

Reperfusion Increases Neutrophils and Leukotriene B₄ Receptor Binding in Rat Focal Ischemia

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Background and Purpose: Neutrophils are critically involved with ischemia and reperfusion injury in many tissues but have not been studied under conditions of reperfusion after focal cerebral ischemia. The present studies were conducted to confirm our previous observations quantifying neutrophils in rat permanent focal stroke using a myeloperoxidase activity assay and to extend them to transient ischemia with reperfusion. In addition, leukotriene B₄ receptor binding in ischemic tissue was evaluated as a potential marker for inflammatory cell infiltration.

Methods: Histological, enzymatic, and receptor binding techniques were used to evaluate neutrophil infiltration and receptor binding in infarcted cortical tissue 24 hours after permanent middle cerebral artery occlusion ($n=25$) or temporary occlusion for 80 ($n=12$) or 160 ($n=22$) minutes followed by reperfusion for 24 hours in spontaneously hypertensive rats.

Results: Sham surgery ($n=26$) produced no changes in any parameter measured. After permanent middle cerebral artery occlusion, neutrophil accumulation was observed histologically, but the infiltration was moderate and typically within and adjacent to blood vessels bordering the infarcted cortex. After temporary middle cerebral artery occlusion with reperfusion, marked neutrophil infiltration was observed throughout the infarcted cortex. Myeloperoxidase activity was increased ($p<0.05$) after permanent occlusion and to a greater extent after temporary occlusion with reperfusion. Myeloperoxidase activity (units per gram wet weight) in ischemic cortex was increased over that in nonischemic (control) cortex 32.2-fold, 54.6-fold, and 92.1-fold for permanent occlusion and 80 and 160 minutes of temporary occlusion with reperfusion, respectively ($p<0.05$). Sham surgery produced no changes in myeloperoxidase activity. Leukotriene B₄ receptor binding also was increased ($p<0.05$) after focal ischemia and paralleled the increases in myeloperoxidase activity. Ischemic cortex-specific receptor binding (femtomoles per milligram protein) was 3.87 ± 0.63 in sham-operated rats and 4.57 ± 0.98 , 8.98 ± 1.11 , and 11.12 ± 1.63 for rats subjected to permanent occlusion and 80 and 160 minutes of temporary occlusion with reperfusion, respectively (all $p<0.05$ different from sham-operated). Cortical myeloperoxidase activity was significantly correlated with the degree of cortical leukotriene B₄ receptor binding ($r=0.66$ and $r=0.79$ in two different studies, $p<0.01$).

Conclusion: These data indicate that neutrophils are involved in focal ischemia and that there is a dramatic accumulation of neutrophils in infarcted tissue during reperfusion that can be quantified using the myeloperoxidase activity assay. Leukotriene B₄ receptor binding increases in infarcted tissue in a parallel manner, which suggests that the increased leukotriene B₄ binding is to receptors located on the accumulating neutrophils. (*Stroke* 1992;23:1337-1348)

KEY WORDS • cerebral ischemia • neutrophils • reperfusion • rats

Polymorphonuclear leukocytes (PMN) and chemoattractant agents that regulate their activity are critically involved with ischemia and reperfusion injury.¹ Strong evidence indicates that these leukocytes, primarily neutrophils, contribute to reperfusion injury/microvascular damage in the stomach,² intestine,^{3,4} heart,⁵⁻⁷ skeletal muscle,⁸ and liver.⁹ Neutro-

phil depletion^{10,11} and inhibition of neutrophil adhesive interactions^{12,13} can protect the myocardium from injury after ischemia and reperfusion. In the central nervous

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system, increased numbers of leukocytes have been identified in the brain in animal models of air emboli-induced focal cerebral ischemia^{14,15} and laser-induced brain injury,¹⁶ after permanent focal ischemia in rats¹⁷ and primates,¹⁸ and in humans in subarachnoid hemor-

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rhage.¹⁹ In addition, neutrophil depletion has been shown to improve the neuronal recovery and/or cerebral perfusion that occurs after complete²⁰ and incomplete²¹ cerebral ischemia and thromboembolic²² and embolic²³ stroke; this recovery includes the reduction of ischemic edema.²⁴ Also, monoclonal antibodies that interfere with leukocyte adhesion to endothelial cells reduce paraplegia²⁵ and improve motor function and blood flow²⁶ after spinal cord ischemia.

Generally, neutrophils accumulate in tissues in response to chemotactic factors associated with tissue injury/inflammatory processes²⁷ and products of hemostatic activity.²⁸ Under these conditions activated neutrophils can release substances noxious to the vascular endothelium and can produce capillary plugging that exacerbates injury.^{29–31} However, to initiate this process the integrin complex of adhesion glycoproteins, CD18, is required for leukocyte adherence to endothelium.³² Basically, the CD11/CD18 receptor complex on activated leukocytes³³ undergoes a receptor-mediated adhesion to intact endothelium by interacting with the corresponding endothelial-leukocyte adhesion molecule³⁴ and intercellular adhesion molecule-1³⁵ on endothelial cells. Among the several known chemotactic agents that regulate this process, leukotriene B₄ (LTB₄) stimulates adhesion³⁶ by inducing a CD18 conformational change on leukocytes³⁷ and by stimulating increased receptor expression (i.e., of CD11/CD18 and the adhesion molecules) on both leukocytes and endothelial cells.³⁸

Recently, a report from this laboratory demonstrated that neutrophil infiltration into the infarcted cortex after middle cerebral artery (MCA) occlusion (MCAO) in rats could be quantified¹⁸ using an assay for the activity of myeloperoxidase (MPO), an enzyme found within the azurophilic granules of these cells.³⁹ However, no previous studies have evaluated and compared the effects of permanent MCAO with those of temporary MCAO and reperfusion on histologically identified and MPO-quantified neutrophil infiltration. Also, the relation between neutrophil infiltration into damaged cerebral tissue and binding of chemotactic mediators to their receptors (i.e., LTB₄ receptors) has not been described.

The purpose of the present experiments was to confirm and extend our previous findings on neutrophil infiltration into rat cerebral tissue 24 hours after permanent focal cerebral ischemia produced by MCAO.¹⁸ Neutrophil infiltration was evaluated by histological techniques and quantified with a modified MPO kinetic assay to demonstrate the increase due to reperfusion after temporary MCAO. In addition, to further extend the evaluation of other potential markers for inflammatory cell infiltration, the effect of MCAO on LTB₄ receptor binding in ischemic versus nonischemic tissue was determined, and the correlation of changes in LTB₄ receptor binding to the degree of neutrophil infiltration (i.e., MPO activity) was determined.

Materials and Methods

Animals were housed and cared for in accordance with *Guide for the Care and Use of Laboratory Animals*, DHEW (DHHS) publication No. (NIH) 85-23, revised 1985, Bethesda, Md., Office of Science and Health Reports, DRR/NIH. Procedures using laboratory animals were approved by the Institutional Animal Care

and Use Committee of SmithKline Beecham Pharmaceuticals, plc.

Surgery was performed in male spontaneously hypertensive rats (SHR; Taconic Farms, Inc., Germantown, N.Y.) weighing 250–330 g. SHR were chosen because of their increased sensitivity to permanent or transient focal ischemia. Procedures for permanent MCAO^{18,40,41} and for temporary MCAO with reperfusion⁴¹ under anesthesia with 60 mg/kg i.p. sodium pentobarbital (Steris Laboratories, Inc., Phoenix, Ariz.), with body temperature maintained at 37°C using a heating pad were as described previously.^{40,41} Briefly, the rats were placed in a stereotaxic head holder (David Kopf Instruments, Tujunga, Calif.) and muscles were dissected, a 2–3-mm craniotomy was made, and the dura was opened over the MCA.⁴² The hooked tip of a Teflon-coated platinum-iridium wire 0.0045 in. in diameter (Medwire, Mount Vernon, N.Y.) mounted on a micro-manipulator was placed under the MCA to pull it 0.5–1.0 mm from the brain surface for occlusion. For permanent right MCAO, the MCA was simultaneously occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein using electrocoagulation (Force 2 Electrosurgical Generator, Valley Lab Inc., Boulder, Colo.). For temporary right MCAO, the MCA was lifted from the brain surface as described above enough to occlude blood flow (i.e., as indicated from cortical microvascular perfusion monitored during the procedure described below) for 0, 80, or 160 minutes and then reperfused for 24 hours. In sham-operated rats (i.e., 0 minutes of MCAO) the dura was opened over the MCA but the artery was not occluded. Following surgery, the muscles and skin were closed in two layers.

Laser-Doppler flowmetry was used to verify occlusion and reperfusion of the MCA by monitoring local cortical microvascular perfusion in the primary ischemic cortex receiving blood from the MCA. The 1-mm-diameter probe of a laser-Doppler perfusion monitor (Periflux PF3, Perimed, Stockholm, Sweden) was positioned on the dorsal surface through a skull hole 2–3 mm in diameter that was drilled above the cortical area receiving blood from the MCA (i.e., centered at 0 mm anteroposterior and 5 mm lateral from the bregma landmark with level skull). Cortical perfusion was monitored before and after MCAO and during reperfusion using the calibrated output of the perfusion monitor connected to an R711 polygraph (Beckman Instruments, Inc., Irvine, Calif.).

Twenty-four hours after surgery, two separate neurological examinations were performed to determine the severity of deficits due to sham surgery or temporary or permanent MCAO. The rats were classified using the neurological grade as described previously⁴³ to assess contralateral forelimb deficits resulting from focal ischemic damage in the ipsilateral cortex (0, no deficit; 1, forelimb flexion when suspended by the tail; 2, reduced forepaw resistance to lateral push; and 3, circling behavior during tail suspension). Also, the Hindlimb Placement Test was used to assess contralateral hindlimb deficits, as described previously.^{18,41} Each rat was held with its face away from the edge of a table and the hindlimb extended downward over the table edge. A normal response (0), observed typically in nonsurgically treated or sham-operated animals or ipsilateral to cerebral surgery, consists of immediate placement of the

TABLE 1. Ischemic Cortex Microvascular Perfusion During MCAO and After Reperfusion in Rats

Group	n	Baseline	During MCAO	After reperfusion
Temporary MCAO				
80 min	9	100	20±4	101±6
160 min	10	100	15±2	102±5
Permanent MCAO (24 hr)				
	11	100	16±3	—

MCAO, middle cerebral artery occlusion. Values are mean±SEM % compared with baseline.

hindlimb back on the table. An abnormal response (1) consists of no limb placement/movement.

After the neurological evaluation, the rats were killed with an overdose of sodium pentobarbital. In some cases, for comparative purposes, the animals were perfused transcardially with 200 ml isotonic saline at 25°C with a pressure of 100 mm Hg to flush all blood components from the vasculature. Within 2–3 minutes after perfusion or following cessation of heartbeat in nonperfused rats, the brains were removed and the forebrains prepared for histological evaluation or for measurements of MPO activity and LTB₄ receptor binding.

For histological evaluation, six coronal forebrain slices 2 mm thick were made from the level of the olfactory bulbs to the cortical–cerebellar junction and were immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemical Co., St. Louis, Mo.) in 1.0 M phosphate buffer (pH 7.4) at 37°C for 20–30 minutes.⁴⁴ Stained tissues then were fixed by infiltration in 10% phosphate-buffered formalin (Lerner Laboratories, Pittsburgh, Pa.). Within the next 24 hours, the two sides of each TTC-stained section were photographed in color by using a Polaroid camera and Polacolor Instant Pack Film (Polaroid Corp., Cambridge, Mass.). Cortical infarct volumes were quantified using image analysis/planimetry as described previously^{40,41} and were expressed as the percent of infarcted tissue in reference to the contralateral (normal) hemispheric volume (i.e., percent hemispheric infarct) by dividing infarct volume by the volume of the contralateral hemisphere and multiplying by 100. After fixation, tissues were prepared for embedding in paraffin by using routine histological procedures.⁴⁵ Tissues were dehydrated through graded ethanol concentrations, cleared in xylene, and infiltrated with Paraplast embedding medium (Monoject Scientific, St. Louis, Mo.). After embedding, 6-μm sections were cut on a microtome (model 820, Reichert Scientific Instruments, Buffalo, N.Y.), stained with hematoxylin and eosin (H&E; Sigma), and later examined and photographed by using an Olympus BH-2 microscope (Hitech Instruments, Newtown Square, Pa.). The profile and degree of neutrophil infiltration into infarcted and uninfarcted cortex were determined and photographed.

For the biochemical determination of MPO activity and LTB₄ receptor binding, the forebrain was dissected from the olfactory bulbs and cut at the cortical–cerebellar junction. The hindbrain and cerebellum were discarded. Based on the location and extent of ischemic damage/infarcts identified in TTC- and H&E-stained forebrain tissue, each forebrain was sectioned into four

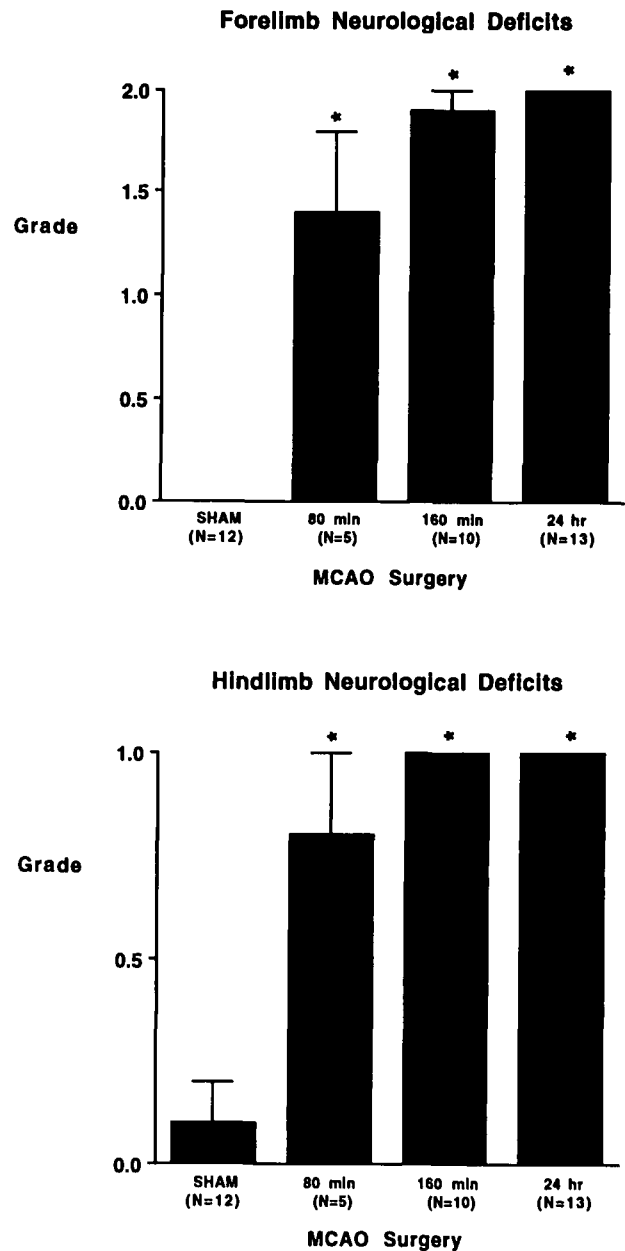


FIGURE 1. Bar graphs showing that increasing duration of middle cerebral artery occlusion (MCAO) (SHAM, 0 minutes) increased the magnitude and decreased the variability of contralateral forelimb (top) and hindlimb (bottom) neurological deficits in rats. * $p < 0.05$ different from SHAM by analysis of variance or χ^2 test. All animals were killed 24 hours after surgery.

segments as diagrammed previously.¹⁸ A segment of the ipsilateral frontoparietal cortex was sliced from the hemisphere ipsilateral to surgery and labeled A. This segment corresponded to the extent of infarction in rats receiving permanent MCAO but was cut similarly for animals receiving sham operation or temporary MCAO. An identical segment was sliced from the contralateral cortical hemisphere and labeled B. The forebrain also was dissected at the midline into two segments, which represented the remaining portion of the hemisphere ipsilateral to surgery (labeled C) and the remaining portion of the hemisphere contralateral to surgery

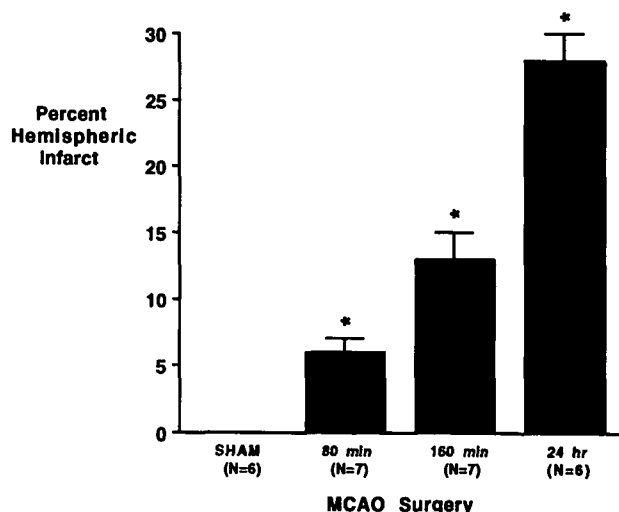


FIGURE 2. Bar graph showing that increasing duration of middle cerebral artery occlusion (MCAO) (SHAM, 0 minutes) increased degree of cortical infarction quantified using image analysis in rats. * $p < 0.05$ different from SHAM by analysis of variance. All animals were killed 24 hours after surgery.

(labeled D). The four forebrain segments were immediately frozen in liquid nitrogen and stored at -80°C for later biochemical analysis.

The method used to quantify MPO activity from rat forebrain segments was as described recently.¹⁸ Briefly, frozen tissue segments were weighed rapidly then homogenized (model PT/1035 Polytron, Brinkmann Instruments, Inc., Westbury, N.Y.; 5-second burst at medium intensity) in 4 ml of 10 mM Tris-HCl, pH 7.4 and 4°C (Sigma). Standardized 1-ml aliquots (25% of the tissue sample) for the MPO activity assay were rehomogenized in 20 ml of 5 mM phosphate buffer, pH 6.0 and 4°C , using a Tissumizer homogenizer (Tekmar Co., Cincinnati, Ohio; three on/off cycles at 5-second intervals) and centrifuged at $30,000g$ for 30 minutes at 4°C . The supernatant was discarded, and the pellet was washed again as described above. After decanting the supernatant, the pellet was extracted by suspension in 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM potassium phosphate buffer at pH 6.0 and 25°C for 2 minutes at a tissue wet weight-to-volume ratio of 1:10. Samples were frozen on dry ice, and three freeze/thaw cycles then were performed, with sonications (10 seconds at 25°C) between cycles. After the last sonication, the samples were incubated at 4°C for 20 minutes and centrifuged at $12,500g$ for 15 minutes at 4°C . MPO activity in the supernatant was assayed as described earlier by Bradley et al.³⁹ The rate at which a colored product formed during the MPO-dependent reaction of *o*-dianisidine (0.167 mg/ml, Sigma) was measured (i.e., the change in absorbance was recorded at 15-second intervals over 2 minutes) at 460 nm using a Beckman DU-7 spectrophotometer with kinetic analysis capability. One unit of MPO activity is defined as the amount that degrades 1 μmol peroxide/min at 25°C . MPO activity (in units) for each forebrain segment was normalized on the basis of grams wet weight tissue.

The method used to quantify LTB_4 receptor binding from the same rat forebrain segments was as described

previously.^{46,47} Briefly, equilibrium [^3H] LTB_4 binding was achieved with optimum incubation conditions (30 minutes at 25°C) using ligand concentrations appropriate to establish maximum effects at saturation. This was accomplished using standardized 3-ml aliquots (75% of the tissue sample) of homogenized segments that were centrifuged at $30,000g$ for 30 minutes at 4°C . The pellets were washed twice with 10 ml of 50 mM Tris-HCl, pH 7.4 and 4°C , and resuspended in 50 mM Tris-HCl at a protein concentration of 2 mg/ml (100 μl membrane preparation or 100–200 μg protein per tube; standard conditions), and 1 nM [^3H] LTB_4 (175–200 Ci/mmol; New England Nuclear–Du Pont Biomedical Products, Clarendon, Calif.) was incubated for 30 minutes at 23°C with or without the addition of 1 μM unlabeled LTB_4 (Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, Pa.) to determine nonspecific and total binding, respectively. The reaction was stopped by the addition of 5 ml of 25 mM Tris-HCl, pH 7.4 and 0°C , and the bound and unbound labeled material was rapidly separated by vacuum filtration through Whatman GF/C filters (Clifton, N.J.) followed by three 5-ml washes of the membranes on the filters with 25 mM Tris-HCl at 0°C . The filter was added to 10 ml of Redit-Protein+ (Beckman), and radioactivity was quantified using a Beckman model LS9800 liquid scintillation spectrometer. Specific binding was calculated as the difference between total binding and nonspecific binding and was expressed as femtomoles per milligram protein. Protein content was determined by the Bio-Rad Labs assay (Richmond, Calif.) using bovine serum albumin as the standard.

All data are presented as mean \pm SEM. Comparisons between two groups (i.e., neurological grade) were made for unpaired data by using Student's *t* test.⁴⁸ In the case of nonparametric data (i.e., proportions scoring 0 or 1 on the Hindlimb Placement Test) the χ^2 test for two independent samples was used.⁴⁹ The four forebrain segments were compared for MPO activity and LTB_4 receptor binding using one-way analysis of variance (ANOVA). Only if the results of ANOVA were significant was Dunnett's *t* test conducted by comparing segment A with all other forebrain segments.⁵⁰ Also, correlation analysis was carried out for matched cortex segments assayed for both MPO activity and LTB_4 receptor binding.⁴⁸ Differences were considered significant if $p < 0.05$.

Results

A sustained decrease in local microvascular perfusion was verified in the primary ischemic area of the cortex during MCAO. Local cortex perfusion, monitored at the cortical surface, was decreased permanently (i.e., verified for recording periods of >40 minutes) after permanent MCAO and was decreased consistently for the whole occlusion period during temporary MCAO, with permanent recovery to the baseline level (i.e., verified for recording periods of >20 minutes) during reperfusion. The degree of decrease in cortex perfusion was similar (i.e., 80–85%) for all groups and was similar to that described previously using this technique.⁴¹ Table 1 lists the results of laser-Doppler flowmetry for 30 rats.

Neurological deficits were produced by MCAO. The neurological grade indicated that MCAO produced significant contralateral forepaw hemiparalysis and

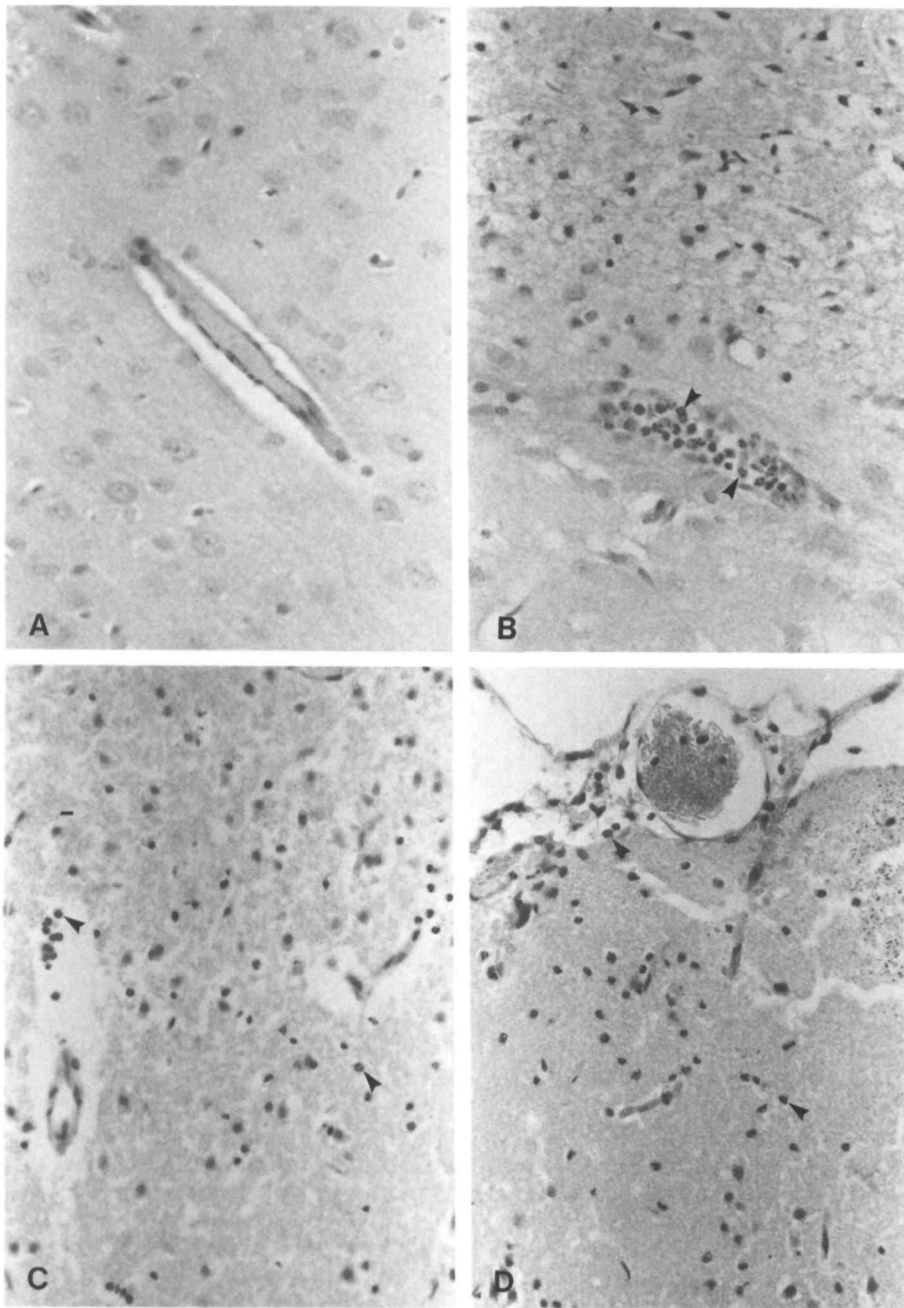


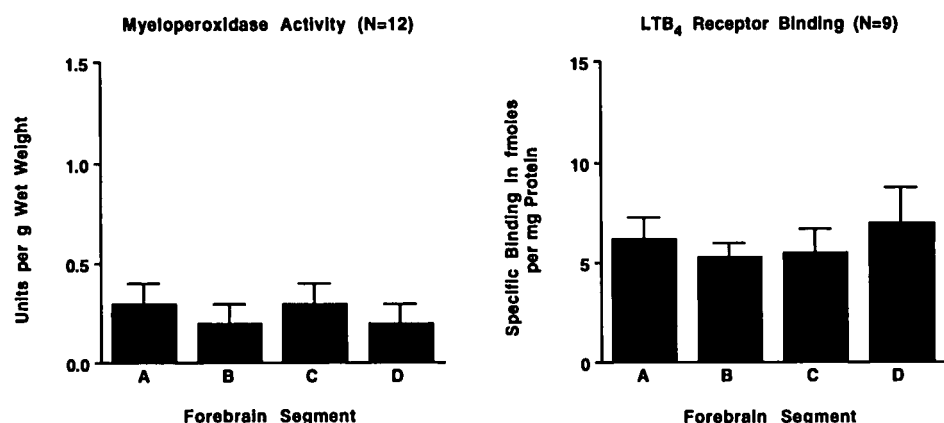
FIGURE 3. Photomicrographs from tissue sections stained with hematoxylin and eosin and depicting degree of neutrophil infiltration into infarcted and uninjured cortex of rats. **A:** Neutrophil infiltration was not observed in contralateral non-ischemic cortex of animals subjected to middle cerebral artery occlusion (MCAO) or in either cortex of sham-operated animals. Here is illustrated uninjured ipsilateral cortex with no neutrophils present following sham surgery. **B:** Moderate neutrophil infiltration was observed following 24 hours of permanent MCAO as determined by presence of neutrophils within and adjacent to blood vessels. Neutrophils were observed in various stages of diapedesis from meningeal and deep cerebral vasculature into peripheral aspects of infarcted cortex. Here are illustrated neutrophils (arrowheads) within blood vessel in infarcted tissue near deep border of lesion 24 hours after permanent MCAO. **C:** Neutrophils (arrowheads) throughout infarcted cortex following 80 minutes of MCAO with 24 hours of reperfusion. **D:** Neutrophils (arrowheads) throughout infarcted cortex following 160 minutes of MCAO with 24 hours of reperfusion. Note marked neutrophil infiltration in C and D as determined by distribution of neutrophils throughout infarcted tissue.

muscle weakness compared with sham-operated rats. In the Hindlimb Placement Test, rats with MCAO typically failed to exhibit a normal limb placement response, demonstrating a disruption of proprioceptive hindlimb control after ischemia compared with sham-operated animals. The increased degree and decreased variability of these deficits related to increased MCAO duration are illustrated in Figure 1 for 40 rats.

Figure 2 illustrates the degree of focal infarction, determined by TTC staining using image analysis, produced by increasing MCAO durations in 26 rats. A graded, increasing degree of cortical infarction was produced by temporary MCAO lasting 80 or 160 minutes and followed by 24 hours of reperfusion and by permanent MCAO compared with sham operation. These infarcts were restricted to the cortex and were located predominantly in the frontal and parietal areas.

Histological preparation/H&E staining of the TTC-stained thick sections also substantiated the infarct volumes quantified using TTC staining. Lighter H&E staining paralleled TTC-staining profiles of infarcted areas and was indicative of liquefactive necrosis, which was absent in sham-operated rats and in normal, non-ischemic regions of animals with MCAO. Evaluation of histological H&E-stained sections under higher magnification revealed the presence of neutrophils in rats subjected to MCAO but not sham surgery. These results are depicted in Figure 3. It was very rare to locate even single neutrophils in the ipsilateral or contralateral cortex of sham-operated animals or in the contralateral cortex of rats with MCAO. However, after 24 hours of permanent MCAO, many neutrophils were identified as isolated cells or as aggregates within meningeal and deeper blood vessels, attached to vessel walls, and at

NONPERFUSED ANIMALS



PERFUSED ANIMALS

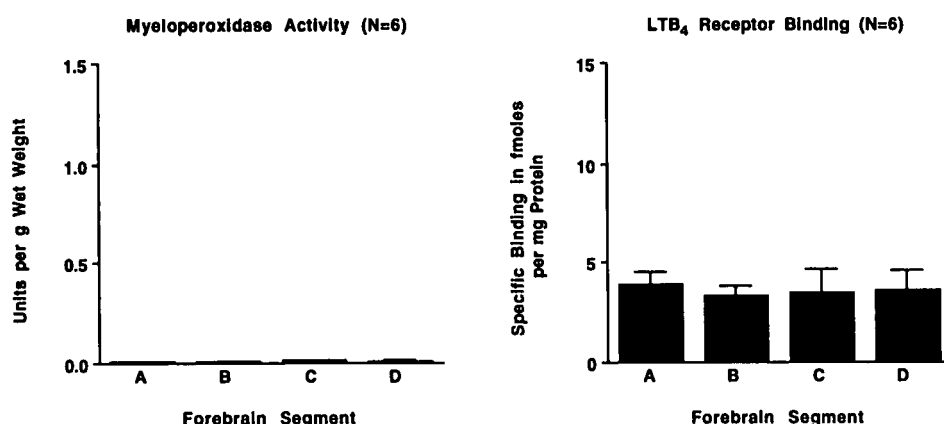


FIGURE 4. Bar graphs of mean \pm SEM myeloperoxidase (MPO) activity (left) and leukotriene B₄ (LTB₄) receptor binding (right) in nonperfused (top) and saline-perfused (bottom) rats subjected to sham surgery. Four forebrain segments (A, ipsilateral to surgery/ischemic cortex; B, contralateral to surgery/nonischemic cortex; C, remaining ipsilateral hemisphere; D, remaining contralateral hemisphere) were removed 24 hours after sham surgery. MPO activity and LTB₄ receptor binding were not significantly different among segments. Perfusing rats with 200 ml isotonic saline, thus washing all blood components from tissue, eliminated MPO activity and reduced LTB₄ receptor binding in these and subsequent studies.

various stages of diapedesis from vessels into the infarcted region. This was considered moderate infiltration since neutrophils were associated only with blood vessels and were not observed throughout the infarcted tissue. In rats subjected to 80 or 160 minutes of temporary MCAO and 24 hours of reperfusion, marked infiltration of neutrophils was observed. Neutrophils were associated with blood vessels, as in rats with permanent MCAO, but also were dramatically distributed throughout the infarcted tissue.

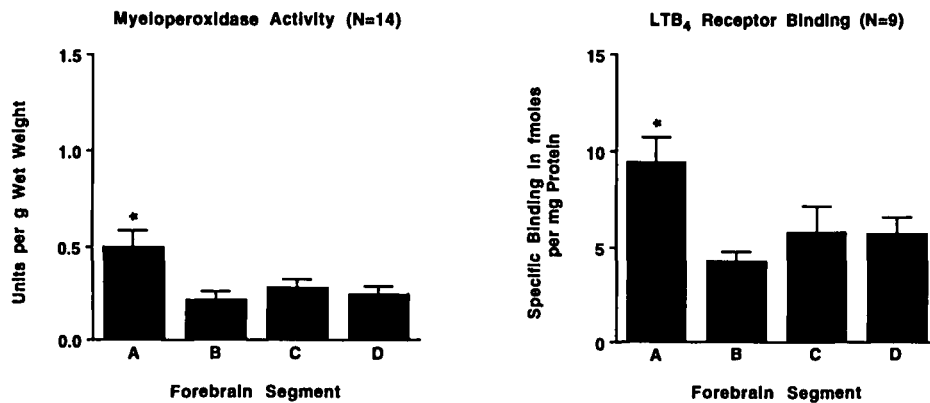
The four forebrain segments dissected from sham-operated and MCAO rats exhibited different profiles of MPO activity and LTB₄ receptor binding. In 18 sham-operated animals (15 that were evaluated using both assays) (Figure 4), no significant differences in MPO activity or LTB₄ receptor binding were observed among segments. Perfusing the rats with 200 ml isotonic saline to remove all blood components eliminated most of the background MPO activity and reduced the background LTB₄ receptor binding. This perfusion effect was observed consistently in subsequent groups. However, in 20 rats with permanent MCAO (15 that were evaluated using both assays) (Figure 5), there were significant ($p < 0.05$) increases in MPO activity and LTB₄ receptor binding in the ischemic cortex (segment A). Similar significant ($p < 0.05$) increases in MPO activity and

LTB₄ receptor binding were also observed for the ischemic cortex of six rats with 80 minutes of temporary MCAO and 24 hours of reperfusion (all matched in both assays) (Figure 6). The most dramatic increase ($p < 0.01$) in MPO activity and LTB₄ receptor binding was observed in the ischemic cortex of five animals (all matched in both assays) with 160 minutes of temporary MCAO and 24 hours of reperfusion (Figure 7). In all cases the increase in MPO activity paralleled the increase in LTB₄ receptor binding. MPO activity and LTB₄ receptor binding for segments B, C, and D were not significantly different. MPO activity in the cortex was also significantly correlated with the degree of LTB₄ receptor binding in assay-matched nonperfused animals ($r = 0.79$, $n = 34$, $p < 0.01$) and in rats perfused with isotonic saline ($r = 0.66$, $n = 44$, $p < 0.01$).

Discussion

Measurements of ischemic cortex perfusion verified permanent occlusion or temporary occlusion and reperfusion of the MCA. The laser-Doppler flowmetry technique used for this purpose accurately measures relative changes in local cerebral blood flow during ischemia, and the decrease observed in cortical perfusion represents severe ischemia.⁵¹⁻⁵⁴ The relatively consistent infarcts of the parietal and motor cortices that occurred

NONPERFUSED ANIMALS



PERFUSED ANIMALS

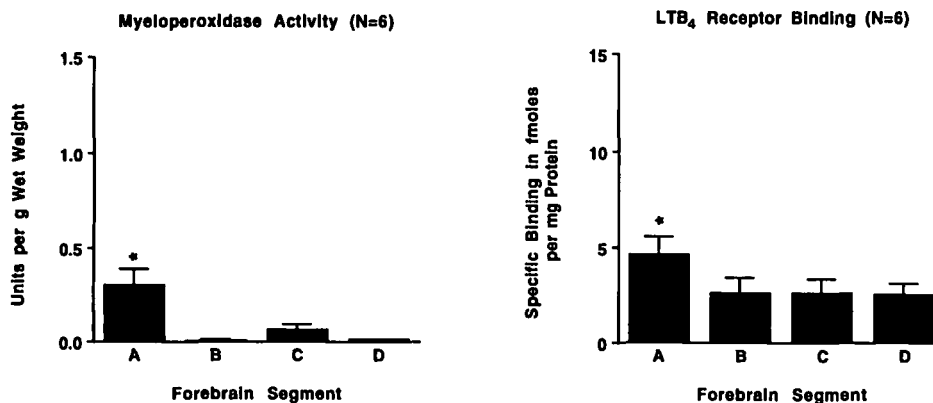


FIGURE 5. Bar graphs of mean \pm SEM myeloperoxidase (MPO) activity (left) and leukotriene B₄ (LTB₄) receptor binding (right) in nonperfused (top) and saline-perfused (bottom) rats subjected to 24 hours of permanent middle cerebral artery occlusion. Four forebrain segments (A, ipsilateral to surgery/ischemic cortex; B, contralateral to surgery/nonischemic cortex; C, remaining ipsilateral cortex; D, remaining contralateral cortex) were removed 24 hours after surgery. MPO activity and LTB₄ receptor binding were significantly increased in segment A in both nonperfused and saline-perfused animals. * $p < 0.05$ by analysis of variance.

after permanent MCAO or temporary MCAO and reperfusion produced significant contralateral sensorimotor neurological deficits measured as the neurological grade and the Hindlimb Placement Test score. The size of the infarct was related to the duration of ischemia (i.e., time of MCAO), and this was reflected, to some degree, in the extent of neurological deficit.

Although neutrophils have been observed in rat¹⁸ and primate¹⁹ ischemic cortex after permanent MCAO, ours is the first study to demonstrate increased neutrophil accumulation after temporary MCAO and reperfusion. Also, the neutrophil marker MPO activity was shown in a previous study to quantify neutrophil infiltration into the infarcted cortex after permanent MCAO.¹⁸ That study also found that increased MPO activity in the ischemic cortex after MCAO was not due to blood trapped following artery occlusion but was related to infiltration/adhesion of neutrophils over time because MPO activity was not increased in brains that were removed immediately after permanent MCAO.¹⁸ Our data are also the first demonstration of increased MPO activity after temporary MCAO with reperfusion and provide further validation of the MPO activity assay for quantifying neutrophil infiltration in focal ischemia. Clearly, neutrophils are present in focal ischemia, and the degree of infiltration is related to the duration of

ischemia and resulting infarct size and is dramatically increased by reperfusion.

In rats perfused with isotonic saline 24 hours after MCAO, baseline MPO activity was practically eliminated in all nonischemic forebrain segments. Therefore, the blood compartment contributes, almost exclusively, to background brain MPO activity in the nonperfused animal and is consistent with the large numbers of neutrophils in the peripheral circulation.¹⁸ In ischemic cortical segments, MPO activity was significantly increased relative to that in nonischemic brain segments, reflecting the increased infiltration of neutrophils that was verified histologically in the ischemic area. Therefore, saline perfusion virtually eliminates background MPO activity and significantly improves the MPO signal that is directly related to the increased numbers of neutrophils that accumulate in ischemic/infarcted cerebral tissue.¹⁸ It was demonstrated previously that MPO activity is due, almost exclusively, to the presence of PMN and not other blood elements.^{18,39,55} Based on previous data,¹⁸ isolated leukocytes exhibit about 0.5 unit MPO activity per 10^6 cells. Therefore, the present increases in MPO activity correspond to about 600,000 PMN/g wet weight for permanent MCAO or 80 minutes of temporary MCAO with reperfusion and more than three times that (almost 2 million neutrophils/g wet

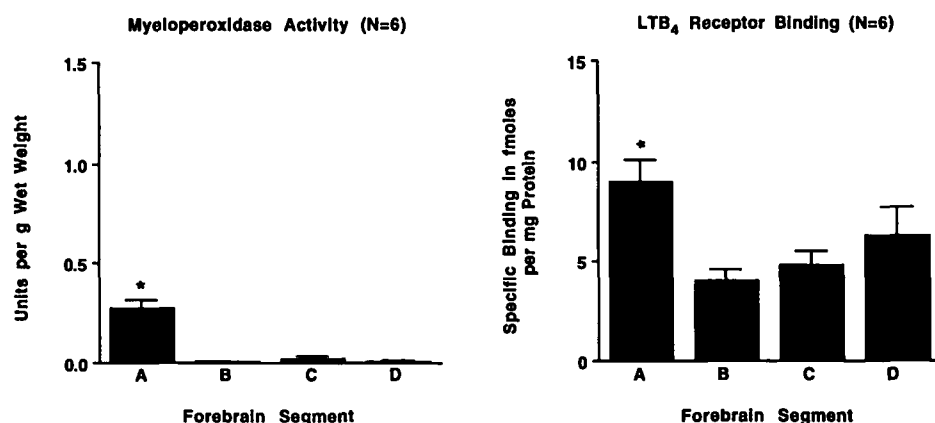


FIGURE 6. Bar graphs of mean \pm SEM myeloperoxidase (MPO) activity (left) and leukotriene B₄ (LTB₄) receptor binding (right) in four forebrain segments (A, ipsilateral to surgery/ischemic cortex; B, contralateral to surgery/nonischemic cortex; C, remaining ipsilateral cortex; D, remaining contralateral cortex) removed from saline-perfused rats after 80 minutes of middle cerebral artery occlusion (MCAO) with 24 hours of reperfusion. MPO activity and LTB₄ receptor binding were significantly increased in segment A (* $p < 0.05$ by analysis of variance). Note that absolute increases are similar to those after permanent MCAO (Figure 5), although infarcts were only about 25% of the size of those after permanent MCAO (Figure 2). Nonperfused animals were not evaluated for this treatment.

weight) following 160 minutes of temporary MCAO with reperfusion. Increased neutrophil accumulation is related to infarct size only in reperfusion rats (i.e., 80 and 160 minutes of MCAO with reperfusion produced infarcts only about 25% and 50%, respectively, the size of those after permanent MCAO).

This study is the first demonstration of elevated LTB₄ receptor binding in cerebral ischemic tissue. The increased LTB₄ receptor binding closely parallels the elevated MPO activity measure of neutrophil infiltration and is increased in ischemic tissue by reperfusion. Perfusing rats with isotonic saline typically reduced, but did not eliminate, background/basal LTB₄ receptor binding, suggesting that some but not all binding is due to vascular blood components and that there is significant LTB₄ binding to brain tissue. However, if changes in brain tissue LTB₄ receptors are responsible for the changes in binding, a completely different profile of binding would be expected that could be related more to infarct size (i.e., permanent MCAO would be expected to exhibit the largest increase). Therefore, the elevated number of LTB₄ receptor binding sites correlates with increased neutrophil infiltration in ischemia and suggests that these receptors are located on neutrophils that have accumulated in the infarcted tissue.

Rat neutrophils have been isolated from peripheral blood and from casein-induced neutrophil-rich peritoneal exudates and exhibit a high-affinity specific binding for [³H]LTB₄.⁵⁶ Although rat neutrophil LTB₄ binding sites share many characteristics with the high-affinity binding site on human neutrophils, human neutrophils show a large number of low-affinity LTB₄ binding sites that are not present on rat PMN.⁵⁷ Rat LTB₄-induced neutrophil aggregation responses are similar in magnitude and specificity to those in human neutrophils, but LTB₄ is not able to elicit the chemotactic response in rat

neutrophils that is characteristic of human neutrophils.^{56,57} Therefore, the role of LTB₄ as a mediator of acute inflammation in rats has been questioned,⁵⁷⁻⁵⁹ but the presence of high-affinity binding sites on rat neutrophils is well established. There has been at least one report of LTB₄-induced chemotaxis by casein-elicited rat neutrophils.⁶⁰ Whether LTB₄ and its binding to LTB₄ receptors is involved in neutrophil aggregation or chemotaxis, endothelial cell adhesion, and/or associated activation and cytodestructive mechanisms in the rat brain cannot be resolved by the present data. However, the increased numbers of LTB₄ binding sites under the present experimental conditions does appear to provide a marker, like MPO activity, of neutrophil infiltration.

Previously, lipoxygenase metabolite production was shown to be unaffected by permanent MCAO.⁶¹ However, in models of temporary global ischemia with reperfusion, levels of lipoxygenase products, including 12-hydroxyeicosatetraenoic acid and LTB₄, are significantly elevated.⁶²⁻⁶⁴ LTB₄ levels are significantly increased in spinal cord injury,⁶⁵ and the source of LTB₄ appears to be infiltrating neutrophils.⁶⁶ Activation of neutrophils via LTB₄ receptor stimulation could contribute to tissue damage. Therefore, in the future LTB₄ levels should be evaluated in temporary MCAO with reperfusion.

Leukocyte accumulation/activation during ischemia and reperfusion can exacerbate tissue injury through rheological effects associated with microvascular occlusion/plugging^{6,30} and the release of a variety of deleterious mediators. They include nonpeptidoleukotrienes that are chemotactic,³⁶ peptidoleukotrienes that increase vascular permeability⁶⁷ and provide potent vasoconstriction,⁶⁸ oxygen free radicals that can increase vascular permeability and damage endothelial and/or brain tissue directly^{4,69} and produce endothelial-dependent vascular smooth muscle contraction,⁷⁰ platelet

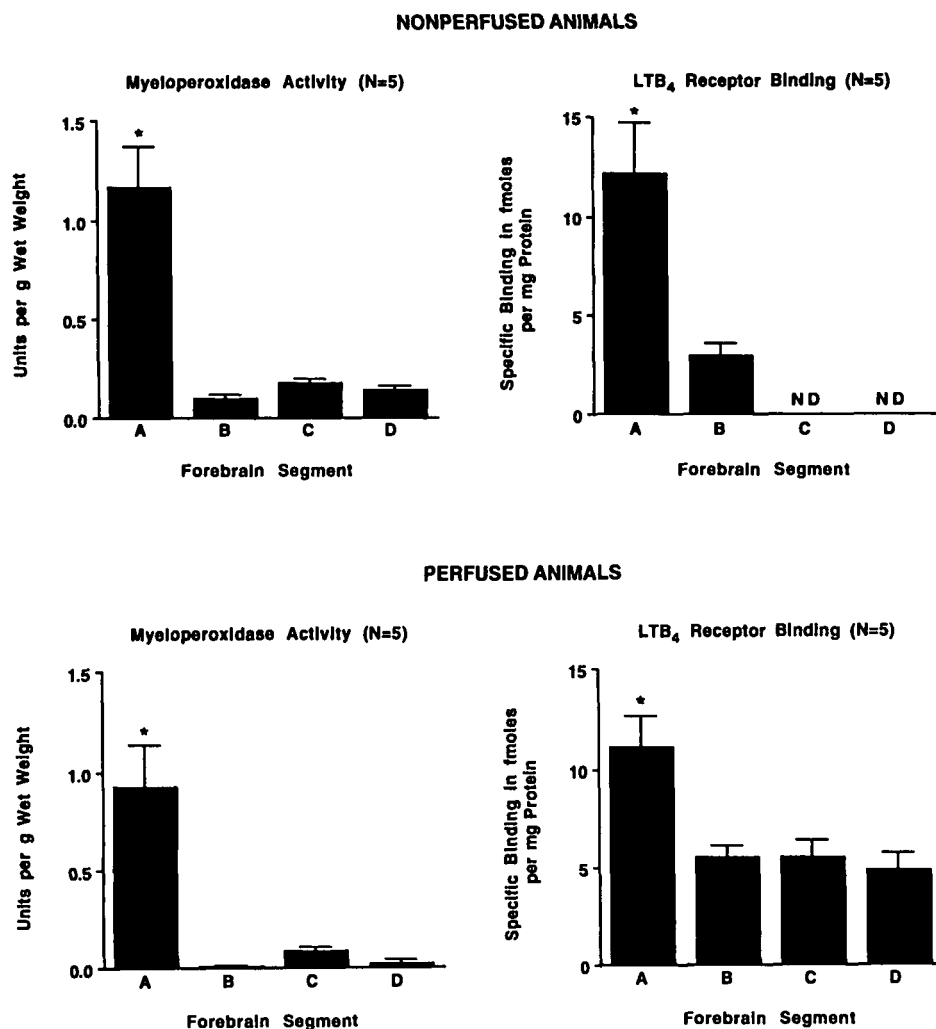


FIGURE 7. Bar graphs of mean \pm SEM myeloperoxidase (MPO) activity (left) and leukotriene B₄ (LTB₄) receptor binding (right) in nonperfused (top) and saline-perfused (bottom) rats. Four forebrain segments (A, ipsilateral to surgery/ischemic cortex; B, contralateral to surgery/nonischemic cortex; C, remaining ipsilateral cortex; D, remaining contralateral cortex) were removed after 160 minutes of middle cerebral artery occlusion (MCAO) with 24 hours of reperfusion. MPO activity and LTB₄ receptor binding were significantly increased in segment A (* $p < 0.01$ by analysis of variance). Note that increases are much larger than those after permanent MCAO (Figure 5), although infarcts were only about 50% of the size of those after permanent MCAO (Figure 2). ND, not determined.

activating factor⁷¹ that increases vascular permeability and neurotoxicity,⁷⁰ and granular, enzymatic constituents including several cytotoxic lysosomal proteases, one being MPO.^{1,31} Acute inflammation is a characteristic of cerebral ischemia in humans,⁷² and potential mechanisms identified for neutrophil-associated ischemic brain injury have been related to rheological effects. Leukocyte filterability is impaired after stroke, apparently due to decreased deformability and/or increased adhesiveness of the cells.^{73–75} Additionally, an increased total leukocyte count has been identified as a predictor for cerebral infarction but not hemorrhagic stroke.⁷⁶

Histological data have suggested that the increase in the number of neutrophils occurs earlier than 24 hours in temporary MCAO with reperfusion.^{77,78} Twenty-four hours was selected for evaluation in the present study because this time provides maximum infarction for both permanent⁷⁷ and temporary^{41,78} MCAO. Certainly a complete time–response profile for MPO activity and LTB₄ receptor binding under these conditions will be necessary to better understand cell infiltration after ischemia in brain versus other tissues and to help identify the potential contribution of neutrophils and other inflammatory cells to ischemic damage. Increased numbers of neutrophils, MPO activity, and LTB₄ receptor binding also have been demonstrated recently in mouse focal ischemia.⁷⁹ In the heart, it appears that

accumulation and activation of leukocytes during the inflammatory process initiated by ischemia and reperfusion is a major determinant of the final extent of absolute tissue injury.^{5,6} The degree of reperfusion injury in the present model will be assessed by us in the future using neutropenic compared with normal animal experimental paradigms.

In summary, neutrophils are recruited in focal ischemia. They can be identified histologically and quantified using an MPO activity assay. One day after MCAO, the degree of neutrophil infiltration in infarcted cortex is related to the duration of occlusion and the resulting infarct size and is dramatically increased by reperfusion. The correlation of LTB₄ receptor binding with MPO activity suggests that the additional binding sites are located on neutrophils that have accumulated in the ischemic tissue.

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Editorial Comment

Neuropathologists have long noted the infiltration of neutrophils in the periphery of cerebral infarcts 6–24 hours after injury. Since irreversible brain injury in humans requires only 3–4 minutes of complete hypoxia, the role of neutrophils was thought to be a secondary phenomenon. Now an amazing range of pharmacological interventions has expanded the window for the onset of irreversible injury well beyond 3–4 minutes, which may allow neutrophils to play a causative role in determining the final extent of injury. Treatment with thrombolytic agents allows reperfusion of ischemic brain, making the question of whether reperfusion injury occurs in brain a clinically important problem. Barone et al have shown that reperfusion substantially increases the influx of neutrophils into ischemic rat brain. Approximately 600,000 neutrophils/g tissue were estimated to be in rat cortex 24 hours after permanent middle cerebral artery occlusion, whereas approximately 2 million neutrophils/g tissue accumulated after reperfusion for 24 hours after 160 minutes of occlusion. Thus, a complication of establishing reperfusion may be the recruitment of additional inflammatory cells.

Considerable attention has been focused on the N-methyl-D-aspartate (NMDA) receptor in selective neuronal loss from ischemia. Remarkably, NMDA antagonists also reduce infarct volume following focal cerebral ischemia.¹ What determines whether only a relatively small population of neurons slowly dies versus the rapid and total collapse of an ischemic region into infarction? Activation of inflammatory cells may be key for propagating injury in the ischemic zone and expansion of the infarct into penumbra. Possibly, some subtle signal to brain endothelium initiating the expression of adhesion molecules may be the critical effect that causes the influx of neutrophils. Furthermore, differentiation of microglia (the precursors of brain macrophages) occurs much earlier in ischemic injury than has been previously suspected.²

Polyethylene glycol-conjugated superoxide dismutase injected into the systemic circulation reduces cerebral infarct volume,³ yet it does not readily cross the blood-brain barrier.^{4,5} There may be a parallel with intestinal ischemia/reperfusion, the first organ system in which superoxide was implicated in free radical injury.