Stimulation of cerebellar fastigial nucleus inhibits interleukin-1β-induced cerebrovascular inflammation

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Galea, Elena, Sara B. Glickstein, Douglas L. Feinstein, Eugene V. Golanov, and Donald J. Reis. Stimulation of cerebellar fastigial nucleus inhibits interleukin-1β-induced cerebrovascular inflammation. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2053–H2063, 1998.—Electrical stimulation of the cerebellar fastigial nucleus (FN) in rat protects the brain against ischemia. We studied whether FN could reduce the cerebrovascular inflammation as a mechanism of protection. FN or dentate nucleus (sham controls) was electrically stimulated for 1 h, and 72 h later rats were either injected with interleukin (IL)-1β into the striata or processed to analyze inflammatory responses in isolated brain microvessels. In striata, IL-1β induced a recruitment of leukocytes that was reduced by 50% by FN stimulation. In isolated microvessels, IL-1β induced the transient and dose-dependent upregulation of the mRNAs encoding for the inducible nitric oxide synthase (NOS-2), intercellular adhesion molecule 1 (ICAM-1), and inhibitory κB-α (IkB-α), an inhibitor of nuclear factor-κB. FN stimulation decreased the upregulation of NOS-2 and ICAM-1 mRNAs, whereas it increased IkB-α mRNA expression. Dentate nucleus stimulation did not mimic the FN actions. These findings suggest that FN stimulation may render brain microvessels refractory to IL-1β by overproduction of IkB-α and support the hypothesis that alteration of microvascular inflammation may contribute to the central neurogenic neuroprotection elicited from the FN.

ELECTRICAL STIMULATION of the cerebellar fastigial nucleus (FN) reduces by over 50% the volume of the infarctions produced by permanent occlusion of the middle cerebral artery (MCAO) (14, 15, 25, 27, 35). This central neurogenic neuroprotection is long lasting and persists, after 1 h of stimulation, for at least 10 days (27). The mechanism by which FN stimulation protects the brain from ischemia is not known. Although FN stimulation acutely elevates regional cerebral blood flow (rCBF) (35), neuroprotection cannot be attributed to changes in rCBF or to differences in regional cerebral glucose utilization or the magnitude of the ischemic insult (15, 35).

Ischemia triggers a cascade of inflammatory reactions in which the parenchymal microvessels, as the interface between brain and blood, play a central role. Thus ischemia releases within the area of damage proinflammatory cytokines, most notably interleukin (IL)-1β (4, 5, 21, 28, 31). These facilitate expression in local microvessels of the calcium-independent isoform of nitric oxide synthase (NOS-2 or inducible NOS) (14, 17, 22) and cell adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1; 29), which, in turn, promote the infiltration of NOS-2-expressing leukocytes (14, 17). The blockade of NOS-2 and adhesion molecules reduces the size of the ischemic infarctions (18, 36), thus indicating that vascular inflammation, which starts as part of the healing reaction to the injury, indeed exacerbates the damage caused by ischemia (for review, see Refs. 4 and 11). Conceivably, FN stimulation may salvage the brain by inhibiting vascular inflammation.

The possibility that FN stimulation may suppress inflammation has been inferred from our previous observation that stimulation of the FN, several days in advance of MCAO, substantially reduces the expression of endothelial NOS-2 and infiltration of leukocytes induced by ischemia (14). However, a caveat of this interpretation arises from the complex cycle that links injury and inflammation, because injury triggers inflammation, which in turns causes more injury. Hence a decrease of inflammation by FN stimulation could reflect either a direct suppression of inflammatory reactions or a decrease in the injury that caused them. To discriminate between the two possibilities, it is therefore necessary to use models of inflammation devoid of cellular death.

Here we sought to establish whether FN stimulation inhibits the inflammatory response of cerebral microvessels, using IL-1β to trigger inflammation per se and not as a consequence of injury. In vivo, we tested whether the FN reduced the accumulation of leukocytes induced by injection of IL-1β in rat striata. In vitro, we asked whether brain microvessels isolated from FN-stimulated brains had a reduced expression of NOS-2 and ICAM-1 on exposure to IL-1β. We report that FN stimulation counteracted the effects of IL-1β both in vivo and in vitro, suggesting that FN stimulation may render the brain microvessels refractory to inflammatory stimuli. This finding indicates that excitation of central neuronal pathways represented in the cerebellum may regulate the inflammatory reactivity of brain microvessels to ischemia.

MATERIALS AND METHODS
Stimulation of the Fastigial or Dentate Nuclei

Procedures for surgery, instrumentation and electrical stimulation of the brain are detailed in other publications from this laboratory (15, 25, 27, 35) and are summarized here.
Instrumentation. Adult male Sprague-Dawley rats were anesthetized (halothane, 1.5–2.5% in 100% O₂) and intubated, and ventilated with an anesthesia-air mixture. The femoral arteries were cannulated to record arterial pressure (AP) and to sample blood. A femoral vein was cannulated to control AP during FN stimulation. Core temperature was maintained at 37°C by a thermostatically controlled infrared lamp connected to a rectal probe. Blood gases [arterial Po₂ (Pao₂) and PCO₂ (Paco₂)], pH, glucose, and hematocrit were measured in aliquots of arterial blood (0.2 ml) sampled just after surgery and periodically during the experiment. Blood gases were maintained at a normal level for rat by adjusting gas mixtures. Typical values from our laboratory (15, 25, 27, 35) are Pao₂ = 95–105 mmHg, Paco₂ = 33–38 mmHg, and pH = 7.35–7.45. These, glucose, and hematocrit (data not shown) were all within normal limits.

Electrical stimulation of brain. Rats were placed in a stereotaxic apparatus, and a hole, 1.5–2.0 mm in diameter, was drilled with a dental drill through the interparietal bone (1 mm lateral to the midline and 1 mm rostral to the occipital suture) for insertion of stimulating electrodes in the cerebellum. The cerebellum was electrically stimulated with cathodal square-wave pulses delivered through a monopolar electrode fabricated from Teflon-insulated stainless steel wire, carried in a stainless steel tube, and exposed at the tip for 100 µm. The anode was a clip attached subcutaneously to a neck muscle. Electrical pulses were generated by a square-wave stimulator, and constant current was passed through a photoelectric stimulus isolation unit. The electrode, mounted on a stereotaxic manipulator, was lowered into the cerebellum with a posterior inclination of 10°.

The area of the FN from which stimulation elicited maximal increases in rCBF (26) is the area from which neuroprotection is produced (25). To localize this site, the electrode was moved through the cerebellum in steps of 0.2 mm during stimulation with 2.5-s trains of pulses of 0.5-ms duration at 50 Hz and a stimulus current of 10–40 µA. The area of the cerebellum explored extended 4.8–5.2 mm anterior to, 0.6–1.0 mm lateral to, and 2.0–0.5 mm above the calamus scriptorius, the stereotaxic zero reference point. Stimulation never elevated AP above 150 mmHg, thereby keeping the AP within the autoregulated range of rCBF for rat. The active site was defined as that point along a track from which AP was elevated 10 mmHg with the lowest stimulus current. Once identified, the electrode was left in place. The FN was then stimulated for 1 h (1 s on-1 s off, 0.5-ms pulse duration, 50 Hz at 5× the threshold current, usually 70–100 µA). AP was maintained by simultaneously withdrawing blood from a femoral artery. At the end of the stimulus epoch blood was reinfused, and small holes, 1.5–2.0 mm in diameter, were drilled 2.7 mm lateral to it. A glass micropipette filled with agent or vehicle was lowered 4.5 mm beneath the cortical surface, and the material was slowly injected by hand over 3 min. The micropipette was left in place for 5 min to minimize back-diffusion and then removed. Sterile saline (0.36 µl) was injected into the left striatum, and IL-1β (10 pg in 0.36 µl sterile saline) was injected into the right striatum. On completion of the procedure, wounds were closed and covered with topical anesthetic, anesthesia was discontinued, and animals were returned to their cages.

Immunohistochemistry. Rats were deeply anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally) and perfused transcardially with heparinized saline followed by 50 ml of 3.75% acrolein-2% paraformaldehyde in phosphate buffer (PB) and 200 ml of 2% paraformaldehyde-PB. Brains were removed from the skulls and postfixed for 4 h in 2% paraformaldehyde. Coronal sections (40 µm) were cut in a Lancer Vibratome and collected in PB. Free-floating sections were incubated 30 min with 0.5–1% borohydride in PB, thoroughly rinsed in PB, and incubated in 0.5% H₂O₂ in PB for 30 min to inactivate endogenous peroxidase activity. After two rinses in 0.1 M Tris-buffered saline (TBS; pH 7.6), sections were blocked with 0.5% BSA in TBS and incubated with an antibody against CD45 (1:1,000; Harlan Sera-Lab, Sussex, UK) in TBS containing 0.3% Triton X-100 and 0.1% BSA. Incubation was performed for 16–18 h at 4°C with gentle shaking. After several washes in TBS, sections were incubated with biotinylated anti-mouse antibody (1:400; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The staining was visualized by the biotin-avidin-peroxidase method (Elite Kit, Vector Laboratories) using diaminobenzidine (DAB) as chromogen.

Quantification of leukocytes. Rats were anesthetized with halothane and killed by decapitation. The brains were removed, immediately frozen in liquid freon, and stored at −80°C until analysis. Brains were sectioned coronally at a 20-µm thickness in a cryostat at −20°C. Sections were collected every 200 µm within a region ±3 mm from bregma and postfixed with 4% paraformaldehyde in 0.1 M PB, pH 7.6. To visualize leukocytes, sections were incubated in DAB and 0.01% H₂O₂ for 4 min at room temperature to label the endogenous peroxidase of leukocytes. Cells were counted with the aid of MCID software (Imaging Research, St. Catharines, ON, Canada) as follows. A field containing labeled leukocytes was digitized, a population of leukocytes was selected, and the size of each cell was represented as a pixel count. Minimum and maximum pixel counts were set so that any object within the digitized image and whose size fell within this pixel range was counted as a single leukocyte. The appropriateness of the defined range was determined by comparing hand-counted to computer-counted results in identical fields, and pixel limits were adjusted until the computer- and hand-counted values agreed. Counts were subsequently performed at this magnification. All objects meeting the criteria were counted in digitized images of sequential fields, which covered the entire brain section. Erythrocytes that leaked into the parenchyma along the injection track were also stained because they too express peroxidase. However, they were excluded by size criteria because they accumulate in dense clusters whereas leukocytes are solitary. To determine the total number of labeled cells per hemisphere, we assumed that the number of leukocytes in the discarded sections averaged those counted in the flanking sections that were analyzed.

Microinjection of IL-1β Into Striatum

Microinjection. Rats were anesthetized with halothane (1.5–2.5% in 100% O₂) blown over the nose. The bregma was exposed, and small holes, 1.5–2.0 mm in diameter, were drilled 2.7 mm lateral to it. A glass micropipette filled with
removal from the skull, brains were placed in PBS, pH 7.6, at 4°C. The following steps were also performed at this temperature. Vessels in the pia-arachnoid and choroid plexus were stripped away. Diencephalon and brain stem were removed to leave cortex, striatum, and hippocampus, the brain areas in which protection by FN stimulation has been observed (25, 27). The parenchyma was gently homogenized in 2 ml of PBS per brain with a glass homogenizer with a loose-fitting pestle. The homogenate was then mixed with 4 ml of 25% dextran in PBS and centrifuged at 5,400 g for 15 min in a swinging-angle rotor. The resulting pellets were saved and the remaining tissue reprocessed once to increase the yield. The pellets, containing blood vessels, erythrocytes, and nuclei, were pooled, resuspended in dextran, and centrifuged again to further clean the vascular fraction of debris. To remove free nuclei and erythrocytes, the suspensions were poured over glass bead columns (Sigma) and washed extensively with PBS. The microvessels, retained by the beads, were separated by agitation in PBS and collected with a plastic pipette.

The purity and contents of the preparation were assessed by immunohistochemistry as described below and biochemically by determining alkaline phosphatase activity, a marker of brain endothelial cells, using previously published methods (13).

To test the response to cytokines, microvessels were resuspended in DMEM (625 µl per brain) and incubated in sterile conditions for 0–24 h at 37°C in a cell culture incubator at 5% CO₂. Cytokines were added at the beginning of the incubation time. IL-1β was obtained from Dr. Craig Reynolds, National Institutes of Health; tumor necrosis factor (TNF)-α was from Genzyme (Cambridge, MA), and interferon (IFN)-γ was from Gibco (Gaithersburg, MD). At the end of the incubations, vessels were harvested by a brief centrifugation.

Immunohistochemistry. Microvessels resuspended in DMEM were plated in polylysine-coated, four-chamber glass slides (50 µl per chamber) and maintained for 30 min at room temperature to allow the vessels to stick to the glass before we proceeded with the fixation. Cells were fixed with 4% paraformaldehyde in PBS (20 min, 4°C) and incubated in H₂O₂-methanol (1:6) to quench endogenous peroxidase (20 min, room temperature). They were then incubated in 0.5% BSA in TBS, blocked with BSA in TBS, and incubated with the primary antibodies overnight at 4°C in TBS containing 0.1% BSA and 0.3% Triton X-100. After two washes in TBS, cells were incubated with biotinylated anti-mouse or anti-rabbit secondary antibody (1:400, Vector Laboratories) for 1 h at room temperature. The staining was visualized by the biotin-avidin-peroxidase method (Elite Kit, Vector Laboratories) using DAB. The primary antibodies used were the following: for factor VIII, a rat polyclonal from Sigma; for perivascular microglia, the TFL-1s5 monoclonal antibody (Courtesy of Dr. William Hickey, Dartmouth Hitchcock Medical Center, Lebanon, NH); for α-actin, a rat polyclonal antibody from Sigma.

RNA isolation and RT-PCR analysis. Total cytoplasmic RNA was isolated from the vessels by homogenization in hyperosmotic Tris·HCl buffer, digestion in proteinase K, extraction in phenol-chloroform, and isopropanol precipitation. The yield of total cytoplasmic RNA was quantitated by the absorbance at 260 nm. To synthesize cDNA, 0.5 µg total cytoplasmic RNA were mixed with 900 ng of random primers in a total volume of 10 µl, heated at 65°C for 2 min, and placed on ice. RT was carried out in a final volume of 20 µl in the presence of 40 mM KCl, 2.5 mM of each deoxynucleotide 5′-triphosphate (dNTP), 10 mM dithiothreitol (DTT), 20 U RNase inhibitor, and 2 U of murine leukemia virus RNAse H-reverse transcriptase (GIBCO, Gaithersburg, MD) at 37°C for 1 h. The reaction was terminated by heating at 95°C for 2 min and diluted to 50 µl with water. Five microliters were amplified by PCR. The PCR reaction mixture contained 200 mM of each dNTP, 50 mM KCl, 10 mM Tris·HCl, pH 8.8, 1.5 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, and 400 ng each of a forward and reverse primer in a final volume of 40 µl. The samples were heated to 98°C, and the reactions were started by the addition of 0.5 U of Taq polymerase (Promega, Madison, WI) in 5 µl of 10 mM Tris·HCl, pH 8.8. PCR conditions were denaturation at 93°C for 30 s, annealing at 60°C for 45 s, and synthesis at 72°C for 45 s. After 40 cycles, samples were maintained for 10 min at 72°C. PCR products were separated by electrophoresis through a 2% agarose gel with ethidium bromide. All PCRs were carried out in a Hybaid thermal reactor controlled by tube temperature.

In some cases the single-point modality of competitive PCR analysis was used to estimate in parallel the relative amounts of mRNA in numerous samples (16). In these experiments, the PCR reactions contained a single amount of the appropriate internal standard and [³²P]dCTP (1.25 µCi per tube, 3,000 Ci/mmol, Amersham). Amounts of internal standard were 0.1 fg for NOS-2, 1 fg for inhibitory αβ1 (iβ1), and 500 fg for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). When the PCRs were completed, the PCR products, corresponding to cDNA and standards, were separated through electrophoresis in 2% agarose and excised out of the gels, and their radioactivity was measured by liquid scintillation counting.


**Statistical Analysis**

Values are expressed as means ± SE. Groups were statistically evaluated by one-way ANOVA followed by Fisher’s test. Differences were considered significant at P < 0.05.

**RESULTS**

**Effect of FN Stimulation on the Inflammation Elicited by Microinjection of IL-1β Into the Brain**

In the course of ischemia, the IL-1β released by brain cells, primarily microglia (5), triggers a chain of inflammatory reactions that include alterations of the brain-blood barrier that lead to infiltration of immune cells. In this study we sought to reproduce these events by injecting IL-1β in brain striata, and, as an index of local microvascular inflammation, we measured the recruitment of leukocytes.

Effect of microinjection of IL-1β into striata. In naïve anesthetized rats, IL-1β (10 pg in 0.36 µl) was microinjected into the right striatum and vehicle into the left. The volume of the injectate was determined, in pilot experiments, to just fill the body of the striatum at the
injection site without spreading into the overlying cerebral cortex. The dose of cytokine was chosen from the range of IL-1β concentrations that in cultured cells produces maximal induction of NOS-2 mRNA (10). One day later, the animals were killed, the brains processed, and the leukocytes identified with antibodies to the leukocyte-specific marker CD45.

Although no staining was observed in the absence of primary antibody (Fig. 1A), injection of vehicle resulted in some leukocyte infiltration at the cannula tip and along the cannula track in the overlying cerebral cortex (Figs. 1B and 2). IL-1β elicited a marked accumulation of CD45-positive cells throughout the treated parenchyma (Figs. 1–G). Occasionally, leukocytes appeared lining vascular walls (Fig. 1G), thus suggesting hematogenous origin. IL-1β did not appear to damage neurons, as determined in sections stained with thionin (data not shown), a finding in agreement with the observation that IL-1β only triggers apoptosis in already damaged cells (28).

To determine the pattern of distribution of leukocytes across the striatum, we counted the total number of leukocytes in sections collected every 200 µM from levels 3 mm rostral to 3 mm caudal to the site of injection, the bregma. In these experiments we took advantage of the endogenous peroxidase activity in leukocytes, which allowed labeling of the cells without primary antibody. This approach produced images of intensely dark cells over a white background that facilitated their identification with the aid of a computer image analysis system. In consecutive sections, the number of cells immunoreactive for CD45 was comparable to the number of cells labeled with endogenous peroxidase activity (Fig. 1, D and E), thus

![Fig. 1. Accumulation of leukocytes in striata of naive rats after injection of vehicle or interleukin (IL)-1β. Leukocytes were labeled either by immunostaining with anti-CD45 antibodies (A–D) or by their peroxidase activity (E–G). A: staining in absence of CD45 antibody. B and C: CD45-positive cells in vehicle-injected side (B), around the injection track (arrows), and in a IL-1β-injected striatum (C). D and E: comparable labeling of leukocytes by immunoreactivity (D) or peroxidase activity (E). Both images were obtained from the same IL-1β-injected area in consecutive sections, and arrows point to a vessel that served as a topographical reference. F: peroxidase-labeled cells at high magnification. In G, leukocytes line the wall of a parenchymal vessel, thus suggesting that they are of hematogenous origin. Magnification is ×125 for A–C and G; ×40 for D and E; and ×250 for F.](http://ajpheart.physiology.org/).


validating the method of quantification. Erythrocytes surrounding the injection track also displayed endogenous peroxidase, but the fact that these cells accumulated in highly dense clusters prevented their identification and quantification as individual cells by the computer.

In striata injected with IL-1β, the greatest numbers of leukocytes accumulated at the injection site, and the number decreased logarithmically rostrally and caudally over 2 mm (Fig. 3), consistent with the diffusion of the cytokine and consequent reduction of its concentration along this path. At all levels, leukocytes were more numerous in the IL-1β-injected sides (Fig. 3). The total number of leukocytes across the striata was higher in the IL-1β-injected side as compared with the side injected with vehicle (P < 0.05; Fig. 4). These results indicate that, although the injection per se triggered inflammation, leukocytes were recruited in the IL-1β-injected side mostly due to specific actions of the cytokine.

Effect of FN stimulation on the inflammatory response to IL-1β. Rats were stimulated in the FN or in the DN (sham stimulation) for 1 h. Three days later, IL-1β or vehicle alone was injected in the striata, and 24 h afterward animals were killed and processed to analyze the leukocyte infiltration.

FN stimulation inhibited the accumulation of leukocytes elicited by IL-1β by 50% (P < 0.05) and decreased the distance from the injection site at which cells were found (Figs. 3 and 4). In contrast, in DN-stimulated rats the rostrocaudal distribution and numbers of infiltrating leukocytes did not differ from naive controls (Figs. 3 and 4). Thus FN stimulation selectively antagonized the proinflammatory action of IL-1β.

Effect of FN Stimulation on Inflammatory Reactions in Brain Isolated Microvessels

A possible mechanism by which FN stimulation can reduce the IL-1β-induced leukocyte infiltration is the inhibition of inflammatory responses to IL-1β in brain microvessels, thereby diminishing the adhesion of circulating leukocytes. To test this possibility we studied the capacity of microvessels isolated from FN-stimulated brains to respond to IL-1β. As a parameter of inflammation we used expression of the mRNAs encoding for NOS-2 and ICAM-1.

Fig. 2. Distribution of leukocytes in a diagrammatic representation of rat brain at level of bregma, 24 h after injection of IL-1β or vehicle alone. Shaded areas depict CD45-positive areas in a representative animal.

Fig. 3. Distribution of leukocytes across striata of naive and fastigial nucleus (FN)- and dentate nucleus (DN)-stimulated animals. Positive numbers represent rostral levels and negative numbers caudal ones, with respect to bregma. Animals were injected with 10 pg of IL-1β in bregma in right striatum and with vehicle in left. Twenty-four hours later, brains were sectioned coronally and total number of leukocytes was determined in sections collected every 200 µm. Values are means ± SE of 6 or 7 animals per group. In all groups, response to IL-1β surpassed that elicited by vehicle. Note that FN stimulation reduced recruitment of leukocytes throughout striatum.
Biochemical and histological characterization of the isolated microvessels. Microvessels were isolated from brains of naive rats. Vascular purity was assessed by comparing alkaline phosphatase activity, a marker of cerebrovascular endothelium, in homogenates of whole brain and in the vascular fraction. Alkaline phosphatase activity, expressed as OD405·mg protein$^1$·30 min$^1$, was $10.0 \pm 1.1$ in whole brain and $34.2 \pm 2.2$ in vessel preparations (means $\pm$ SE, $n = 4$). The threefold enrichment is characteristic of highly purified vascular preparations (13).

We also characterized the cellular composition of the preparation immunocytochemically using other cell-selective markers. Analysis with an antibody against factor VIII, an endothelial marker, indicated that the vascular preparations contained a heterogeneous pool of branched segments with cross sections ranging from 2 to 40 µm (Fig. 5A), presumably including all the elements of the brain vascular bed: arterioles, capillaries, and venules. Five to ten percent of the vascular segments, measuring 10–40 µm, were arterioles because they were positively labeled by the smooth muscle cell marker $\alpha$-actin (Fig. 5B). The remaining 90% can hence be defined as microvessels devoid of smooth muscle cells, i.e., capillaries and postcapillary venules. Staining with the specific antibody TFL-1s5 (12) revealed the presence of perivascular microglia, cells with the nuclei situated in the middle of a very long and narrow cytoplasm, resting on the abluminal surface of intermediate-size segments (Fig. 5C).

Thus the major anatomic components of the preparation are capillaries, whereas the principal cellular component is endothelium. However, some microglia and perivascular smooth muscle are also present. Because all these cells can participate in inflammation in vivo and in vitro, they are all potential cellular loci for the inflammatory responses observed in this study.

Effect of proinflammatory cytokines on NOS-2 and ICAM-1 mRNA expression. To establish whether brain microvessels can respond to inflammatory stimuli in vitro, vessels isolated from normal brains were incubated with the proinflammatory cytokines IL-1$\beta$ (20 ng/ml), TNF-$\alpha$ (10 ng/ml), or IFN-$\gamma$ (20 U/ml) or with a combination of the three. Five hours later, expression of NOS-2 and ICAM-1 mRNAs was assessed by RT-PCR (Fig. 6A). The concentration of the cytokines used was expected to produce a maximal response, as determined previously (10).

In the absence of cytokines, vessels expressed little or no NOS-2 mRNA, in agreement with the absence of this mRNA species in normal healthy brains (12). Although each of the three cytokines increased expression of NOS-2 mRNA, IL-1$\beta$ was the most potent inducer. ICAM-1 mRNA was, in contrast to NOS-2, constitutively expressed (29) and upregulated only by IL-1$\beta$. Surprisingly, the combination of the three cytokines, which usually acts synergistically (10), was less effective than IL-1$\beta$ alone.

Fig. 4. Total number of leukocytes in striata of naive and FN- and DN-stimulated animals. Animals were injected with 10 pg of IL-1$\beta$ in bregma in right striatum and with vehicle in left. Twenty-four hours later, brains were processed to determine total number of leukocytes as described in MATERIALS AND METHODS. Values are means $\pm$ SE of 6 or 7 animals per group. In all groups, injection of IL-1$\beta$ resulted in a statistically significant increase in number of leukocytes recruited compared with injection of vehicle alone. In FN-stimulated animals, recruitment was reduced by 50% compared with sham-stimulated and naive rats. *$P < 0.05$, vehicle- vs. IL-1$\beta$-injected sides within each group of animals. $^P < 0.05$, IL-1$\beta$-injected sides in FN animals vs. IL-1$\beta$-injected sides in naive and DN rats (ANOVA).

Fig. 5. Cellular composition of rat brain isolated microvessels. A: endothelial cells (factor VIII positive). B: smooth muscle cells ($\alpha$-actin positive). C: perivascular microglia (TFL-1s5 positive). Vessels ranged from 2 to 40 µm. In A, arrowheads point to examples of largest (arterioles) and smallest vascular segments (capillaries). B shows a branched arteriole and transition (arrowhead) to a capillary devoid of smooth muscle cells. In C, arrowheads point to nuclei of perivascular microglia. Magnification is $\times 70$ for A and B and $\times 140$ for C.

Fig. 6. Effect of proinflammatory cytokines on NOS-2 and ICAM-1 mRNA expression. A: RT-PCR analysis of NOS-2 and ICAM-1 mRNA expression in rat microvessels incubated with various cytokines for 5 hours. B: Western blot analysis of NOS-2 protein expression in rat microvessels incubated with various cytokines for 5 hours.
These studies indicated that the inflammatory response in cerebral microvessels, defined by cytokine-mediated induction of NOS-2 and ICAM-1, remained viable after their acute isolation, and that IL-1β was the more potent inducer. Because in vivo FN stimulation antagonizes the actions of IL-1β, this was the cytokine chosen to compare the inflammatory reactivity of naive and FN-conditioned microvessels in vitro.

Effect of FN stimulation on the induction of NOS-2 mRNA. In side-by-side studies, microvessels isolated from naive and FN-stimulated brains were incubated with IL-1β, and NOS-2 mRNA was measured by competitive RT-PCR. To determine the time course of the response, vessels were incubated with 20 ng/ml of the cytokine, and expression of NOS-2 mRNA was analyzed 0–24 h thereafter. To study the dose dependency, vessels were incubated for 5 h with 0.2–200 ng/ml IL-1β. In microvessels from naive animals, IL-1β caused the transient upregulation of the NOS-2 mRNA compared with expression in the absence of cytokine, which was considered basal. The upregulation occurred as early as 2 h, peaked at 5 h (fivefold increase over basal, *P* < 0.05), and gradually decreased to basal levels by 24 h (Fig. 7A). Upregulation was dose dependent (Fig. 7B); although 0.2 ng/ml had no effect, the induction triggered by 2 ng/ml was significantly different from the basal (*P* < 0.05), and increased linearly up to 200 ng/ml.

Stimulation of the FN significantly reduced, by 50% (*P* < 0.05), the induction of NOS-2 mRNA by IL-1β at 5 and 8 h (Fig. 7A). At 5 h, the response to IL-1β was reduced over a range of doses from 2 to 200 ng/ml (*P* < 0.05) (Fig. 7B). In contrast, stimulation of the DN had no effect (Fig. 8). Thus FN stimulation specifically reduced the induction of NOS-2 mRNA.

Effect of FN stimulation on induction of ICAM-1 mRNA by IL-1β. Vessels from naive and FN-stimulated brains were incubated with 20 ng/ml of the cytokine for 0–24 h, and expression was determined by RT-PCR (Fig. 7C). Although not quantitative, these experiments showed that IL-1β induced expression of ICAM-1 mRNA, which was greatest after a 5-h exposure to IL-1β and declined thereafter. Expression was reduced...
at all times in vessels from FN- (Fig. 7C) but not DN-stimulated rats (Fig. 8).

Effect of blockade of nuclear factor-κB on the induction of ICAM-1 and NOS-2 mRNAs. IL-1β activates the transcription factor nuclear factor (NF)-κB, and there are κB-binding sites in the promoters of the NOS-2 and ICAM-1 genes (6, 34). To establish whether induction of NF-κB contributed to the upregulation by IL-1β of NOS-2 and ICAM-1 mRNAs, we tested the effect of the specific NF-κB inhibitor N-benzyloxycarbonyl-Ile-Glu(O-t-6u)-Ala-leucinol (ZIE) (30). Vessels were incubated with IL-1β (20 ng/ml, 5 h) in the presence of ZIE. At a concentration of 1 µM, ZIE suppressed the upregulation of ICAM-1 and NOS-2 mRNAs by IL-1β, and 0.1 µM ZIE had a moderate effect (Fig. 9, A and B). Quantitative analysis of the NOS-2 mRNA induction confirmed that the highest concentration of ZIE resulted in over 80% inhibition (P < 0.05), and the lowest concentration inhibited NOS-2 expression by 25%, although the effect was not statistically significant (Fig. 9B). The results suggest that IL-1β upregulated microvascular NOS-2 and ICAM-1 by a pathway mediated by NF-κB.

Effect of FN stimulation on the expression of IkB-α mRNA. One mechanism by which FN stimulation might inhibit an NF-κB-mediated induction of NOS-2 and ICAM-1 mRNAs would be the upregulation of IkB-α, the inhibitory subunit of the NF-κB complex. IkB-α upregulation has recently been reported to reduce transcription of NF-κB-dependent genes (3, 23). We investigated the time course and dose dependence of IkB-α mRNA expression elicited by IL-1β (20 ng/ml for 0–24 h and 5 h with 0.2–200 ng/ml) and the effect of FN stimulation thereon.

IkB-α mRNA was constitutively expressed in microvessels of naive rats and elevated in a time- and dose-dependent manner by IL-1β (20 ng/ml) (Fig. 10). In vessels from stimulated rats, the response to IL-1β was increased by 30–50%, the increase being statistically significant (P < 0.05) at 2- and 5-h incubation times, and at 2–20 ng/ml IL-1β. FN stimulation by itself did not appear to significantly increase basal levels of expression of IkB-α (Fig. 10).

These results indicate that IL-1β time and dose dependently induced IkB-α mRNA expression and that the response was increased by FN stimulation. The fact that basal levels of IkB-α mRNA were not clearly altered by FN stimulation suggests that FN stimulation acts permissively by priming the vascular response.

DISCUSSION

Electrical stimulation of the cerebellar FN reduces the volume of a focal ischemic infarction elicited by MCAO in rat (14, 15, 25, 27, 35). It also reduces induction of the proinflammatory molecule NOS-2 in
cerebral microvessels in the territory of salvage (14), an effect that might contribute to neuroprotection considering the deleterious consequences of NOS-2 expression (18). Here we sought to determine in vivo and in vitro whether the neurogenic downregulation of expression of NOS-2 could result from reduction in the cytokine IL-1β stimulating gene transcription. The second requirement, the effect that might contribute to neuroprotection, was demonstrated by the observation that within 2 h vessels displayed expression of NOS-2, ICAM-1, and IκB-α mRNAs in response to IL-1β, thus confirming that brain microvessels can undergo inflammation in vitro (32). The reduced expression after 8 h of incubation could be due to a gradual

Although we did not elucidate the identity of the leukocytes, they were probably neutrophils, the characteristic infiltration during early phases of stereotypical inflammatory reactions, and perhaps perivascular microglia, which, as previously shown in the blood-retina barrier (8), are rapidly activated and migrate away from the vessels in response to IL-1β. Our observations differ from studies in mice showing that microinjection of IL-1β into striatum or hippocampus fails to increase leukocyte infiltration, even though it does so in the periphery, a result interpreted to indicate brain-specific resistance to leukocyte extravasation (1, 2). The difference between these and our study may reflect variations in dosages (although comparisons are precluded by differences in the IL-1β units used in each study) or species variation.

Stimulation of the FN 72 h earlier reduced, by ~50%, the number of leukocytes recruited by intraatrial IL-1β, and this action was specific because comparable stimulation of the DN was without effect. The possibility that FN stimulation delayed rather than reduced the leukocyte infiltration cannot be ruled out. However, our observation (14) that FN stimulation inhibits expression of NOS-2 at all posts ischemic times analyzed suggests that the effect of the FN endures.

The preceding finding that FN stimulation decreases leukocyte infiltration could be attributed to an FNd induced direct downregulation of inflammatory responses to IL-1β at the blood-brain barrier. To test this idea, we studied cerebrovascular inflammation in isolated preparations so that changes in responses can be unequivocally attributed to the vessels and not to the parenchymal environment. To validate the approach, we determined whether the isolated vessels maintained a cellular organization comparable to in vivo and were metabolically viable. The cellular integrity was studied by immunohistochemistry with cell-specific markers, which confirmed the presence of endothelial cells, smooth muscle cells, and perivascular microglia and revealed that capillaries and postcapillary venules were the predominant components of the preparations. Although perivascular astrocytes were also present (19), they are not expected to contribute to the inflammatory responses in vitro because they retain the end feet but not the nuclei after the isolation, thus precluding gene transcription. The second requirement, the metabolic viability, was demonstrated by the observation that within 2 h vessels displayed expression of NOS-2, ICAM-1, and IκB-α mRNAs in response to IL-1β, thus confirming that brain microvessels can undergo inflammation in vitro (32). The reduced expression after 8 h of incubation could be due to a gradual
loss of cellular viability over time, to increased instability of the mRNAs, and/or to development of cellular tolerance.

IL-1β is a potent inducer of gene transcription via activation of NF-κB, a dimer of two members of the Rel protein family that is maintained in the cytoplasm by association to inhibitory proteins, including IκB-α. On stimulation by IL-1β, IκB molecules are phosphorylated and degraded, thus allowing the translocation of NF-κB to the nucleus and the binding to κB DNA motifs in the promoter of specific genes including NOS-2 (34), ICAM-1 (6), and IκB-α (20). These observations, together with our finding that the effect of IL-1β was reversed by the specific NF inhibitor ZIE, suggest that the increase in expression of ICAM-1, NOS-2, and IκB-α mRNAs by IL-1β was due to NF-κB-dependent transcriptional activation.

FN stimulation reduced the magnitude of the induction of NOS-2 and ICAM-1. It also facilitated the induction of IκB-α, indicating that the attenuated expression of the other mRNAs cannot be attributed to altered cellular viability in FN-conditioned microvessels. The changes in mRNA expression were seen throughout the time course and over the entire dosage range of IL-1β, and thus they are not due to shifts in the timing nor dose-response characteristics of the response. The increase in IκB-α is of particular interest, for it raises the question of whether it is causally related to the inhibition of expression of the other two proinflammatory molecules. Upregulation of IκB-α mRNA may enhance production of a protein that, in turn, may stabilize NF-κB in the cytoplasm to decrease transcription of the NOS-2 and ICAM-1 genes. Recent evidence indeed supports that overproduction of inhibitory subunits may be a biological mechanism to inhibit NF-κB activation. Thus glucocorticoids induce transcription of the gene encoding for IκB-α and translation of the protein, which reassociates with NF-κB, thus reducing its migration to the nucleus (3). In lymphocytes, stimulation of protein kinase A may block the translocation of NF-κB by retarding the degradation of IκB-α (23), and overexpression of IκB-α protein suppresses inflammatory responses in endothelial cells (33). Although we have not proven that NF-κB is blocked in FN-conditioned vessels or that the IκB-α protein is increased, the upregulation of IκB-α mRNA suggests that activation of the FN might modify signal transduction mechanisms by which IL-1β leads to NF-κB activation.

That vascular inflammation contributes to cellular death after ischemia is strongly supported by the findings that blockade of IL-1β (28) or ICAM-1 (36) or inhibition of NOS-2 activity (18) decreases the size of the infarctions. A plausible scenario is that IL-1β, produced in the brain after ischemia by microglia (5), would act on the brain vessels to promote expression of NOS-2 and adhesion molecules, which, in turn, would facilitate the transmigration of activated leukocytes. The NO released by NOS-2, together with other oxygen radicals produced by the infiltrated cells, is highly cytotoxic and may kill neurons and astrocytes weakened by the ischemic episode. In rendering the vessels less responsive to IL-1β, the FN may reduce expression of NOS-2 and infiltration of immune cells, thereby blocking the ensuing cellular death and providing a link between neuroprotection and inhibition of inflammation.

Stimulation of the FN induces a wide range of autonomic, behavioral and motor responses that are anatomically represented in discrete areas within the nucleus (26). The neuroprotective effect is evoked only from the rostral ventral pole, and is abolished after excitotoxic lesions of the FN (Glickstein, Golanov, and Reis, unpublished observations), indicating that intrinsic neurons, not fibers, account for this role. Assuming that the anti-inflammatory function is represented in the same neural structures as neuroprotection, it too would arise from intrinsic neurons of the rostral ventral pole. However, the nature and direction of the projections remain to be determined, as do the neurotransmitters or humoral factors that would act locally on the microvessels.

In conclusion, this study demonstrates that electrical stimulation of the FN alters the cerebrovascular inflammatory responses to IL-1β. This finding supports the views that brain microvessels are under neuronal control of the cerebellum and related networks, and that reduction of vascular inflammation may contribute to the FN-elicited central neurogenic neuroprotection.

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REFERENCES


