An Essential Role of Interleukin-1 β in Mediating NF- κ B Activity and COX-2 Transcription in Cells of the Blood–Brain Barrier in Response to a Systemic and Localized Inflammation But Not During Endotoxemia

Nathalie Laflamme, Steve Lacroix, and Serge Rivest

Laboratory of Molecular Endocrinology, Centre de Recherche de l'Université Laval Research Center and Department of Anatomy and Physiology, Laval University, Québec, Canada G1V 4G2

When released into the bloodstream, proinflammatory cytokines have the ability to trigger the transcription of different genes in cells of the blood–brain barrier (BBB), including members of the nuclear factor kappa B (NF- κ B) family and cyclooxygenase-2 (COX-2), the limiting enzyme for the formation of prostaglandins (PGs). The present study investigated the possibility that interleukin-1 β (IL-1 β) plays an essential role in these events during a systemic inflammatory response. Both wild-type and IL-1 β -deficient mice were killed at different times after two different immunogenic stimuli, i.e., intraperitoneal lipopolysaccharide (LPS) injection and intramuscular turpentine injection, used here as a model of systemic localized inflammatory insult. The inhibitory factor κ B α (I κ B α , index of NF- κ B activity) and COX-2 transcripts were detected throughout the brain by means of *in situ* hybridization. Systemic LPS injection caused a strong and rapid expression of I κ B α in endothelial cells lining the BBB of large and small blood vessels and thereafter within parenchymal microglia across the brain. This treatment also provoked a transient expression of COX-2 along

cells of the vascular system, and the expression pattern and intensity of the signal for both transcripts were essentially the same in wild-type and IL-1 β -deficient animals. In contrast, the induction of these genes that was quite selective to the cells of the BBB in response to intramuscularly turpentine insult was completely abolished in IL-1 β -deficient mice. Indeed, a late and prolonged expression of I κ B α and COX-2 mRNAs was found along the cerebral blood vessels in response to the sterile and localized inflammation in wild-type mice, whereas such induction was absent in the brain of IL-1 β -deficient animals. These results indicate that IL-1 β has an obligatory role in the activation of NF- κ B molecules and PGs within endothelial cells of the BBB in an experimental model of intramuscularly turpentine-induced inflammation but not during endotoxemia.

Key words: blood vessels; circumventricular organs; endothelial cells; *in situ* hybridization histochemistry; inflammation; interleukin-1 β -deficient mice; immunocytochemistry; lipopolysaccharide; proinflammatory cytokines; microglia; macrophages; septic shock; transcription factor

Systemic administration of the endotoxin lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a powerful immune challenge associated with an increase in the circulating levels of different proinflammatory cytokines, such as tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), and IL-6 (Andersson et al., 1992). It is not well known how cell activation is triggered after binding between the LPS binding protein–LPS complex and the glycosylphosphatidylinositol-anchored membrane CD14 (mCD14), although there is now evidence that activation of tyrosine kinase leads to transduction signal and cytokine gene transcription through the nuclear factor kappa B (NF- κ B). NF- κ B is normally present in the cytoplasm forming an inactive complex with an

inhibitor known as I κ B α . After extracellular stimulation by growth factors, mitogens and cytokines that activate mitogen-activated protein (MAP) kinases, I κ B α is phosphorylated by NF- κ B-inducible kinase (NIK)/I κ B kinase (IKK), ubiquitinated and degraded by cytoplasmic proteasome (Baeuerle, 1998; Baeuerle and Baltimore, 1996). Free active NF- κ B (the most common complex is the p50/p65 heterodimer) is then translocated into the nucleus, where it is able to regulate transcription of various genes in binding to its κ B consensus sequence. After its degradation, I κ B α is rapidly resynthesized to act as an endogenous inhibitory signal for NF- κ B, and monitoring I κ B α expression is an effective tool to investigate the activity of the transcription factor within the CNS.

Circulating LPS and proinflammatory cytokines have the ability to cause robust transcriptional activity of I κ B α in different structures of the brain (Quan et al., 1997; Laflamme and Rivest, 1999). The endotoxin injected either intravenously or intraperitoneally caused a rapid and prolonged expression of I κ B α mRNA in the endothelium of the brain blood vessels and parenchymal microglia, whereas the effects of circulating IL-1 β and TNF- α were rapid (within 30–60 min) and vanished 3 hr after the injection (Laflamme and Rivest, 1999). On the other hand, a selective expression of I κ B α was detected along the cerebral endothelium of intramuscular turpentine-injected rats (Laflamme

Received Aug. 18, 1999; revised Sept. 30, 1999; accepted Oct. 20, 1999.

This research was supported by the Medical Research Council of Canada (MRCC). S.R. is an MRCC Scientist. S.L. is currently an MRCC Postdoctoral Fellow in the Department of Neurosciences at the University of California, San Diego (La Jolla, CA). We thank Dr. Alain Israel (Institut Pasteur, Paris) for the gift of the plasmid containing the mouse I κ B α cDNA, Dr. K. Peri (Ste-Justine Hospital Research Center, Montreal, Canada) for COX-2 cDNA, and Dr. H. Zheng (Merck Research Laboratories, Rahway, NJ) for the generous gift of IL-1 β -deficient mice.

Correspondence should be addressed to Dr. Serge Rivest, Laboratory of Molecular Endocrinology, Centre de Recherche de l'Université Laval Research Center and Department of Anatomy and Physiology, Laval University, 2705, boulevard Laurier, Québec, Canada G1V 4G2. E-mail: serge.rivest@crchul.ulaval.ca.

Copyright © 1999 Society for Neuroscience 0270-6474/99/1910923-08\$05.00/0

and Rivest, 1999), a model of sterile and localized inflammatory insult that provokes a robust swelling at the site of injection (Fantuzzi and Dinarello, 1996). The rapid and transient induction of $\text{IkB}\alpha$ indicates a strong NF- κB activity in cells of the blood–brain barrier (BBB) by circulating proinflammatory cytokines, which may lead to the transcription of target genes.

One potential candidate is the gene encoding cyclooxygenase-2 (COX-2), the limiting enzyme for the formation of prostaglandins (PGs). Two putative NF- κB motifs from the COX-2 promoter were found to bind p50/p65 NF- κB heterodimers in an IL-1 β -dependent manner, and the two NF- κB subunits synergistically activate a –917/+49 COX-2 promoter construct (Crofford et al., 1997; Sorli et al., 1998). The NF- κB site (nucleotide –223 to –214) is also involved in LPS-induced expression of COX-2 in monocytic differentiated U937 cells (Inoue and Tanabe, 1998), whereas hypoxia induces COX-2 transcription via p65 binding to 3' NF- κB consensus element in the enzyme upstream promoter region in vascular endothelial cells (Schmedtje et al., 1997). Of interest is the fact that systemic LPS, intravenous injection of IL-1 β and TNF- α , and intramuscular turpentine insult cause a profound transcriptional activation of the gene encoding COX-2 along the brain vascular cells (Lacroix and Rivest, 1998). It is therefore likely that circulating proinflammatory molecules stimulate PG production via transcriptional activation of COX-2 through NF- κB signaling pathways within cells of the BBB.

Although the release of proinflammatory cytokines by systemic phagocytes may contribute to activate the cerebral endothelial cells in response to systemic LPS, the effects of the endotoxin may be direct and independent of the production of other circulating molecules. The endothelium does not express mCD14, but these cells were shown to play a major role in the pathogenesis of Gram-negative bacterial sepsis via free soluble CD14. In fact, LPS can trigger tyrosine phosphorylation of MAP kinases within endothelial cells despite the lack of mCD14 (Arditi et al., 1995). However, a direct action on cells of the BBB may not take place during a systemic and localized inflammatory response, such as intramuscular turpentine injection. This model of localized tissue damage provokes a specific induction of IL-1 β and IL-6 without any detectable IL-1 α and TNF- α production, suggesting the existence of a common cascade of cytokine release, characteristic of sterile inflammation in which IL-1 β and IL-6 might play a critical role (Fantuzzi and Dinarello, 1996). Interestingly, IL-1 β -deficient mice respond normally after systemic LPS injection, whereas the mutant mice exhibit an impaired acute-phase inflammatory response and are completely resistant to fever development when challenged with turpentine (Zheng et al., 1995). Because intravenous IL-1 β injection is capable of triggering the transcription of $\text{IkB}\alpha$ and COX-2 in cells of the BBB, whereas intravenous injection of high doses of IL-6 has no effect (Lacroix and Rivest, 1998; Laflamme and Rivest, 1999), we hypothesized that IL-1 β has a leading role in triggering NF- κB signaling events in cells lining the vascular wall of the CNS irrigating system. We show here an elegant pattern of $\text{IkB}\alpha$ expression in the mouse brain in response to different systemic immunogenic challenges and that IL-1 β is responsible for the effects of intramuscular turpentine insult but not endotoxemia.

MATERIALS AND METHODS

Animals. Interleukin-1 β knock-out mice were generously provided by Dr. H. Zheng (Merck Research Laboratories, Rahway, NJ) and generated with their control littermates as described previously (Zheng et al., 1995). Adult male mice of both groups [~20–30 gm body weight (b.w.)] were acclimated to standard laboratory conditions (14/10 light/dark cy-

cle; lights on at 6:00 A.M. and off at 8:00 P.M.; room temperature at 23 $\pm$ 1°C) with access to mouse chow and water *ad libitum*. Each mouse was only used once for experimentation, and all protocols were approved by the Animal Welfare Committee of Laval University. A total of 53 mice were assigned to different protocols each, corresponding to different treatments and postinjection times. Paired vehicle-treated mice of both wild-type and mutant groups were also killed at corresponding times after the injection.

Experimental protocols. The bacterial endotoxin LPS (100 $\mu\text{g}/100$ gm) diluted in 100 μl of sterile pyrogen-free saline or the vehicle solution was administered into the peritoneal cavity. Two other groups of mice were injected into the left thigh muscle with 50 $\mu\text{l}/100$ gm b.w. of turpentine (TU 109, CAS 8006-64-2; Spectrum Chemical, Gandena, CA) or 0.9% of sterile pyrogen-free saline. This experimental model of sterile inflammation (i.e., an inflammatory response developing in the absence of any microbial stimulus) induces a local tissue damage that is responsible for the development of a systemic acute-phase response.

At various times after the systemic treatments (from 30 min to 12 hr, depending on the challenge), animals were deeply anesthetized via an intraperitoneal injection of a mixture of ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borax buffer, pH 9.5, at 4°C. For the combination of immunocytochemistry [especially for COX-2-immunoreactive (IR) cells] with *in situ* hybridization, another group of mice was perfused with saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2. In this particular case, brains were removed from the skull, post-fixed for 2 hr, and then placed in 20% sucrose diluted in 4% paraformaldehyde–sodium phosphate buffer for 12–15 hr.

Single *in situ* hybridization histochemistry. Rapidly after the transcardial perfusions, brains were removed from the skulls, post-fixed for 2–8 d, and then placed in a solution containing 10% sucrose diluted in 4% paraformaldehyde–borax buffer overnight at 4°C. The frozen brains were mounted on a microtome (Reichert-Jung; Cambridge Instruments, Deerfield, IL), and cut into 20 μm coronal sections from the olfactory bulb to the end of the medulla. The slices were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, and 20% glycerol) and stored at –20°C. Hybridization histochemical localization of each transcript was performed on every fifth section of the whole rostrocaudal extent of each brain using ^{35}S -labeled cRNA probes. All solutions were treated with diethylpyrocarbonate (Depc) and sterilized to prevent RNA degradation. Tissue sections mounted onto gelatin and poly-L-lysine-coated slides were desiccated overnight under vacuum, fixed in 4% paraformaldehyde for 20 min, and digested with proteinase K (10 $\mu\text{g}/\text{ml}$ in 0.1 M tris HCl, pH 8.0, and 50 mM EDTA, pH 8.0, at 37°C for 25 min). Thereafter, the brain sections were rinsed in sterile Depc water, followed by a solution of 0.1 M triethanolamine (TEA), pH 8.0, acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated through graded concentrations of alcohol (50, 70, 95, and 100%). After vacuum drying for a minimum of 30 min, 90 μl of hybridization mixture (10⁷ cpm/ml) was spotted on each slide, sealed under a coverslip, and incubated at 60°C overnight (~15–20 hr) in a slide warmer. Coverslips were then removed, and the slides were rinsed in 4 $\times$ SSC at room temperature. Sections were digested by RNase A (20 $\mu\text{g}/\text{ml}$, 37°C, 30 min), rinsed in descending concentrations of SSC (2, 1, and 0.5 $\times$), washed in 0.1 $\times$ SSC for 30 min at 60°C (1 $\times$ SSC: 0.15 M NaCl and 15 mM sodium citrate buffer, pH 7.0), and dehydrated through graded concentrations of alcohol. After being dried for 2 hr under vacuum, the sections were exposed at 4°C to x-ray films (Eastman Kodak, Rochester, NY) for 17–65 hr (depending on the probe), defatted in xylene, and dipped into NTB2 nuclear emulsion (diluted 1:1 with distilled water; Eastman Kodak). Slides were exposed for 7 (IkB α mRNA) or 20 (COX-2 mRNA) d, developed in D19 developer (Eastman Kodak) for 3.5 min at 14–15°C, washed 15 sec in water, and fixed in rapid fixer (Eastman Kodak) for 5 min. Tissues were then rinsed in running distilled water for 1–2 hr, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with distrene plasticizer xylene (DPX) mounting medium.

cRNA probe synthesis and preparation. The Bluescript SK plasmid containing the 1.114 kb full-length coding sequence of the mouse IkB α cDNA (kindly provided by Dr. Alain Israel, Institut Pasteur, Paris, France) was linearized with *Bam*HI and *Hind*III for the antisense and sense riboprobes, respectively. The pGEM4 plasmid containing the COX-2 cDNA fragment (kindly provided by Dr. K. Peri, Ste-Justine Hospital Research Center, Montreal, Canada) was linearized with *Hin*-

dIII and *Eco*RI for the antisense and sense riboprobes, respectively. The length of COX-2 cDNA fragment was 176 bp consisting of nucleotides 124–300 of the complete published cDNA sequence (Feng et al., 1993). Radioactive cRNA copies were synthesized by incubation of 250 ng of linearized plasmid in 6 mM MgCl₂, 40 mM Tris, pH 7.9, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.2 mM ATP–GTP–CTP, 200 μ Ci of α -³⁵S-UTP (catalog #NEG 039H; DuPont NEN, Boston, MA), 40 U of RNasin (Promega, Madison, WI), and 20 U of T7 (I κ B α antisense and COX-2 sense probes), SP6 (COX-2 antisense probe), or T3 (I κ B α sense probe) RNA polymerase for 60 min at 37°C. Unincorporated nucleotides were removed using ammonium-acetate precipitation method; 100 μ l of DNase solution (1 μ l DNase, 5 μ l of 5 mg/ml tRNA, 94 μ l of 10 mM Tris/10 mM MgCl₂) was added, and 10 min later, a phenol-chloroform extraction was performed. The cRNA was precipitated with 80 μ l of 5 M ammonium acetate and 500 μ l of 100% ethanol for 20 min on dry ice. The pellet was washed with 500 μ l of 70% ethanol, dried, and resuspended in 100 μ l of 10 mM Tris/1 mM EDTA. A concentration of 10⁷ cpm probe was mixed into 1 ml of hybridization solution [500 μ l of formamide, 60 μ l of 5 M NaCl, 10 μ l of 1 M Tris, pH 8.0, 2 μ l of 0.5 M EDTA, pH 8.0, 50 μ l of 20 $\times$ Denhart's solution, 200 μ l of 50% dextran sulfate, 50 μ l of 10 mg/ml tRNA, 10 μ l of 1 M DTT, (118 μ l Depe water; volume of probe used)]. This solution was mixed and heated for 5–10 min at 65°C before being spotted on slides.

Combination of immunocytochemistry with *in situ* hybridization. Immunocytochemistry was combined with the *in situ* hybridization histochemistry protocol to determine the types of cells that express I κ B α and COX-2 transcript and protein. Among the antibodies selected for this study, anti-von Willebrand factor (vWF) was used to stain the endothelial cells of the vasculature, whereas anti-ionized calcium binding adapter molecule 1 (iba1) labeled cells of myeloid lineage (macrophages and microglia). Dual labeling for COX-2 protein and I κ B α or COX-2 mRNA was accomplished using an affinity-purified polyclonal antibody raised against the mouse COX-2 peptide. Every fifth brain section was processed by using the avidin–biotin amplification bridge method with peroxidase as a substrate. Briefly, slices were washed in sterile Depex-treated 50 mM potassium PBS (KPBS) and incubated 2 hr at room temperature with either vWF, iba1, or COX-2 antibody diluted in sterile KPBS plus 0.4% Triton X-100 plus 1% bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, MO) plus 0.25% heparin sodium salt USP (ICN Biomedicals, Aurora, OH). Sheep anti-vWF (catalog #CL20176A-R, lot AB22–74; Cederlane Laboratory, Hornby, Ontario, Canada), rabbit anti-iba1 (generously provided by Dr. Y. Imai, National Institute of Neuroscience, Kodaira, Tokyo, Japan) (Imai et al., 1996) and COX-2 polyclonal antibody raised in goat (catalog #SC1747, lot L148; Santa Cruz Biotechnology, Santa Cruz, CA) were diluted at 1:1000 or 1:8000 (iba1). After incubation with the primary antibodies, brain slices were rinsed in sterile KPBS and incubated with a mixture of KPBS plus 0.4% Triton X-100 plus 1% BSA plus 0.25% heparin plus biotinylated secondary antibodies (rabbit anti-sheep IgG for vWF, goat anti-rabbit for iba1, and horse anti-goat IgG for COX-2; 1:1500 dilution) (Vector Laboratories, Burlingame, CA) for 60 min. Sections were then rinsed with KPBS and incubated at room temperature for 60 min with an avidin–biotin–peroxidase complex (Vectastain ABC elite kit; Vector Laboratories). After several rinses in sterile KPBS, the brain slices were reacted in a mixture containing sterile KPBS, the chromogen 3,3'-diaminobenzidine tetrahydrochloride (0.05%), and 0.003% H₂O₂.

Thereafter, tissues were rinsed in sterile KPBS, immediately mounted onto gelatin and poly-L-lysine-coated slides, desiccated under vacuum for 30 min, fixed in 4% paraformaldehyde for 20 min, and digested by proteinase K (10 μ g/ml in 100 mM Tris HCl, pH 8.0, and 50 mM EDTA, pH 8.0) at 37°C for 25 min. Prehybridization, hybridization, and posthybridization steps were performed according to the above description with the difference of dehydration (50, 70, 95, 100% alcohol), which was shortened to avoid decoloration of immunoreactive cells (brown staining). After being dried for 2 hr under the vacuum, sections were exposed at 4°C to x-ray film (Eastman Kodak) for 17 hr, defatted in xylene, and dipped into NTB2 nuclear emulsion (diluted 1:1 with distilled water; Eastman Kodak). Slides were exposed for 9 (I κ B α mRNA) or 20 (COX-2 mRNA) d, developed in D19 developer (Eastman Kodak) for 3.5 min at 15°C, and fixed in rapid fixer (Eastman Kodak) for 5 min. Tissues were then rinsed in running distilled water for 1–2 hr, rapidly dehydrated through graded concentrations of alcohol, cleared in xylene, and cover-slipped with DPX. The presence of I κ B α and COX-2 transcript was detected by the agglomeration of silver grains in perikarya, whereas vWF, iba1, and COX-2 immunoreactivity within the cell cytoplasm and

Figure 1. Time-related influence of endotoxin LPS injection on the expression of the mRNA encoding I κ B α in the blood vessels and parenchymal elements of the mouse brain. Animals were killed 0.5, 1.5, 3, and 6 hr after intraperitoneal treatment with LPS (100 μ g/100 gm b.w.) or the vehicle (*Veh*) solution. These dark-field photomicrographs of dipped coronal sections (20 μ m) into nuclear emulsion NTB2 exhibit positive signal first within blood vessels and then within small cells scattered across the brain parenchyma. Magnification: *left panels*, 25 $\times$; *right panels*, 50 $\times$.

ramifications (microglia) was indicated by a brown homogeneous coloration. Determination of the double-labeled cells was performed visually for each cell exhibiting clear brown cytoplasm and a number of silver grains within the cell body delineating convincing hybridized message.

RESULTS

Time-related induction of I κ B α by intraperitoneal LPS

A robust hybridization signal was detected over the large blood vessels of the leptomeninges, choroid plexus, circumventricular organs, and those penetrating the brain parenchyma 30 min after the intraperitoneal LPS injection (Fig. 1). However, small capillaries also exhibited I κ B α -positive cells at that time, although the intensity of the signal varied among capillaries and regions of the brain. Ninety minutes after LPS injection, the message became very strong within the whole brain vasculature and within scattered small cells across the brain parenchyma. We have reported previously that these cells were microglia because they were

Table 1. Qualitative analysis of the hybridization signal for I κ B α and COX-2 transcripts in the brain of IL-1 β -deficient (KO) and wild-type (WT) mice in response to intraperitoneal LPS injection and intramuscular turpentine abscess

	I κ B α mRNA				COX-2 mRNA			
	Blood vessels		Parenchymal cells		Blood vessels		Parenchymal cells	
	WT	KO	WT	KO	WT	KO	WT	KO
Vehicle	–	–	–	–	–	–	–	–
LPS, 3 hr	++/+++	++/+++	++/+++	++	++	++	–	–
LPS, 6 hr	–/+	–/+	++	++	–	–	–	–
Turpentine	++	–	–	–	+/++	–	–	–

In situ hybridization histochemistry was accomplished via ³⁵S-labeled cRNA probes encoding either I κ B α or COX-2 gene. The relative intensity of I κ B α and COX-2 mRNA signals in the microvasculature (Blood vessels) and throughout the brain parenchyma (small scattered cells; Parenchymal cells) was assessed on dipped emulsion slides under microscopic evaluation and graded according to the scale of undetectable (–), low (+), moderate (++) , strong (+++), or very strong (++++). The qualitative signals represent the means of four (WT Vehicle), eight (KO Vehicle), three (WT LPS, 3 hr), six (KO LPS, 3 hr), four (WT LPS, 6 hr), five (KO LPS-6 hr), four (WT Turpentine, 7.5 hr), and four (KO turpentine, 7.5 hr) mice. Note that hybridization signal of both transcripts were comparable with background levels at 12 hr after LPS injection in WT and IL-1 β KO mice.

Figure 2. Effects of the bacterial endotoxin LPS or turpentine injection on the expression of I κ B α mRNA in the brain of wild-type (WT) and IL-1 β -deficient (KO) mice. These dark-field photomicrographs of dipped NTB2 emulsion slides depict I κ B α mRNA induction in vascular and nonvascular elements of the brain in mice killed 3 hr after intraperitoneal LPS injection or 7.5 hr after intramuscular turpentine administration. Note that LPS caused induction of the inhibitory factor within blood vessels as well as dispersed small cells in the brain of both mouse groups (*middle panels*), whereas the systemic and localized inflammatory insult provoked I κ B α transcription only in cells associated with the blood–brain barrier in wild-type but not IL-1 β knock-out mice (*right panels*). Magnification, 25 $\times$.

immunoreactive to the complement receptor type 3 on phagocytes (CD11b/c) (Laflamme and Rivest, 1999). The intensity of the signal declined along the endothelium of the blood vessels 3 hr after the endotoxin administration, but it remained high within the small scattered cells of the brain parenchyma. At 6 hr, the mRNA encoding I κ B α returned to basal levels in arteries, capillaries, and venules, although small scattered positive cells were still detected across the brain (Fig. 1, *bottom panels*). The message was undetectable in the cerebral vascular elements and parenchymal microglia 12 hr after the single injection with the bacterial endotoxin (data not shown) and at all postinjection times with the vehicle solution (Fig. 1, *top panels*).

The pattern of I κ B α expression and the intensity of the signal was essentially the same in wild-type and IL-1 β -deficient mice in all of the animals evaluated in this study (Table 1). Figure 2 shows representative examples of I κ B α expression in cells of the vascular and parenchymal elements of the brain in wild-type and IL-1 β -deficient mice 3 hr after intraperitoneal LPS injection

(*middle panels*). Both mouse groups exhibited a moderate-to-strong signal for the inhibitory factor transcript along the endothelium, as well as within small scattered cells across the brain parenchyma, a message that returned to basal levels at 12 hr after injection in wild-type as well as IL-1 β -deficient mice.

Effect of intramuscular turpentine injection on I κ B α expression

In contrast to systemic LPS administration, intramuscular turpentine insult stimulated I κ B α transcription only in cells associated with the BBB and not within scattered small cells across the brain parenchyma as seen in LPS-challenged mice (Fig. 2, *top right panel*). We have observed similar selective I κ B α mRNA induction in vascular cells of the brain in the rats killed up to 2 d after intramuscular turpentine injection in which the signal peaked at 6 and 12 hr (Laflamme and Rivest, 1999). Of interest is the lack of positive signal in vascular elements of the brain in IL-1 β -deficient mice after the sterile and localized systemic inflammatory insult,

Figure 3. Representative examples of COX-2 expression in blood vessels of wild-type (WT) and IL-1 β -deficient (KO) mice in response to systemic LPS or turpentine injection. Animals were killed 3 hr after intraperitoneal LPS injection (*middle panels*) and 7.5 hr post-turpentine injection into the left hind paw (*right panels*). These dark-field photomicrographs show *in situ* hybridization signals for COX-2 mRNA through the brain microvasculature in wild-type mice receiving either LPS or turpentine challenge and in knock-out animals during endotoxemia. The mRNA encoding COX-2 was undetectable in the cerebral vascular cells of IL-1 β knock-out mice in response to the systemic and localized inflammatory insult (*right bottom panel*). Magnification, 25 $\times$.

whereas the signal was intense along the cerebral endothelium of wild-type animals (Fig. 2, *right panels*). Indeed, the mRNA encoding I κ B α was comparable with background levels in cells of the BBB of all the IL-1 β -deficient animals that received the same dose of turpentine and were killed together with their wild-type littermates. Injection of sterile saline in the left thigh muscle (control treatment) did not activate transcription of I κ B α in the brain of either wild-type or IL-1 β knock-out mice (data not shown).

COX-2 expression in response to systemic inflammation

Although intraperitoneal LPS caused NF- κ B activation in both vascular- and parenchymal-associated cells of the brain of both wild-type and IL-1 β -deficient mice, COX-2 mRNA was induced only within cells of the BBB during endotoxemia (Table 1, Fig. 3). Indeed, a positive signal was detected along the leptomeninges, choroid plexus, as well as large and small blood vessels penetrating the different regions of the brain 3 hr after the systemic injection with the endotoxin. The pattern of expression and the intensity of the COX-2 hybridization signal in these structures were comparable in wild-type and IL-1 β -deficient mice in response to intraperitoneal LPS challenge (Fig. 3, *middle panels*). In contrast, intramuscular turpentine insult caused COX-2 transcriptional activation only in vascular-associated cells of wild-type and not in the brain of IL-1 β -deficient animals (Fig. 3, *right panels*). The systemic and sterile inflammatory challenge induced COX-2 expression in the cerebral endothelium of control littermates, whereas no positive cells were found along the BBB of all IL-1 β knock-out mice. Only constitutive expression was observed in the brain of these latter and vehicle-injected mice that exhibited positive signal in neurons of different regions, such as the hippocampal formation, piriform cortex, and amygdala (data not shown).

I κ B α and COX-2 transcripts are expressed in endothelial cells

To determine the phenotype of cells expressing the induced genes in vascular-associated elements, immunocytochemistry was combined with *in situ* hybridization on the same coronal sections of wild-type mice. Figure 4 shows different examples of such dual-labeling procedures and presents evidence that I κ B α and COX-2 transcripts are colocalized within vWF-IR cells. Indeed, the large majority of I κ B α - and COX-2-positive cells were found over those that stained for endothelial markers (Fig. 4, *top and middle panels*), although isolated I κ B α cells were also detected across the brain parenchyma and these were immunoreactive to a microglial marker (Laflamme and Rivest, 1999). Of interest is the fact that COX-2-IR cells were positive for I κ B α mRNA in response to LPS administration (Fig. 4, *middle right panel*), which suggests a potential interaction between NF- κ B and COX-2 molecules in the endothelium of the brain blood vessels.

A polyclonal antibody directed against iba1 molecule was used to label cells of myeloid origin, such as small ramified microglia that were found across the brain parenchyma (Fig. 4, *bottom panels, white arrows*). Positive iba1-IR cells were also present along blood vessels, although there was no clear overlap with COX-2-expressing cells (*silver grains, black arrows*). Indeed, the expression pattern of the mRNA encoding COX-2 was different from the perivascular microglial cells that were dispersed along the vascular walls (*white arrowheads*). Other antibodies (Mac-1 and F4/80) were used to label these cells, which also failed to show convincing colocalization with COX-2 transcript (data not shown).

DISCUSSION

The present study shows a strong transcriptional activation of both I κ B α and COX-2 genes in the mouse brain in response to

vWF-ir/COX-2 mRNA **vWF-ir/COX-2 mRNA** **vWF-ir/I κ B α mRNA** **COX-2-ir/I κ B α mRNA** **iba1-ir/COX-2 mRNA** **iba1-ir/COX-2 mRNA**

Figure 4. Phenotype of I κ B α - and COX-2-expressing cells during endotoxemia. Endothelial cells were labeled by immunohistochemistry using an antisera against the von Willebrand factor (vWF-ir), whereas a COX-2 antibody (COX-2-ir) was used to perform the dual-labeling with I κ B α -expressing cells (middle right panel). Cells of myeloid origin were labeled by the immunoperoxidase technique using an antisera directed against ionized calcium binding adapter molecule (iba1-ir, bottom panels). I κ B α or COX-2 mRNA was thereafter hybridized on the same sections by means of a radioactive *in situ* hybridization technique (silver grains). Note the presence of the mRNA encoding COX-2 within endothelial cells (top panels, black arrowheads) but not those positive for iba1 molecule (bottom panels, white arrowheads). Note also the expression of I κ B α mRNA in the endothelium of the brain microvasculature and within cells positive for COX-2 protein (middle panels). Black arrowheads, Dual-labeled cells (endothelial–COX-2 mRNA, top panels; endothelial–I κ B α mRNA, middle left panel; COX-2-IR–I κ B α mRNA, middle right panel); white arrowheads, single iba1-IR cells along the vascular walls (perivascular microglia); white arrows, single iba1-IR cells within the brain parenchyma (parenchymal microglia); black arrows, single COX-2-expressing cells along the cerebral endothelium. bv, Blood vessel. Magnification, 100 $\times$.

different models of systemic immunogenic stimuli. The bacterial endotoxin caused an interesting pattern of I κ B α expression; a robust signal was first detected in blood vessels and then within microglial cells across the brain parenchyma, whereas COX-2 was only induced along vascular cells of the brain. Dual-labeling procedure provided the anatomical evidence that both I κ B α and COX-2 transcripts were expressed over cells that stained for endothelial markers. These results contrast with the report showing that COX-2-IR proteins were present essentially within perivascular microglia of the rat brain (Elmquist et al., 1997a). We have used different antibodies (Mac-1, F4/80, and iba1) to label cells of myeloid lineage in the mouse brain and found very few perivascular microglia with convincing hybridization signal for COX-2 mRNA. It is possible that antibodies and the technique used in this study were not sensitive enough to detect all perivascular microglial cells or that species differences exist between mice and rats. In agreement with the present study, however, are the recent reports showing that LPS induces COX-2 mRNA in cerebral endothelial cells and not those of myeloid origin in the rats (Matsumura et al., 1998a,b; Quan et al., 1998; Cao et al., 1999). The dosage or strain of LPS may be important factors in determining the cell type(s) in which COX-2 is induced, and the distribution of the protein may not entirely match the mRNA.

The patterns of expression and intensity of signal in response to intraperitoneal LPS injection were similar in IL-1 β -deficient mice and their control littermates. In contrast to the effects of the endotoxin that induced I κ B α in vascular and parenchymal elements of the brain, intramuscular turpentine injection stimulated the inhibitory factor only within cells of the BBB in wild-type mice. This model of sterile and localized inflammation also provoked a transcriptional activation of the gene encoding COX-2 in the endothelium of the brain blood vessels in wild type, whereas IL-1 β -deficient mice did not exhibit notable induction of both I κ B α and COX-2 genes. Together, these data provide the evidence that IL-1 β is responsible for triggering the endothelium of the brain capillaries during a systemic and localized inflammatory response and not during endotoxemia. The fact that both I κ B α and COX-2 genes are induced within the same cells of the BBB suggests that IL-1 β may stimulate COX-2 transcription through NF- κ B signaling pathways, an event that may be of great importance in the production of PGs in the brain during the acute-phase response.

We have reported previously a robust and transient transcriptional activation of both COX-2 and I κ B α genes in cells of the BBB by an intravenous IL-1 β injection (Lacroix and Rivest, 1998; Laflamme and Rivest, 1999). The binding of IL-1 β to its cognate type I receptor leads to the formation of the IL-1 receptor-associated kinase (IRAK)/TNF receptor-associated factor 6 (TRAF6) complex, which activates NIK/IKK kinases involved in the phosphorylation and degradation of I κ B α (Baeuerle, 1998). NF- κ B is then translocated into the nucleus and may bind to its κ B consensus sequence on target genes (Baeuerle and Baltimore, 1996). The nuclear factor binding to the COX-2 promoter is able to influence the enzyme transcription in response to different immunogenic ligands, including IL-1 β (Crofford et al., 1997; Sorli et al., 1998). Here, we show that both I κ B α and COX-2 are expressed within the same cells, indicating a potential interaction between the transcription factor and COX-2 expression in the cerebral endothelium of immune-challenged animals. Obviously, this remains speculative from these anatomical data, and the signaling pathways that lead to the NF- κ B nuclear translocation

and COX-2 transcription have yet to be determined in the cells of the BBB. *In vivo* approaches are quite limited in the investigation of the intracellular events taking place within specific cellular populations of the CNS, but they provide an essential integration of the systems that interact during the acute-phase response. In this regard, the results that I κ B α and COX-2 transcripts were induced only in wild-type and not in IL-1 β -deficient mice in response to intramuscular turpentine injection strongly support the hypothesis that the circulating proinflammatory cytokine, when released by the site of inflammation (here the left hind paw), has a key role in leading to the NF- κ B signaling pathway and COX-2 transcription in the brain endothelial cells.

The physiological outcomes of such molecular mechanisms are numerous, and the well characterized role of PGs in mediating fever during the acute-phase response and circulating IL-1 β has been studied in various species (for review, see Elmquist et al., 1997b). Of interest is the data that IL-1 β -deficient mice respond normally after systemic LPS injection, whereas the mutant mice exhibit an impaired acute-phase inflammatory response and are completely resistant to fever development when challenged with turpentine (Zheng et al., 1995). The data presented in this study are in agreement with this original report, because COX-2 and NF- κ B activity is maintained in IL-1 β -deficient mice that received the bacterial endotoxin but not after intramuscular turpentine injection. It is possible that the effects of the bacterial endotoxin in IL-1 β -deficient mice are mediated by the production of other proinflammatory cytokines, such as TNF- α , which has the ability to stimulate I κ B α and COX-2 in cerebral endothelial cells (Lacroix and Rivest, 1998; Laflamme and Rivest, 1999). However, cytokine production induced by LPS appears essentially intact in TNF $^{-/-}$ mice, and these animals show the same symptoms of toxicity as wild-type mice after high doses of LPS (Marino et al., 1997). It would then be surprising to see a suppressed NF- κ B/COX-2 induction within the endothelium of the brain blood vessels in TNF $^{-/-}$ mice treated systemically with LPS. The bacterial endotoxin may in fact activate directly endothelial cells via its soluble receptor CD14 and trigger tyrosine phosphorylation of MAP kinases despite the lack of mCD14 (Arditi et al., 1995). Such a mechanism may obviously not take place in response to a localized systemic insult, and intermediate molecules produced at the site of inflammation and released into the bloodstream must transmit the information to the brain.

The present results highlight a central role for IL-1 β as a mediator of the acute-phase response in an experimental model of localized inflammation associated with a increase in the circulating levels of IL-1 β and IL-6 but not IL-1 α and TNF- α . Circulating IL-6 may not contribute to the induction of COX-2 and I κ B α during systemic inflammation, because high doses of recombinant IL-6 failed to stimulate these genes in cerebral vascular cells (Lacroix and Rivest, 1998; Laflamme and Rivest, 1999). Moreover, mice deficient in either IL-1 α or IL-1 receptor antagonist genes did not exhibit a suppressed acute-phase response upon injection with turpentine (Horai et al., 1998). This latter study also reported regional differences in the induction of COX-2 in large regions of the brain by Northern blot analysis (Horai et al., 1998). As shown here and in other reports (Cao et al., 1996; Elmquist et al., 1997a; Lacroix and Rivest, 1998), COX-2 mRNA and protein are induced only in cells associated with the brain vasculature in response to systemic immunogenic challenges, and regional changes may simply correspond to the structures that are either highly or poorly vascularized.

Together, these data suggest that circulating IL-1 β targets directly the cerebral endothelium to induce NF- κB nuclear translocation in response systemic and localized inflammatory processes. Induction of the IL-1R1-Toll/IRAK/TRAF6/NIK/IKK pathway may then lead to COX-2 gene transcription and brain PGE₂ production by microvascular-associated cells. These intracellular mechanisms are likely to be the critical link between the circulating immunogenic molecules and parenchymal elements of the brain to activate the neuronal circuits needed to restore the homeostatic balance. Induction of fever and autonomic and neuroendocrine functions, such as the increase in plasma glucocorticoid levels, may be the determinant functional consequences of the molecular mechanisms that take place in cells of the BBB.

REFERENCES

- Andersson J, Nagy S, Björk L, Abrams J, Holm S, Andersson U (1992) Bacterial toxin-induced cytokine production studied at the single-cell level. *Immunol Rev* 127:69–96.
- Arditi M, Zhou J, Torres M, Durden DL, Stins M, Kim KS (1995) Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases p44, p42, and p41 in vascular endothelial cells in a soluble CD14-dependent manner. Role of protein tyrosine phosphorylation in lipopolysaccharide-induced stimulation of endothelial cells. *J Immunol* 155:3994–4003.
- Baeuerle PA (1998) Pro-inflammatory signaling: last pieces in the NF- κB puzzle. *Curr Biol* 8:R19–R22.
- Baeuerle PA, Baltimore D (1996) NF κB : ten years after. *Cell* 87:13–20.
- Cao C, Matsumura K, Yamagata K, Watanabe Y (1996) Endothelial cells of the rat brain vasculature express cyclooxygenase-2 mRNA in response to systemic interleukin-1 β : a possible site of prostaglandin synthesis responsible for fever. *Brain Res* 733:263–272.
- Cao C, Matsumura K, Ozaki M, Watanabe Y (1999) Lipopolysaccharide injected into the cerebral ventricle evokes fever through induction of cyclooxygenase-2 in brain endothelial cells. *J Neurosci* 19:716–725.
- Crofford LJ, Tan B, McCarthy CJ, Hla T (1997) Involvement of nuclear factor κB in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. *Arthritis Rheum* 40:226–236.
- Elmqvist JK, Breder CD, Sherin JE, Scammell TE, Hickey WF, Dewitt D, Saper CB (1997a) Intravenous lipopolysaccharide induces cyclooxygenase 2-like immunoreactivity in rat brain perivascular microglia and meningeal macrophages. *J Comp Neurol* 381:119–129.
- Elmqvist JK, Scammell TE, Saper CB (1997b) Mechanisms of CNS response to systemic immune challenge: the febrile response. *Trends Neurosci* 20:565–570.
- Fantuzzi G, Dinarello CA (1996) The inflammatory response in interleukin-1 β -deficient mice: comparison with other cytokine-related knock-out mice. *J Leukoc Biol* 59:489–493.
- Feng L, Sun W, Xia Y, Tang WW, Chanmugam P, Soyoola E, Wilson CB, Hwang D (1993) Cloning two isoforms of rats cyclooxygenase: differential regulation of their expression. *Arch Biochem Biophys* 307:361–368.
- Horai R, Asano M, Sudo K, Kanuka H, Suzuki M, Nishihara M, Takahashi M, Iwakura Y (1998) Production of mice deficient in genes for interleukin (IL)-1 α , IL-1 β , IL-1 α / β , and IL-1 receptor antagonist shows that IL-1 β is crucial in turpentine-induced fever development and glucocorticoid secretion. *J Exp Med* 187:1463–1475.
- Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S (1996) A novel gene *iba1* in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun* 224:855–862.
- Inoue H, Tanabe T (1998) Transcriptional role of the nuclear factor κB site in the induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. *Biochem Biophys Res Commun* 244:143–148.
- Lacroix S, Rivest S (1998) Effect of acute systemic inflammatory response and cytokines on the transcription of the genes encoding cyclooxygenase enzymes (COX-1 and COX-2) in the rat brain. *J Neurochem* 70:452–466.
- Laflamme N, Rivest S (1999) Effects of systemic immunogenic insults and circulating proinflammatory cytokines on the transcription of the inhibitory factor κB α within specific cellular populations of the rat brain. *J Neurochem* 73:309–321.
- Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, Basu S, Old LJ (1997) Characterization of tumor necrosis factor-deficient mice. *Proc Natl Acad Sci USA* 94:8093–8098.
- Matsumura K, Cao C, Ozaki M, Morii H, Nakadate K, Watanabe Y (1998a) Brain endothelial cells express cyclooxygenase-2 during lipopolysaccharide-induced fever: light and electron microscopic immunocytochemical studies. *J Neurosci* 18:6279–6289.
- Matsumura K, Cao C, Ozaki M, Morii H, Nakadate K, Watanabe Y (1998b) Electron microscopic evidence for induction of cyclooxygenase-2 in brain endothelial cells. *Ann NY Acad Sci* 856:278–280.
- Quan N, Whiteside M, Kim L, Herkenham M (1997) Induction of inhibitory factor $\kappa\text{B}\alpha$ mRNA in the central nervous system after peripheral lipopolysaccharide administration: an *in situ* hybridization histochemistry study in the rat. *Proc Natl Acad Sci USA* 94:10985–10990.
- Quan N, Whiteside M, Herkenham M (1998) Cyclooxygenase 2 mRNA expression in rat brain after peripheral injection of lipopolysaccharide. *Brain Res* 802:189–197.
- Schmedtje Jr JF, Ji YS, Liu WL, DuBois RN, Runge MS (1997) Hypoxia induces cyclooxygenase-2 via the NF- κB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 272:601–608.
- Sorli CH, Zhang HJ, Armstrong MB, Rajotte RV, MacLough J, Robertson RP (1998) Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. *Proc Natl Acad Sci USA* 95:1788–1793.
- Zheng H, Fletcher D, Kozak W, Jiang M, Hofmann KJ, Conn CA, Soszynski D, Grabiec C, Trumbauer ME, Shaw A, Kostura MJ, Stevens K, Rosen H, North RJ, Chen HY, Tocci MJ, Kluger MJ, VanDerPloeg LHT (1995) Resistance to fever induction and impaired acute-phase response in interleukin-1 β -deficient mice. *Immunity* 3:9–19.