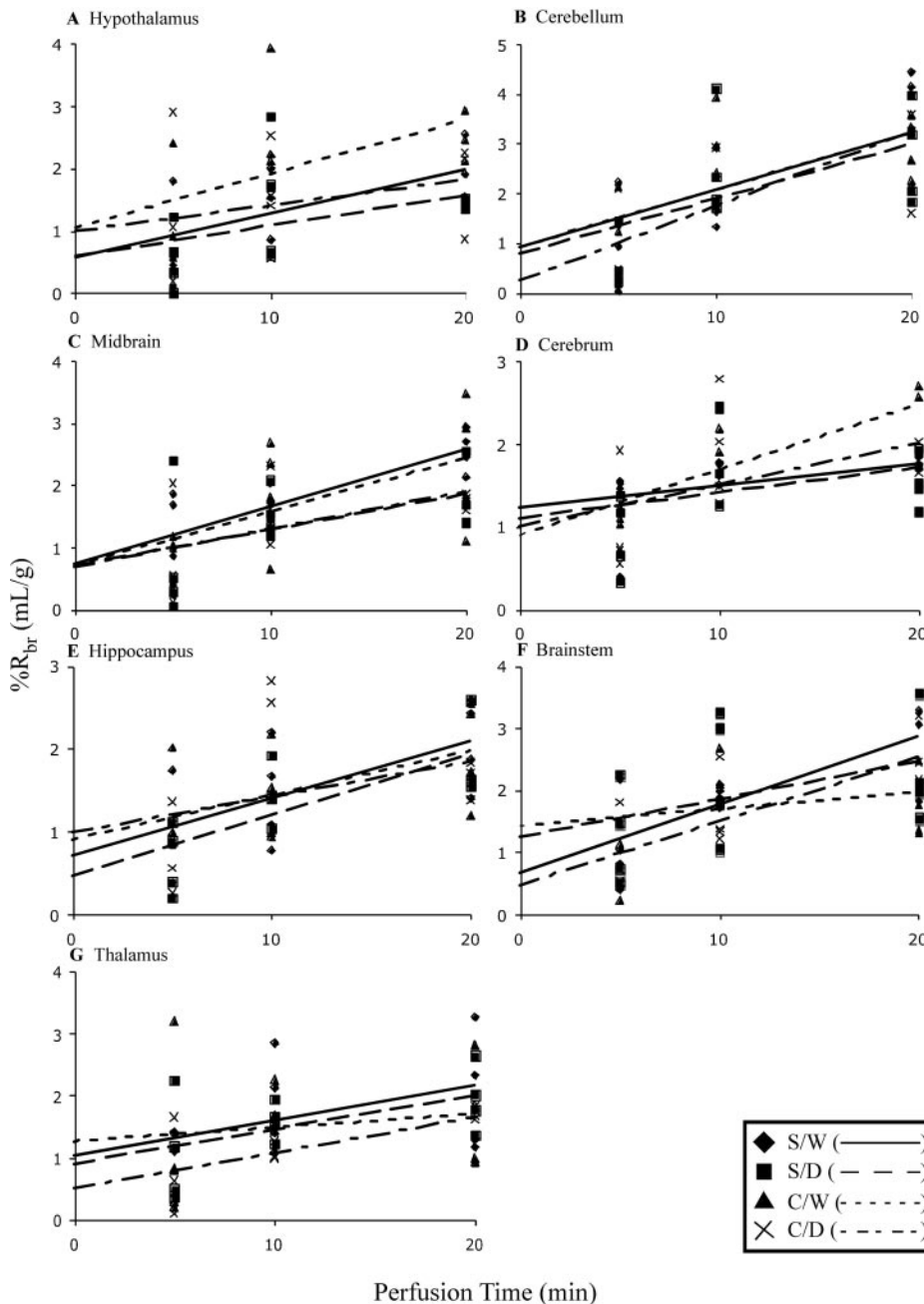
* $p < 0.01$ and # $p < 0.05$, compared to all other experimental groups within the brain region.

Fig. 4. Regional changes in [14 C]sucrose permeability in all experimental groups. The marker of paracellular permeability, [14 C]sucrose, was measured in multiple perfusion times for S/W (solid line), S/D (long dash), C/W (short dash), and C/D (variable dash) groups. Values are brain radioactivity (% R_{br}) in the hypothalamus (A), cerebellum (B), midbrain (C), cerebrum (D), hippocampus (E), brainstem (F), and thalamus (G) of $n = 4-6$ animals per time point. Unidirectional transport of [14 C]sucrose was increased in the cerebellum and decreased in the brainstem. Diclofenac administration attenuated the cerebral change and reversed that in the brainstem. The initial volume of distribution (V_D) was not altered in any region.

of this up-regulation attenuated the change in BBB function. Regionally, λ -carrageenan induced a tighter paracellular space (i.e., limited paracellular diffusion) within the brainstem, which correlated with decreased claudin-5 ex-

pression. Several studies have described the importance of claudin-5 regulation in barrier systems. Both increased and decreased expression have been demonstrated with a disruption of the paracellular space, highlighting the inte-

	Whole Brain	Isolated Microvessels	kD
CNS Cell Markers			
GFAP (glia)			50
α smooth muscle actin (pericytes)			42
GLUT-1 (endothelial cell marker)			44
TJ's			
ZO-1			220
Occludin			65
Claudin-5			22

Fig. 5. Protein analysis of microvessel purity. Expression of markers of various NVU members and tight junction proteins was examined in whole-brain and microvessel isolates. The glial (GFAP) and pericyte (α smooth muscle actin) markers were lost in the process of microvessel isolation, which enhanced the expression of the endothelial marker, GLUT-1. In addition, expression of the TJ proteins ZO-1, occludin, and claudin-5 were significantly heightened in the microvessel isolation process. Western blots are representative of $n = 3$ naive rats, and microvessels are isolated independently.

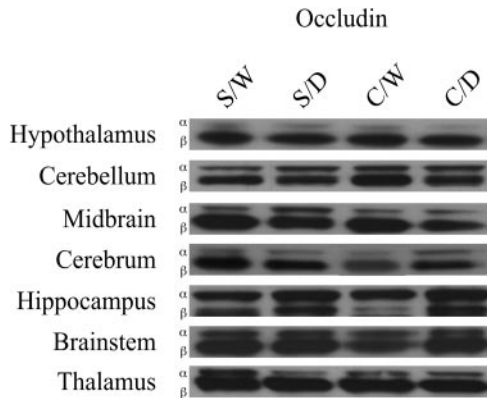


Fig. 6. Occludin protein expression. Expression of occludin was examined 3 h postpaw injection in microvessels isolated from various brain regions. Western blots showing protein expression demonstrate representative results from at least three independent experiments. OD of each band was measured and normalized to S/W control expression on each membrane. No significant changes were noted in either the α or β band of occludin in any brain region. $n = 9$ animals, pooled in three groups of three, for all experimental groups (S/W, S/D, C/W, and C/D).

gral role and complex regulation of claudin-5 (Coyne et al., 2003; Nitta et al., 2003; Brooks et al., 2005, 2006; Kitajima et al., 2006). It is interesting to note that the recovery of BBB function by diclofenac within the brainstem does not correlate with a change in claudin-5 expression, demonstrating a more multifaceted interaction occurring within the BBB, which controls paracellular permeability.

The BBB is situated at the level of endothelial tight junctions of the cerebral microvessels (Reese and Karnovsky, 1967; Brightman et al., 1970), and it is critical for regulating and maintaining the brain microenvironment and, thus, proper neuronal function. Moreover, the BBB is a dynamic structure that is part of the NVU, a functional unit composed of the endothelial cells interacting with smooth muscle cells, groups of neurons, and their associated astrocytes and pericytes (Abbott et al., 2006). Certain members of the NVU, such as neurons and glial cells, are clearly involved in pain transmission. Neurons are responsible for mediating the action

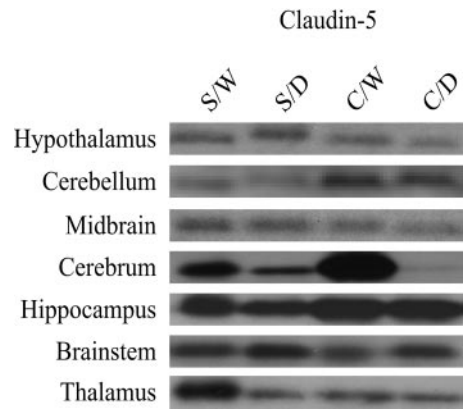


Fig. 7. Claudin-5 protein expression. Expression of claudin-5 was examined 3 h postpaw injection in microvessels isolated from various brain regions. Western blots showing protein expression demonstrate representative results from at least three independent experiments. OD of each band was measured and normalized to S/W control expression on each membrane. Expression seems to be up-regulated in the cerebellum and hippocampus, but it only reached statistical significance ($p < 0.05$) in the cerebrum. The λ -carrageenan-induced up-regulation of protein was reversed with diclofenac administration. Claudin-5 expression seems to be down-regulated in the midbrain and thalamus, but OD semiquantitation was only significant ($p < 0.05$) in the brainstem. Diclofenac did not affect this protein decrease. $n = 9$ animals, pooled in three groups of three, for all experimental groups (S/W, S/D, C/W, and C/D).

potential from the peripheral site of injury into the CNS for processing. Some, but not all, neurons are part of the NVU where they are also able to communicate "pain" signals to the astrocytes of the NVU and the endothelium directly, because both cell types express receptors for, and are modulated by, the autonomic nervous system (Kalaria et al., 1989; Hawkins and Davis, 2005). The local release of neurotransmitters can potentially regulate endothelial TJs, smooth muscle surrounding the endothelial cells, and astrocytes as well as activate microglia (Watkins et al., 2003; Hawkins and Davis, 2005).

Active astrocytes and microglia have been implicated in the maintenance of neuropathic pain (Watkins et al., 2003; Abbott et al., 2006). Astrocytes form a glial network through their extensive gap junction connections, and they have the potential to spread the sensation of pain along to the NVU. Stimulated astrocytes release numerous inflammatory mediators with known BBB perturbing effects, such as interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-1 β , and prostaglandins (Abbott et al., 2006). Microglia are inactive in their resting state, but they are able to rapidly respond to alterations in CNS structural integrity and subtle changes in their microenvironment (Hansson and Ronnback, 2003). Activation of the microglia is a multifaceted process with changes in morphology and release of inflammatory agents, such as nitric oxide, IL-1, and prostaglandins, each of which also affects the NVU (Watkins et al., 2003; Abbott et al., 2006).

Cytokines seem to be key modulators of BBB function, demonstrating disruption both in vivo and in vitro. Continuing to study the physiological/inflammatory pain, Huber et al. (2006a) demonstrated a temporal up-regulation of various proinflammatory cytokines within the peripheral circulation after injection of λ -carrageenan, including interferon- γ and IL-1 β . IL-1 β , injected into the striatum, is associated with neutrophil-dependent disruption of the BBB, associated with

a loss of occludin and ZO-1 from the TJ (Blamire et al., 2000). Chronic IL-1 β expression induces neutrophil recruitment, vasodilation, and marked breakdown of the NVU with endothelial cell disruption and activation of astrocytes and microglia (Ferrari et al., 2004). Interferon- γ seems to work synergistically with TNF- α in disrupting endothelial cells and barrier function, probably by inducing the up-regulation of TNF receptors, enhancing the effects of TNF- α (Wang et al., 2006).

TNF- α induces BBB breakdown, and it has been shown to induce COX-2 expression and subsequent PGE₂ release from the cerebral endothelial cells. Indicative of barrier dysfunction, in vitro high transmembrane electrical resistance disruption induced by TNF- α is attenuated by a COX-inhibitor (Mark et al., 2001). These works indicate that the effects of TNF- α on the BBB are mediated, at least in part, through COX activity. Inflammatory pain has been shown to up-regulate COX-2 expression and activity within the CNS as well, corresponding with time periods (of λ -carrageenan-induced pain) with known BBB perturbations. Further linking these two systems, the cerebral endothelial cells were demonstrated as a source of COX-2 within the CNS, producing PGE₂ release into the cerebrospinal fluid (Ibuki et al., 2003; Guay et al., 2004).

In the present study, we have demonstrated a regionally specific molecular and functional regulation of the BBB. The dynamic regulation of claudin-5 occurs in the cerebrum and brainstem, where control of the paracellular space is altered. Diclofenac, a classic NSAID that blocks COX activation and subsequent PGE₂ production, attenuates some of the molecular and functional BBB changes caused by inflammatory pain. In health, the BBB is critical to the maintenance of a homeostatic microenvironment by controlling the passage of solutes into and out of the CNS. During the pathological insult of pain, the barrier is rapidly modulated to increase permeability while not foregoing overall structural integrity.

There are many possible mechanisms for this BBB perturbation, which are also involved in the onset and maintenance of pain including cytokines, neurotransmitters, prostaglandins, proteases, kinins, and even activated members of the NVU itself, such as microglia. The intracellular mechanisms for these actions are still being investigated, but these data support a key role for COX activity in TJ dysregulation. Elucidation of the complete and particular mechanisms may prove to be novel and unique therapeutic targets for the treatment of various pain states and neuroinflammatory conditions.

Modulation of the BBB by pain has both pros and cons. Increased permeability will probably alter drug delivery. With an open barrier, there is increased access for drugs and toxins to the CNS, and CNS-specific peptides may leak into the periphery. With increased access to the CNS, some agents may have heightened efficacy, perhaps outside of the therapeutic window. Increased access may be detrimental to overall patient health due to increased CNS toxicity. Leakage of CNS-specific peptides into the periphery may lead to an autoimmune response that can later lead to CNS diseases, as with multiple sclerosis. Alternatively, a greater understanding of the structural and functional modulation of the BBB by pain has the potential to lead to novel approaches for the control and treatment of painful disease states and to new therapeutic avenues for the treatment of CNS diseases, such

as epilepsy, Alzheimer's dementia, and cancers within the CNS.

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