Interleukin-1β mRNA Expression in Ischemic Rat Cortex

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Background and Purpose: Interleukin-1β is a proinflammatory cytokine produced by blood-borne and resident brain inflammatory cells. The present study was conducted to determine if interleukin-1β mRNA was produced in the brain of rats subjected to permanent focal ischemia.

Methods: Rat interleukin-1β cDNA, synthesized from stimulated rat peritoneal macrophage RNA by reverse transcription and polymerase chain reaction and cloned in plasmid Bluescript KS+, was used to evaluate the expression of interleukin-1β mRNA in cerebral cortex from spontaneously hypertensive rats and normotensive rats subjected to permanent middle cerebral artery occlusion. Interleukin-1β mRNA was quantified by Northern blot analysis and compared with rat macrophage RNA standard. To correct for gel loading, blots were also analyzed with cyclophilin cDNA, which encodes an abundant, conserved protein that was unchanged by the experimental conditions.

Results: Interleukin-1β mRNA produced in the ischemic zone was significantly increased from 6 hours to 120 hours, with a maximum of 211±24% of interleukin-1β reference standard, ie, 0.2 ng stimulated rat macrophage RNA, mRNA compared with the level in nonischemic cortices (4±2%) at 12 hours after ischemia (P<.01; n=6). Interleukin-1β mRNA at 12 hours after ischemia was markedly elevated in hypertensive rats 0.2 ng rat, levels found in two normotensive rat strains. Neurological deficits were also apparent only in the hypertensive rats.

Conclusions: Brain interleukin-1β mRNA is elevated acutely after permanent focal ischemia and especially in hypertensive rats. These data suggest that this potent proinflammatory and procoagulant cytokine might have a role in brain damage following ischemia. (Stroke. 1993;24:1746-1751.)

Key Words • cerebral ischemia • cytokines • neuronal damage • rats

Interleukin-1β (IL-1β) is a cytokine with multiple proinflammatory, procoagulant, and cell growth modulatory actions. The presence of IL-1β in the central nervous system is believed to reflect synthesis by diverse cells such as endothelium, microglia, astrocytes, and neurons.

Interleukin-1β acts via specific brain IL-1 receptors that demonstrate significant spatial distribution. As in peripheral organs, the proinflammatory and prothrombotic actions of IL-1β are expected to promote acute neuropathological changes at excessive levels in the brain. Brain ischemia has been shown to be associated with an acute inflammatory response, but the nature of the inflammatory mediators involved in brain ischemia is still unknown. While numerous reports have dealt with theputative roles of various inflammatory mediators such as thromboxane A2, leukotrienes, and platelet-activating factor in brain injury, few attempts have been made to explore the role of proinflammatory cytokines in brain injury. Evidence supporting the involvement of cytokines in central nervous system injury includes demonstrations of IL-1β mRNA expression in brain tissue 3 hours after endotoxin administration, release of IL-1 in mechanically injured brain produced by intraparenchymal implantation of a microdialysis probe, expression of IL-1β mRNA in rat brain in response to direct intraparenchymal administration of the neurotoxin kainate, or combination of endotoxin and interferon.

IL-1β mRNA expression was also shown in a rat model of transient global brain ischemia induced by permanent bilateral vertebral artery occlusion followed by bilateral carotid occlusion with reperfusion. However, cytokine transcription in permanent focal ischemia has not been previously evaluated. The purpose of the present study was to determine whether the initiation phase of IL-1β production, ie, the transcription of its mRNA, takes place after permanent middle cerebral artery occlusion (MCAO); furthermore, this study also aimed to explore the differences, if any, between rats carrying risk factors for stroke (eg, hypertension) and normotensive rats.

Materials and Methods

To provide IL-1β mRNA for use as a standard and to create the necessary IL-1β cDNA probe for Northern...
 blot analysis, rat peritoneal macrophages were collected by lavage with phosphate-buffered saline and incubated with 5 μg/mL Escherichia coli lipopolysaccharide (Sigma Chemical Co, St Louis, Mo) at 37°C for 4 hours. Cells were lysed in 4 mol/L guanidinium thiocyanate, and total RNA was isolated by centrifugation over 5 mol/L CsCl. IL-1β cDNA was synthesized by reverse transcription and polymerase chain reactions (RT-PCR; Boehringer Mannheim Biochemica, Indianapolis, Ind) using the 5' and 3' synthetic oligonucleotide primers 5'-CACCGGCCTTGGCAAGTGTCGAGCAG-3' and 3'-AAACAGCTTATCCGGTCAGGTCCAGATCTCGCCGGAC-5', respectively. The primers were engineered to contain a NotI restriction site for cloning purposes. The fragment obtained by RT-PCR was digested with NotI and ligated into Bluescript KS+ (Stratagene, La Jolla, Calif). The identity of the cDNA insert was confirmed by sequencing and agrees with the known sequence. For Northern blot hybridization, the 0.9-kb rat IL-1β cDNA insert was used (isolated by NotI digestion of Bluescript K+ that contained the IL-1β cDNA fragment and purified by agarose gel electrophoresis). Similarly, a human 0.9-kb EcoRI cyclophilin cDNA fragment was prepared and also used for hybridization according to Bergsma et al. Both rat IL-1β and cyclophilin cDNA fragments were labeled using an oligolabeling kit (Pharmacia LKB Biototechnology, Piscataway, NJ) via random primer extension using Klenow DNA Polymerase I and purified using a Nuctrap pull column (Stratagene).

Focal ischemia or sham surgery was carried out in spontaneously hypertensive rats (SHR; Taconic Farms, Germantown, NY) and in two normotensive rat strains (F-344 or Wistar-Kyoto; Taconic Farms) (weight, 250 to 300 g). Animals were anesthetized with sodium pento- barbital (Sernolin Laboratories, Inc, Phoenix, AZ; 60 mg/kg IP) and prepared for surgery as previously described. Briefly, via a 2- to 3-mm craniotomy the right middle cerebral artery was isolated on the hooked tip of a platinum-iridium wire (0.0045-in diameter; Medwire, Mount Vernon, NY) and then simultaneously occluded and cut dorsal to the lateral olfactory tract (n = 18, SHR; n = 4, F-344; n = 4, Wistar-Kyoto). In sham-operated animals (n = 3; SHR) the same surgical procedures were exercised, but the artery was not occluded, and animals were killed at 12 hours. In addition, nonoperated animals (n = 3; SHR) were also studied.

Two separate neurological examinations were performed on the rats before euthanasia to determine the severity of motor deficits. Contralateral forelimb deficits were measured using a neurological grade as previously described, while contralateral hind-limb deficits were measured using a standard hind-limb placement test. At various times post-MCAO rats were overdosed with pentobarbital, and forebrains were removed and dissected as described previously. A segment of the ipsilateral frontoparietal cortex was sliced from the hemisphere; an identical segment was sliced from the contralateral cortical hemisphere (nonischemic control). The segments were immediately frozen in liquid nitrogen and stored at −80°C.

For Northern blot analysis of IL-1β mRNA in the focal ischemic and nonischemic tissue, total RNA from cortical tissues (50 μg) and total RNA obtained from peritoneal macrophages (ie, 0.2 μg isolated as described above and used in the Northern blots as an IL-1β mRNA reference standard for quantitation purposes) was fractionated through a 1% agarose gel, containing 6% formaldehyde in MOPS (3-[N-morpholino]-propane-sulfonic acid; Sigma). The RNA was then transferred onto a nitrocellulose membrane (Keene, NH; BA83, 0.2 μm) by capillary blotting in 20× saline–sodium phosphate–ethylenediaminetetraacetic acid (EDTA) (SSPE) (1× SSPE is 150 mmol/L NaCl, 2 mmol/L NaHPO4, and 1 mmol/L EDTA). RNA was immobilized on the filter, prehybridized, and then hybridized to the 32P-deoxyctydine triphosphate–labeled rat IL-1β cDNA probes at 42°C for 14 to 18 hours in the buffer containing 50% formamide, 6× SSPE, 5× Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS), 100 μg/mL denatured salmon sperm DNA, and 10% dextran sulfate and then washed to a final stringency of 0.5× SSPE, 0.1% SDS, at 50°C for 15 minutes. The membrane was exposed to Amersham Hyperfilm-MP for 2 days with intensifying screen and developed by a Kodak M35A-OMAT processor. All the membranes were stripped in boiling 0.05× SSPE and rehybridized to the 32P-deoxyctydine triphosphate–labeled human cyclophilin cDNA to control for differences in RNA loading.

Radioactivity of the hybridized blots was counted by a Betascope 603 blot analyzer (Betagen Corp, Waltham, Mass). Cortical tissue IL-1β mRNA was quantitated for between-blot comparisons as the percent relative radioactivity of the reference standard (ie, macrophage RNA loaded on the same gel normalized to the actual amount of RNA loaded that was determined from the cyclophilin mRNA counts from the same cortical samples). For within-blot comparisons of ischemic cortex IL-1β, percent relative radioactivity was normalized directly in relation to the loaded RNA determined from the cyclophilin mRNA in each sample.

All data are expressed as mean±SEM. Statistical analysis of the data was performed using analysis of variance and Tukey’s multiple comparison tests. Significant differences were accepted at P < .05.

Results

Neurological deficits after focal ischemia occurred in SHR (Fig 1) but not in normotensive animals. Both forelimb and hind-limb dysfunction in SHR were observed. Partial spontaneous recovery was evidenced at 5 days for forelimb scores and at 1 and 5 days for hind-limb scores.

Fig 2 illustrates the results of a representative Northern blot of rat cortical RNA 12 hours after MCAO. IL-1β mRNA is significantly more abundant in ipsilateral (top IL-1β lanes 1 through 6) ischemic cortices than in the contralateral cortices (bottom IL-1β lanes 1 through 6) of the same animals. RNA from macrophages was used as the reference standard for quantitation purposes (Fig 2); correcting for total loaded RNA was done by measurement of the cyclophilin mRNA (cyclophilin lanes 1 through 6) from the ischemic (top) and nonischemic (bottom) regions.

Fig 3 depicts the quantitated IL-1β mRNA levels in the cortices taken at various time points after MCAO. IL-1β mRNA was not detectable in cortical samples from nonoperated rats (0±0%, n = 3). Sham surgery produced no significant effect. In the contralateral
nonischemic cortex after MCAO, a weak trend for expression of IL-1β was detected that did not reach statistical significance. Permanent MCAO dramatically increased the IL-1β mRNA level in the ipsilateral ischemic cortex compared with the contralateral control cortex from 6 hours after MCAO and up to 5 days later. Peak IL-1β mRNA expression was noticed 12 hours after MCAO (P<.01), but significantly elevated levels were still monitored 5 days later (P<.05). To examine whether the elevated levels of IL-1β found in the ischemic cortices are specific to SHR only, we have monitored IL-1β mRNA in three groups (n=4 per group) of rats studied simultaneously (ie, killed at 12 hours after MCAO and quantitated together). The two normotensive strains, WKY and F-344 rats, responded with much less IL-1β mRNA expression compared with the SHR group (Fig 4). However, the levels of IL-1β mRNA in both normotensive rat strains were significantly elevated over background levels.

Discussion

This article demonstrates that IL-1β mRNA in rat brain is significantly increased in ischemic cerebral tissue. The time course of IL-1β mRNA expression demonstrated a trend toward increased IL-1β mRNA expression as early as 1 hour after permanent MCAO, a definite increase at 6 hours, and persistently elevated IL-1β mRNA levels up to 5 days after ischemia. The early IL-1β mRNA expression, ie, 1 to 6 hours, precedes the time of leukocyte infiltration (measured by myeloperoxidase accumulation) from blood vessels outside the ischemic zone, which only begins to occur after 12 hours and then dramatically increases for a period of 5 days after infarction in this model.17 These data are further supported by a detailed histological study showing significant elevation of neutrophils in ischemic cortex at 48 hours after ischemia.18 Our data also suggest that IL-1β mRNA expression in focal ischemic brain tissue is not specific to the SHR strain since significant IL-1β mRNA expression has been clearly demonstrated in the two normotensive rat strains. The lesser IL-1β mRNA expression in the two normotensive rat strains may represent the lesser vulnerability of brain cortex of normotensive rats to permanent MCAO,6 as also reflected in the present study by lack of neurological deficits in the normotensive rats and previous reports on infarct volume.6

The data presented in this study are in accord with recent reports demonstrating IL-1β mRNA expression in several different brain injury models.9-13 Thus, Minami et al13 have shown acute biphasic IL-1β mRNA expression in various brain regions after transient global ischemia. However, several important differences between the two studies must be pointed out. First, the present data represent transcription of IL-1β in permanent focal ischemia. Second, the previous study13 has
Fig 3. Bar graph illustrates quantitative analysis of the time course for interleukin-1β (IL-1β) mRNA production in cerebral cortices ipsilateral or contralateral to permanent middle cerebral artery occlusion (MCAO) (n=3 to 6 per time point) or sham surgery (n=3 killed at 12 hours). The β-emissions from bands on hybridized Northern blots were counted using a Betascope counter. Data (mean±SE) from different blots are presented as percentage of loaded rat macrophage IL-1β mRNA (reference standard) normalized to the actual amount of loaded RNA (see "Materials and Methods"). *P<.05 compared with contralateral cortex; **P<.01 compared with contralateral cortex and compared with ipsilateral cortex of 5-day MCAO. No detectable IL-1β mRNA was observed in either cortex of nonoperated rats (n=3; data not shown). Vertical bars represent SEM.

not used a reference message (eg, cyclophilin), nor did it standardize its hybridization probe against a single IL-1β mRNA–containing sample such as rat macrophage IL-1β mRNA. Third, and most importantly, the present study provides quantitated data, whereas only qualitative (visual) representative gels were provided in the global ischemia study.13 These differences may underlie the lack of significant IL-1β mRNA in the brain between 1 and 7 days after global brain ischemia, whereas significant IL-1β mRNA was clearly demonstrated in our study at 5 days after MCAO. The lack of standardization and quantification of IL-1β mRNA in the global ischemia report also makes it difficult to correlate the magnitude of the IL-1β mRNA expression in the focal ischemic brain cortex described here to the changes described previously in the global ischemia model.13

The significance of the IL-1β mRNA expression described in the present study must be interpreted with caution because no evidence has been provided to indicate that translation of the message into the functional cytokine has occurred either at the time of the IL-1β mRNA expression or thereafter. However, the de novo expression of IL-1β mRNA in the nonperfused ischemic cortex supports the possibility that endogenous IL-1β might be produced in ischemic brain. This possibility draws further credence from reports demonstrating the capacity of the brain to synthesize cytokines, including IL-1, in several other brain injury models. For example, mechanical injury10 resulted in significant IL-1 production 24 hours after injury, which compares favorably with the earlier IL-1β mRNA shown in our study. Also in this latter study, IL-1 production was significantly lower at 7 days after injury, again in accord with the significant diminution of IL-1β mRNA observed in our study. In any case, it will be necessary to directly monitor IL-1β production in focal brain ischemia to precisely relate the transcriptional event to the cytokine production.

The cellular elements expressing IL-1β mRNA in the ischemic brain have not been elucidated in the present study. However, IL-1 has been shown to be produced by microglial cells in vitro19 and in vivo20,21 and by astrocytes in vitro.22,23 Furthermore, IL-1 has been detected in the cerebrospinal fluid of rats.24 It is also noteworthy that a peripheral source for IL-1 might gain access into the brain since this cytokine was shown to be transported from the blood into the brain25; therefore, even

Fig 4. Bar graph illustrates quantitative analysis of ischemic cortex interleukin-1β mRNA production in groups (n=4) of hypertensive (spontaneously hypertensive rats [SHR]) and normotensive (Wistar-Kyoto [WKY] and Fisher) rats 12 hours after middle cerebral artery occlusion. Data from one blot are presented as relative change normalized to the actual amount of loaded RNA (see "Materials and Methods"). *P<.05 compared with SHR; **P<.01 compared with WKY rats.
if IL-1β is produced in the brain, this may not preclude a role for peripheral IL-1 in brain injury.

If one accepts the possibility that the increase in IL-1β mRNA shortly after ischemic stroke indeed translates into a robust production of this potent cytokine, one must also anticipate its consequences; these include activation of microglia and astrocytes26 and transformation of the endothelium into a proinflammatory and prothrombotic27 state that may propagate thrombosis and the extension of the ischemic zone. Finally, it is important to point out that IL-1β mRNA is only one of many genes that are overexpressed in the brain after injury; most notably, acute immediate genes such as c-fos,28 heat-shock protein,28,29 and the tumor suppressor gene product p5330 have been shown to be overexpressed in the brain in response to ischemia.

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Editorial Comment

Recent advances and research interest in molecular biology have prompted stroke researchers and neuroscientists to study gene induction and expression in ischemic brain. A good example of this interest is the induction and expression of the 70-KD stress (heat-shock) gene at both transcriptional and translational levels in neurons, glia, and endothelial cells that have been intensively investigated in various models of permanent and temporary cerebral ischemia. Along a similar line, using a rat interleukin (IL)-1β cDNA synthesized from stimulated rat peritoneal macrophage RNA, Liu et al have now reported the induction and expression of IL-1β mRNA in the ischemic zone in both hypertensive and normotensive rats after permanent focal ischemia. Furthermore, the brain level of IL-1β mRNA is increased markedly in hypertensive rats over the levels found in two different strains of normotensive rats. A couple of important implications resulting from this interesting study might need elaboration. First, the elevated levels of IL-1β mRNA in the brain cortex in hypertensive rats correlate with the severity of the neurological deficits after permanent focal ischemia, which suggests that the elevated levels of cytokine mRNA...
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