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Concomitant Cortical Expression of TNF-α and IL-1β mRNAs Follows Early Response Gene Expression in Transient Focal Ischemia

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ABSTRACT

The expression of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) mRNAs was significantly increased in the rat ischemic cortex following temporary occlusion of the middle cerebral artery (TMCAO) with reperfusion. Northern blot analysis demonstrated that the induction of TNF-α and IL-1β mRNAs occurred as early as 1 h after reperfusion, exhibiting a 4.6-fold increase ($p < 0.05$, $n = 4$) and 6.8-fold increase ($p < 0.05$, $n = 4$) in the ischemic cortex over control, respectively. TNF-α mRNA reached its peak at 3 h (8.0-fold, $p < 0.05$), whereas IL-1β mRNA reached its peak at 6 h (29.5-fold, $p < 0.05$). Both cytokine mRNA levels remained elevated for up to 2 d after reperfusion. In contrast to the time course of these cytokine mRNAs, c-*fos* and zif268 mRNAs, two early response genes, displayed a greater and earlier time-response profile. The early induction of c-*fos* and zif268 mRNAs in temporary brain ischemia with reperfusion suggests their roles in transcriptional regulation. The later concomitant expression of TNF-α and IL-1β suggests that these cytokines play an important role in the inflammatory response associated with focal ischemia.

Index Entries: TNF-α; IL-1β; transient focal brain ischemia; stroke; inflammation.

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INTRODUCTION

Tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) are pleiotropic cytokines associated with disease development and host defense response (Tracey and Cerami, 1993). TNF-α stimulates acute phase protein secretion, and enhances the permeability of endothelial cells where it also induces expression of adhesion molecules and prothrombotic activities (Nawroth and Stern, 1986; Warren, 1990). The increased expression of these two cytokines has been observed in a number of brain injuries and other central nervous system (CNS) diseases involving inflammatory and immune reactions. In the CNS, TNF-α, IL-1β, and other cytokines have been implicated in the pathogenesis of multiple sclerosis, meningitis, brain tumors, and cerebral edema (Tracey and Cerami, 1993).

Brain ischemia has been shown to be associated with an acute inflammatory response, characterized by polymorphonuclear leukocyte (PMN) infiltration into the ischemic cortex (Hallenbeck et al., 1986; Barone et al., 1991, 1992b; Clark et al., 1993; Garcia et al., 1994). The recruitment of circulating PMN into ischemic tissue requires the interaction of microvascular endothelial cells with leukocytes via specific adhesion molecules (Beekhuizen and van Furth, 1993; Thornhill and Haskard, 1993). Very recently, the induced expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and P-selectin in vascular endothelial cells has been demonstrated following focal ischemia in our laboratory and by others (Wang et al., 1994a; Okada et al., 1994). However, the factors mediating the induction of the adhesion molecules in focal brain ischemia are not clear. In vitro studies suggest that proinflammatory cytokines such as TNF-α and IL-1β may play such a role to mediate the expression of adhesion molecule expression in both endothelial cells and leukocytes (Nawroth and Stern, 1986; Mantovani et al., 1992; Beekhuizen and van Furth, 1993; Thornhill and Haskard, 1993). We previously demonstrated the induced expression of both TNF-α and IL-1β mRNAs in rat ischemic cortex following permanent occlusion of the middle cerebral artery (PMCAO) (Liu et al., 1993a, 1994). IL-1β mRNA expression was also shown in a rat model of transient global forebrain ischemia (Minami et al., 1992; Wiebner et al., 1993). In the present study, we evaluated the expression pattern of TNF-α and IL-1β mRNAs in another rat focal ischemic model, temporary occlusion of middle cerebral artery (TMCAO) with reperfusion. Such an ischemic insult was shown to result in a larger and earlier cortical inflammatory response following reperfusion than that reported in permanent focal ischemia (Barone et al., 1992b, 1993).

c-*fos* and zif268 (also named as NGFI-A, egr-1, Krox-24, TIS-8) are two critical immediate early genes that are rapidly and transiently induced in response to environmental stimuli (Herschman, 1991; Sheng and Greenberg, 1990). We previously demonstrated their early and transient induction profile following PMCAO in the rat (Wang et al., 1994b). The induced ex-

pression of c-*fos* mRNA has also been reported following cerebral transient ischemia in rats (Onodera et al., 1989; An et al., 1993) and gerbils (Kindy et al., 1991). However, the expression patterns of these immediate early genes have not been studied in the TMCAO model. Therefore, in the present study, for comparative purposes, we also evaluated the time-response profiles of c-*fos* and zif268 mRNAs in addition to TNF-α and IL-1β following TMCAO with reperfusion.

MATERIALS AND METHODS

Male spontaneously hypertensive rats (SHR; Taconic Farms, Germantown, NY) at 18 wk old, weighing 250–330 g were used in this study to generate cerebral focal ischemia or sham surgery. The protocol for TMCAO has been described in detail previously (Barone et al., 1991, 1992a). Briefly, the middle cerebral artery was lifted from the brain surface at the level of inferior cerebral vein to occlude blood flow for 160 min and then allowed to reperfuse. In sham-operated rats the dura was opened over the artery but it was not occluded. Rats were overdosed with pentobarbital and forebrains were removed and dissected at various times following MCAO with reperfusion. Tissue samples from the ischemic and nonischemic cortex were dissected from the ipsilateral (ischemic) and contralateral (nonischemic) hemispheres. Ischemic cortex tissue samples always included infarcted and some adjacent, noninfarcted tissues as described previously (Barone et al., 1992a, b). The samples were immediately frozen in liquid nitrogen and stored at $-80°C$.

Total cellular RNA was prepared by using acid guanidinium thiocyanate, phenol, and chloroform extraction procedure (Chomczynski and Sacchi, 1987). RNA samples (40 μg/lane) were electrophoresed through formaldehyde-agarose slab gels (Lehrach et al., 1977), and transferred to GeneScreen Plus membranes (DuPont-New England Nuclear, Boston, MA). Rat TNF-α and IL-1β cDNAs were generated by RT/PCR and subcloned into a vector as described in detail previously (Liu et al., 1993a, 1994). For Northern blot analysis, TNF-α, IL-1β, c-*fos* (Mohn et al., 1991; a kind gift of R. Taub, University of Pennsylvania, PA), zif268 (Christy et al., 1988; a kind gift of B. A. Christy, University of Texas Health Science Center at San Antonio, TX), or ribosomal protein L32 (rpL32) (Meyuhas and Perry, 1980; a kind gift of R. P. Perry, Institute for Cancer Research, Fox Chase, PA) cDNA insert were released with restriction enzymes, isolated by electrophoresis and uniformly labeled with [α-^{32}P]dATP and/or [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Corp., Arlington Heights, IL) using a random-printing DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). As a relatively weak signal was observed for the TNF-α probe, a double labeling with two isotopes for this probe was used in the present study. Hybridization was carried out overnight with 1×10^6 cpm/ml of probe at 42°C in 5X SSPE (750

m*M* NaCl, 50 m*M* NaH_2PO_4, pH 7.6, 5 m*M* EDTA), 50% formamide, 5X Denhardt's solution, 2% SDS, and 200 μg/mL boiled salmon sperm DNA (Wang et al., 1992). The membranes were washed in 2X SSPE, 2% SDS at 65°C for 1–2 h with a change for every 30 min, then autoradiographed at −80°C with a Cronex Lightning-Plus intensifying screen for various times depending on the signal intensity. Probes were stripped from the membranes by boiling in 10 m*M* Tris, pH 7.5, 1 m*M* EDTA, pH 8.0, 1% SDS for 20 min prior to rehybridization with the other probe (Wang et al., 1992).

PhosphorImager was used to quantitate the band intensities of the Northern blots using ImageQuant-TM Software v3.0 (Molecular Dynamics, Sunnyvale, CA). The amount of RNA loaded on each lane was normalized to a rpL32 probe, which displayed a constant mRNA level under the various conditions and at the different time-points following focal ischemia (Wang et al., 1994a, b). The relative mRNA level for each probe was calculated after normalizing to the rpL32 signal in each sample.

Statistical evaluation was performed based on the Northern blots using four complete sets of samples at each time point ($n = 4$). The results were analyzed by a repeated measures analysis of variance, followed by Bonferroni-adjusted post-hoc *t*-test (Milliken and Johnson, 1984), which ensures that the overall false positive error rate for a set of multiple comparisons is less than or equal to 0.05. The data were expressed as mean +/− standard error, and significance was accepted for $p < 0.05$ by multiple comparisons of the relative mRNA levels at each time point vs sham samples.

RESULTS

The expression of TNF-α mRNA in rat focal ischemic (ipsilateral) but not the nonischemic (contralateral) cortex was observed following temporary MCAO with reperfusion. Only a very low, basal level of TNF-α mRNA expression was detected in samples from sham-operated animals. A representative Northern blot for TNF-α mRNA expression was shown in Fig. 1A and the quantitated data from the corresponding Northern blot, after normalizing with rpL32 mRNA for samples loaded in each lane, were depicted on Fig. 1B. A significantly elevated level of TNF-α mRNA was observed as early as 1 h after reperfusion (4.6-fold increase in the mean value compared to sham sample, $n = 4$, $p < 0.05$). The mRNA reached a peak level at 3 h (8.0-fold increase, $p < 0.05$), and maintained at a high level for up to 12 h (4.6-fold increase, $p < 0.05$) following MCAO with reperfusion. The elevated TNF-α mRNA level was observed as late as 2 d after reperfusion (2.3-fold increase).

The expression pattern for IL-1β mRNA was very similar to that of TNF-α mRNA under the same conditions and samples. A representative Northern blot for IL-1β mRNA expression in sham, ischemic, and nonischemic cortex is illustrated in Fig. 2A and the quantitated Northern blot data ($n = 4$) are shown graphically in Fig. 2B. A significant increase in the

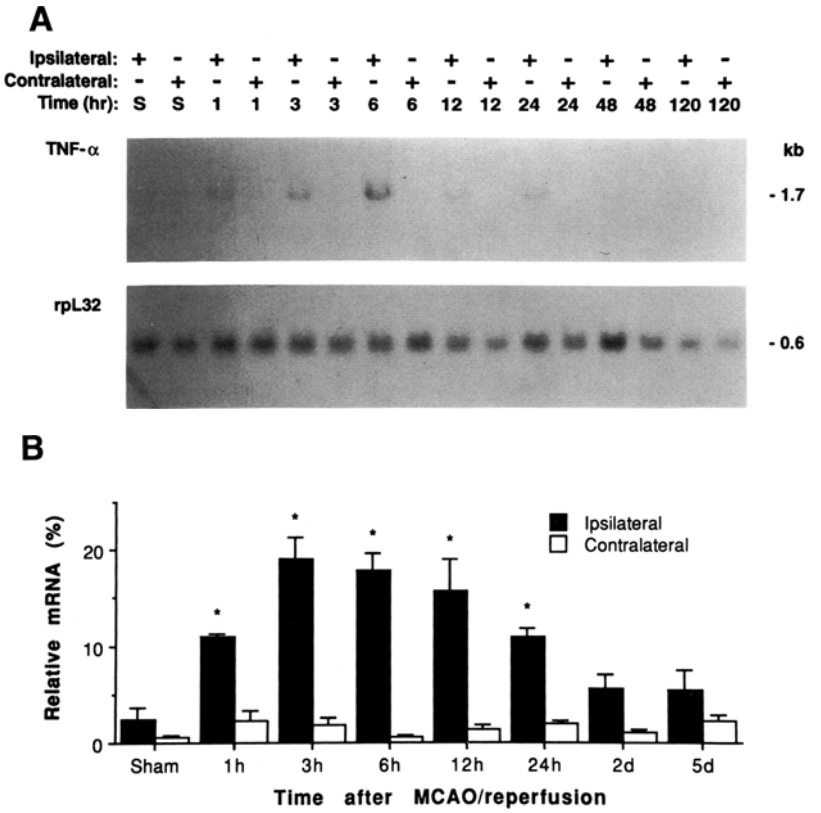

Fig. 1. Northern blot analysis of TNF-α mRNA induction in rat ischemic cortex following temporary MCAO with reperfusion. (**A**) A representive Northern blot for TNF-α and rpL32 probes to samples isolated at various time points and conditions from rats subjected to transient occlusion (160 min) of the middle cerebral artery followed by reperfusion. Total cellular RNA (40 μg/lane) was resolved by electrophoresis, transferred to a nylon membrane, and hybridized to the indicated cDNA probe as detailed in Materials and Methods. The mRNA size was determined by comparing with the migration of RNA ladder (BRL) and the number of kilobases (kb) is marked on the right. Ipsilateral and contralateral cortex samples (denoted by +) from individual rats following sham surgery (S) or following 1, 3, 6, 12, 24, 48, and 120 h of TMCAO are depicted. (**B**) Quantitated Northern blot signals for TNF-α cDNA probe. The quantitation was carried out using PhosphorImager analysis. The samples loaded in each lane were normalized to the values of rpL32 mRNA signals. The normalized values for each probe were displayed graphically with a sum of 100% total. Data are presented as the mean values for four separate experiments in spontaneously hypertensive rats ($n = 4$) for each time point. $^*p < 0.05$, vs sham samples by a repeated measures analysis of variance, followed by Bonferroni-adjusted post-hoc *t*-test.

IL-1β mRNA was observed in the ipsilateral (ischemic) cortex compared to the nonischemic cortex or sham-operated animals at 1 h (6.8-fold increase, $p < 0.05$, $n = 4$). IL-1β mRNA peaked at 6 h (29.5-fold increase, $p < 0.05$) and then slowly decreased but maintained at an elevated level for up to 2 d (6.6-fold high, $p < 0.05$) following temporary MCAO with reperfusion (Fig. 2).

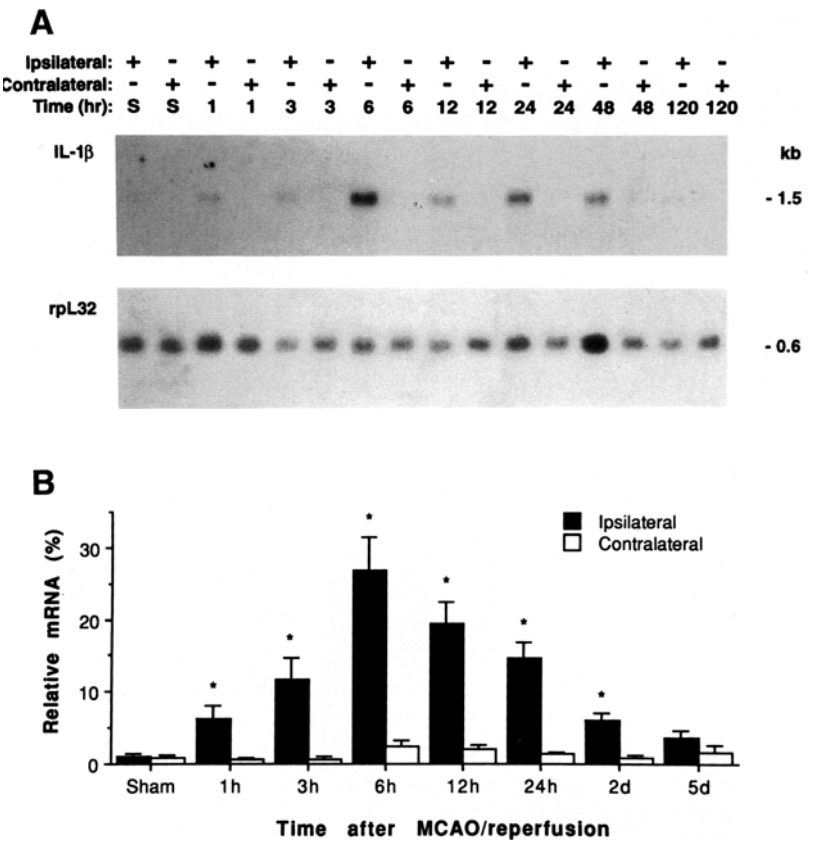

Fig. 2. Northern blot analysis of IL-1β mRNA induction in rat ischemic cortex following temporary MCAO with reperfusion. The figure is illustrated as described in the Fig. 1 legend, except that a rat IL-1β cDNA probe is used here.

As illustrated in Figs. 3 and 4, the mRNA expression patterns for c-*fos* and zif268 were much greater initially, and more transient than that of TNF-α and IL-1β following TMCAO with reperfusion. Within as little as 1 h following reperfusion, c-*fos* and zif268 mRNAs were remarkably induced with a 62-fold ($p < 0.05$, $n = 4$) and eightfold ($p < 0.05$, $n = 4$) increase compared to the sham-operated samples, respectively. Both mRNAs decreased sharply to a low elevated level at 6 h after reperfusion (Figs. 3 and 4). As noted, zif268 exhibited a constitutive mRNA expression in normal brain as reflected by a relative high level of mRNA in sham surgery samples, which is thought to be driven in part by central norepinephrine (NE) and serotonin (5-HT) inputs (Bhat et al., 1992), whereas c-*fos* mRNA displayed an interesting extended but low level elevated expression pattern for up to 2 d after reperfusion (8.1-fold increase compared to ipsilateral sham cortex, $p < 0.05$).

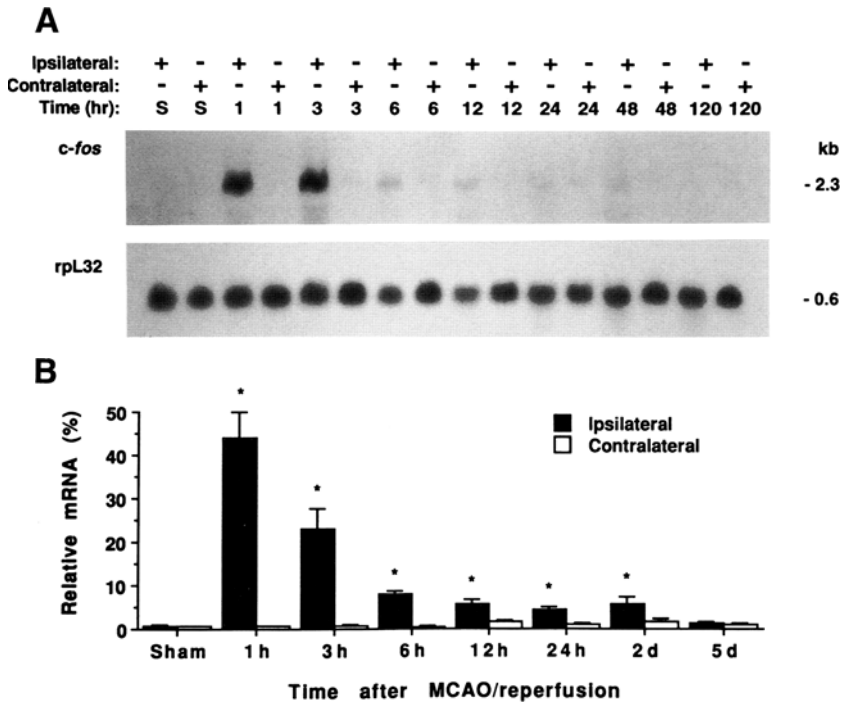

Fig. 3. Northern blot analysis of c-*fos* mRNA induction in rat ischemic cortex following temporary MCAO with reperfusion. *See* details in Fig. 1 legend.

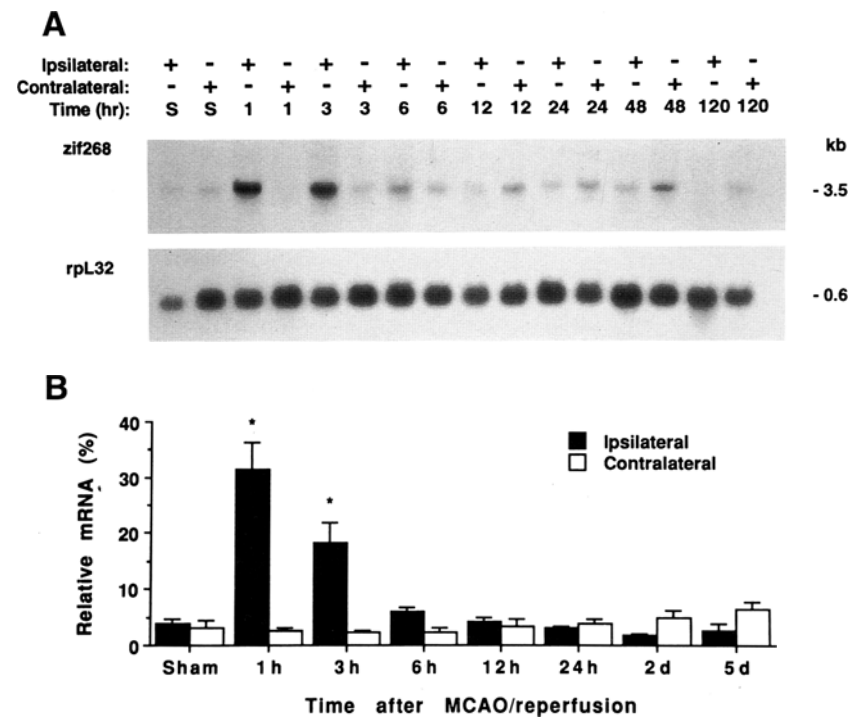

Fig. 4. Northern blot analysis of zif268 mRNA induction in rat ischemic cortex following temporary MCAO with reperfusion. The figure is illustrated as described in Fig. 1 legend.

DISCUSSION

In the present study, we demonstrated a marked increase in the expression of both TNF-α and IL-1β mRNAs very soon after TMCAO with reperfusion (Figs. 1 and 2). The mRNA expression profile of these two proinflammatory cytokines in this TMCAO model exhibited a similar pattern compared to that in the PMCAO model (Liu et al., 1993a, 1994), except that following TMCAO in the present study they increased earlier. An increased number of PMNs in the ischemic cortex are also observed earlier following TMCAO (significantly increased after 6 h) than following PMCAO (not increased until 12 h) (Barone et al., 1993; Clark et al., 1993). Many leukocytes, including monocytes, are observed within the infarcted tissue over 5 d of permanent ischemia or reperfusion. The peak expression of TNF-α mRNA is observed at 3 h (eightfold increase, $p < 0.05$) in this model, whereas the peak occurred at 12 h following PMCAO. IL-1β mRNA reached a peak level at 6 h (29.5-fold increase, $p < 0.05$) and remained elevated for up to 2 d (6.6-fold increase, $p < 0.05$) in the reperfusion model. In contrast, the expression of IL-1β mRNA following PMCAO reached a peak level with a larger delay of 12 h, and maintained a longer elevated level for up to 5 d (Liu et al., 1993a). Overall, our present and previous data strongly suggest that cytokine expression in focal stroke precedes and facilitates the infiltration of leukocytes into the ischemic cortex.

The present observation for the increased IL-1β mRNA expression in response to temporary focal ischemia is also in accord with previous report in rat transient forebrain ischemia model (Minami et al., 1992; Wiebner et al., 1993). However, the IL-1β mRNA induction profiles in these studies are different. Minami et al. (1992) have shown acute biphasic IL-1β mRNA expression in various brain regions and an induction profile in the cerebral cortex that is more transient after transient global ischemia. Wiebner et al. (1993) using *in situ* hybridization have shown a surprisingly late induction for IL-1β mRNA (i.e., expression at 7 d after transient forebrain ischemia). The reasons for the different mRNA induction profiles are not clear, except that different times of ischemia were applied for four-vessel occlusion (15 vs 30 min) compared to 160 min TMCAO in our model.

It is of interest that the inducation profile for TNF-α and IL-1β mRNAs in the present report is remarkably similar to that of ICAM-1 mRNA following TMCAO with reperfusion (Wang et al., 1994a). This similarity suggests a functional correlation between inflammatory cytokines and adhesion molecules and the involvement of these molecules in the inflammatory response in the ischemic tissue. TNF-α, IL-1β, and other cytokines have been shown to induce the expression of ICAM-1, VCAM-1, ELAM-1, and other adhesion molecules on endothelial cells in vitro and in vivo (Dustin et al., 1986; Sobel et al., 1990; Wellicome et al., 1990; Mantovani et al., 1992; Wong and Dorovini-Zis, 1992; Beekhuizen and van Furth, 1993; Thornhill and Haskard, 1993). It is likely that the increased expression of

the proinflammatory cytokines, such as TNF-α and IL-1β, in cerebral focal ischemia can contribute to the elevated expression of adhesion molecules such as ICAM-1 and ELAM-1 on microvascular endothelial cells, which in turn facilitates the recruitment of circulating leukocytes into the focal ischemic tissue. Indeed, TNF-α microinjection into the cortex does produce vascular neutrophil accumulation similar to that observed in focal ischemia (Liu et al., 1994). Thus, the temporally coordinated expression profile for inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6; Liu et al., 1993a, 1994; Wang et al., 1994b; and present observations), chemokines (e.g., CINC/KC; Liu et al., 1993b), and adhesion molecules (e.g., ICAM-1; Wang et al., 1994a; Okada et al., 1994) in focal ischemic tissue strongly suggest their role(s) in the inflammatory reaction contributing to ischemic tissue injury.

Certainly more work identifying the cell sources for increased messenger expression and protein levels need to be carried out in the future. However, the early induction profile of TNF-α and IL-1β mRNA prior to the infiltration of PMN into the ischemic tissue (which occurs at 6–12 h post-MCAO) suggests that these cytokines are initially produced from within the focal ischemic tissue. TNF-α has been localized in ischemic neurons following PMCAO using immunohistochemistry (Liu et al., 1994). *In situ* hybridization study suggests that IL-1β mRNA is most likely to be produced by macrophage or activated microglia in the ischemic brain tissue (Buttini et al., 1994). Early increases in messenger expression appear to reflect infarcted and adjacent tissue responses, whereas later increases appear to correspond to the presence of leukocytes in the infarcted cortex as pointed out previously (Liu et al., 1994).

The expression of c-*fos* and zif268 mRNAs in TMCAO with reperfusion has not been previously investigated. The present study demonstrated that TMCAO in the rat resulted in an immediately marked but transient induction for c-*fos* and zif268 mRNAs. This larger early induction profile following TMCAO is very similar to that demonstrated in transient forebrain ischemia models in rats (Onodera et al., 1989; An et al., 1993) and gerbils (Kindy et al., 1991), but differs from PMCAO in rats (Wang et al., 1994b), which exhibited a more delayed increase that did not reach a peak level until 3 h. Differing from all other ischemia models studied, the elevated c-*fos* mRNA following TMCAO was extended for up to 2 d although it displayed a much lower intensity relative to the peak elevation. The mechanism for this extended c-*fos* mRNA expression is not known.

Early response genes (e.g., c-*fos* and zif268) are usually characterized as transcriptional regulators that control the delayed response gene expression, whereas the delayed response genes (e.g., TNF-α and IL-1β) function as effector molecules playing a biological or pathological role. The results presented in this report extended previous observation for c-*fos* and zif268 gene expression in response to ischemic injury and suggested their transcriptionally regulatory role in focal ischemia. The demonstration of a coordinated expression of TNF-α and IL-1β in the ischemic cortex prior to PMN

infiltration suggests their roles in the leukocyte recruitment and the inflammatory response to focal ischemia, and provides potential targets for blocking the proinflammatory reaction in stroke, although it remains to be further evaluated. The functional significance for the upregulation of IL-1β in focal ischemia has been suggested by a recent study demonstrating that administration of IL-1 receptor antagonist (IL-1ra) into the brain can significantly reduce ischemic injury (Rothwell and Ruton, 1993).

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