Effect of Phorbol Myristate Acetate on Cerebral Blood Flow in Normal and Neutrophil-Depleted Rats

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Background and Purpose: Recent evidence suggests a possible role for leukocytes in ischemic brain injury. This study examined the effect of activation of endogenous circulating leukocytes on cerebral blood flow in normal and neutrophil-depleted rats.

Methods: Leukocytes were activated by rapid injection of either 50 μg/kg phorbol 12-myristate 13-acetate, a protein kinase C activator, or an equimolar amount of the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine, into the right carotid artery. Control rats received an equal volume of dimethyl sulfoxide in saline vehicle. H2-clearance cerebral blood flow was measured in each of the three groups and in vinblastine-treated, neutrophil-depleted rats after carotid artery injection of phorbol.

Results: Phorbol 12-myristate 13-acetate dramatically decreased circulating leukocyte and platelet counts from 5 to 120 minutes after infusion and decreased regional cerebral blood flow in the ipsilateral parietal cortex from a baseline of 119±14 mL·min⁻¹·100 g⁻¹ (mean±SEM) to 49±5 mL·min⁻¹·100 g⁻¹ at 30 minutes (P<.05). Decreased flow persisted for the 2-hour study. Neither N-formyl-methionyl-leucyl-phenylalanine nor vehicle had an effect on cerebral blood flow. In the neutrophil-depleted rats the initial decrease in cerebral blood flow at 30 and 60 minutes after infusion of phorbol was observed, but cerebral blood flow was restored to 70% to 80% of its baseline value (P>.05 versus baseline) by 90 to 120 minutes.

Conclusions: The early phorbol 12-myristate 13-acetate–induced decrease in cerebral blood flow may be due to the effects of protein kinase C activation on vascular smooth muscle or on platelet aggregation, whereas the persistent decrease in cerebral blood flow appears to be mediated in part by neutrophil activation. (Stroke. 1993;24:1977-1982.)

Key Words • cerebral blood flow • leukocytes • protein kinase C • rats

Experimental and clinical evidence suggests that leukocytes, including neutrophils and monocytes, are involved in the pathogenesis of ischemic brain damage. However, their quantitative contribution and the mechanisms by which they exacerbate injury are still undefined and controversial.1 Experimentally, leukocytes were first implicated in the “no-reflow” phenomenon after global cerebral ischemia in rabbits.2 Subsequently, in animal models of cerebral air embolism, radiolabeled neutrophil accumulation was observed in low cerebral blood flow (CBF) areas, and neutrophil depletion improved postischemic CBF and recovery of somatosensory evoked responses.3-5 Neutrophil depletion also improved postischemic CBF and decreased infarct size in other selected models of cerebral and spinal cord ischemia.6-8 Clinically, a peripheral white blood cell (WBC) count greater than 10×10⁹/L increased stroke risk by 1.6-fold over 2 years compared with that in persons with a WBC count of 4×10⁹/L.9

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Increased poststroke WBC count was also correlated with greater neurological deficits.10 Leukocyte rheology may be an important factor; WBC filterability is reduced both immediately and for 3 to 4 months after a stroke.11,12 Thus, the accumulation and depletion of neutrophils are often associated with an exacerbation and amelioration, respectively, of ischemic brain damage. However, the question remains as to what specific pathophysiological events are directly attributable to neutrophils.

In myocardial, intestinal, or skeletal-muscle ischemia, neutrophils worsen tissue injury by obstructing or plugging the microvasculature13-15 and by releasing inflammatory mediators, such as oxygen free radicals,16-18 arachidonic acid–derived eicosanoid products,19 and platelet-activating factor.20-22 In models of acute lung injury, intravascular activation of endogenous circulating leukocytes triggers a cascade of events including the accumulation of leukocytes in the pulmonary vasculature, pulmonary arterial vasoconstriction, and increased vascular permeability.23-26

In an attempt to elucidate the specific pathophysiological events that may be ascribed to leukocyte activation in the cerebral vasculature in the absence of other metabolic and physiological events that are associated with cerebral ischemia, we studied the effects of activating leukocytes in the normal cerebral circulation of the
rat with two different activators: phorbol 12-myristate 13-acetate (PMA), a croton oil–derived phorbol ester that directly activates protein kinase C and stimulates leukocytes to aggregate, produce superoxide anion, and release degradative enzymes from secretory granules,27,28 and the chemotactic peptide N-formyl-methionyl-leucyl-phenyalanine (fMLP), which acts via specific cell-surface receptors on leukocytes to increase intracellular free calcium and cause cellular activation.29–31 Our results show that leukocyte activation in the normal cerebral circulation does not alter CBF; however, leukocytes contribute to sustaining CBF reduction in the presence of established hypoperfusion.

Materials and Methods

This protocol was approved by the Animal Care and Use Committee of the University of Pittsburgh. Thirty-four adult male Wistar rats (Hilltop Laboratories, Scottsdale, Pa) weighing 375 to 500 g were randomly assigned to one of four experimental groups: (1) carotid PMA infusion (n=8); (2) carotid fMLP infusion (n=5); (3) carotid infusion of dimethyl sulfoxide (DMSO) in saline vehicle (n=8); and (4) carotid PMA infusion after neutrophil depletion by pretreatment with vinblastine (n=8). Five additional rats received carotid PMA infusion, and the time course of changes in circulating blood elements was determined.

Rats were anesthetized with 4% halothane in O2, the trachea was intubated, and the lungs were mechanically ventilated with 1% halothane/66% N2O/33% O2. Bicillin (100 000 U) and gentamicin (10 mg/kg) were administered intramuscularly. Femoral venous and arterial catheters were inserted using aseptic techniques. To deliver leukocyte-activating drugs to the right cerebral hemisphere, a PE-50 catheter was inserted retrogradely into the right external carotid artery, with its tip at the bifurcation of the common carotid artery. The distal external carotid, superior thyroid, occipital, and pterygopalatine arteries were ligated.32 The carotid catheter was kept patent by a continuous infusion of normal saline at 0.75 mL/h. Pancuronium bromide, 0.1 mg·kg⁻¹·h⁻¹·IV, was administered for sustained muscle relaxation. Mean arterial blood pressure was continuously monitored. Rectal temperature was maintained at 37.0±0.5°C with a heated water blanket.

The dural calvarium was exposed by a midline incision, and the rats were fixed in a stereotaxic device (David Kopf, Tujunga, Calif). Platinum microelectrodes (tip diameter, 25 μm) were inserted 1 mm into the right parietal cortex through a small burr hole. The dura remained intact, and the craniotomy was sealed with agar gel to prevent diffusion of H2 gas from the surface of the brain. The platinum microelectrodes were fabricated from 25-μm-diameter platinum-iridium wire embedded in sharpened glass capillary tubing and sealed with epoxy resin. The reference, silver chloride electrode was embedded in the outer layer of the microelectrode housing. Polarization voltage was +250 mV with the output signal monitored on a chemical microsensor (Diamond Electrotech, Ann Arbor, Mich) and chart recorder. Electrodes were tested before use, and only those showing an immediate and measured response in an H2-saturated solution were used. After completion of surgical procedures the inspired halothane concentration was reduced to 0.4% with 66% N2O in O2.

Local CBF (ICBF) was measured by standard H2-clearance techniques.33 H2 gas (5% to 10%) was introduced into the ventilator circuit, and the electrode signal was monitored until a stable saturation baseline was obtained. ICBF was determined by the clearance rate of H2 washout and is reported in milliliters per minute per 100 grams, as calculated by the half-time method from the clearance curves.33 The first 15-second portion of each curve was disregarded to eliminate the arterial recirculation effect.34 Blood flow was measured hourly beginning 30 minutes after insertion of the microelectrode. Baseline ICBF in each animal was the average of the ICBF values obtained at 2.5 and 3.5 hours after microelectrode placement, the time required for ICBF stabilization after microelectrode insertion.35

PMA and fMLP (Sigma, St Louis, Mo) were each dissolved in DMSO, divided into 50-μL aliquots, and stored at −70°C. For each CBF study, either PMA (50 μg/kg) or an equimolar amount of fMLP (35 μg/kg) was diluted in normal saline at room temperature to a final volume of 1.5 mL (final DMSO concentration, 0.2% to 0.4%). Similar doses of PMA have been shown to produce profound effects on circulating leukocytes and platelets23 and have been used in studies of leukocyte activation in the pulmonary circulation.23,24 An equimolar amount of fMLP was used for comparison.

The leukocyte-activating drug (1.5 mL of either PMA or fMLP dissolved in 0.2% to 0.4% DMSO in saline) or an equal volume of vehicle (1.5 mL of 0.4% DMSO in saline) was infused via the right carotid artery as a bolus at a rate of 5.73 mL/min (over approximately 15 seconds) to ensure uniform delivery of 1.5 mL to the right cerebral hemisphere. This rate of infusion has been shown to provide optimum and uniform distribution of the drug throughout the right hemisphere territory of the rat internal carotid artery.36 ICBF was then determined at 5, 30, 60, 90, and 120 minutes after drug infusion. Arterial blood gas measurements were obtained at the same times. Care was taken to maintain the blood gas tensions within the normal physiological range. A blood sample for hematologic measurements was obtained at 15 minutes. Complete blood cell counts were determined by a Coulter counter (Coulter Co, Hialeah, Fla) and by manual differential counts of WBCs and platelets.

To further assess the contribution of neutrophils to the observed PMA-induced ICBF effects, eight rats were neutrophil-depleted with vinblastine sulfate. Rats were anesthetized with 1% to 2% halothane, femoral venous catheters were inserted, and 0.5 mg/kg vinblasticine sulfate was administered intravenously. Bicillin (100 000 U) and gentamicin (10 mg/kg) were given intramuscularly to prevent infection. Catheters were removed, and the rats recovered and were returned to their cages. Five days later at the nadir of neutropenia, the rats were prepared for carotid artery PMA infusion and ICBF studies as described above.

All data in the text and figures are presented as mean±SEM. CBF data (H2 clearance) and hematologic data obtained after carotid infusion of PMA were compared with their respective baseline values by repeated-measures ANOVA and the Student-Newman-Keuls multiple comparison test. Groups were compared by t test with correction for multiple comparisons. A value of P<.05 was considered statistically significant.
Results

Mean arterial blood pressure, rectal temperature, and arterial blood gas values were similar in all groups before and after drug infusion. Group mean blood pressure ranged from 108±8 to 149±6 mm Hg and rectal temperature from 37.1±0.3°C to 38.0±0.1°C. Arterial pH ranged from 7.37±0.01 to 7.46±0.04 and PCO2 from 32.7±1.5 to 38.5±1.6 mm Hg with no difference between groups. Arterial PO2 was greater in the fMLP group than the other groups at baseline and at 60, 90, and 120 minutes, but it remained within the normal physiological range (112.1±8.6 to 154.9±9.2 mm Hg). Neither fMLP nor PMA produced a decrease in arterial Pco2.

Baseline ICBF was similar in all groups (Fig 1, time 0) and was unaltered by infusion of either vehicle or fMLP. PMA, however, caused a marked and sustained 50% to 60% decrease in ICBF between 30 and 120 minutes after infusion compared with baseline (Fig 1).

Although a wide range of baseline circulating leukocyte counts was observed (Fig 2), the important comparison occurred within groups between baseline and 15 minutes after drug infusion. PMA infusion markedly decreased circulating leukocytes as reflected by the total WBC count and absolute neutrophil count (ANC) (Fig 2 left and 2 right, respectively, 15 minutes after drug infusion). The time course of these hematologic effects of PMA administration in five rats is shown in Fig 3. PMA decreased circulating WBC, neutrophil, and platelet counts throughout the 120 minutes of the study. Platelet counts were dramatically decreased for the duration of the study, whereas ANC and WBC counts tended to recover. In contrast to PMA, fMLP did not significantly decrease the peripheral ANC or WBC count 15 minutes after infusion (Fig 2 left and 2 right).
Profound selective neutropenia was produced by vinblastine sulfate. Total WBC count in normal rats was 7.00 ± 0.33 × 10^9/L compared with 2.33 ± 0.68 × 10^9/L in vinblastine-treated rats (P < .05). ANC was 5.42 ± 0.36 × 10^9/L in normal rats versus 0.18 ± 0.11 × 10^9/L in vinblastine-treated rats (P < .05). Platelet and mononuclear cell (absolute monocyte and absolute lymphocyte) counts were unchanged by vinblastine. Neutrophil depletion did not prevent the early PMA-induced decrease in ICBF (Fig 4), although in the neutropenic rats, ICBF returned to 70% to 80% of baseline by 90 to 120 minutes. This ICBF was not significantly different from the baseline value.

**Discussion**

The time course of the effects of PMA infusion on the circulating blood elements relative to the effects on ICBF is revealing. Total WBC, neutrophil, and platelet counts were dramatically reduced 5 minutes after PMA infusion but were without effect on ICBF. By 30 minutes, however, a 60% decrease in ICBF occurred. This early reduction in ICBF may be due to direct activation of protein kinase C and thereby an increase in vascular smooth muscle tone. This is consistent with the observations of Busija and Leffler, who reported that superfusion of phorbol ester over the brain of newborn pigs activated protein kinase C and constricted pial arterioles. In our study the early decrease in ICBF after PMA infusion did not appear to be due to leukocyte activation, since it was unaffected by neutrophil depletion.

In vivo intravenous injection of PMA profoundly increased pulmonary artery pressure and vascular resistance, an effect not blocked by neutrophil depletion. Phorbol esters such as PMA readily incorporate into membranes and irreversibly activate protein kinase C, which in turn phosphorylates key membrane proteins and receptors, thus triggering turnover of inositol phospholipids and increases in intracellular free calcium.

In vitro, phorbol esters constrict veins and arteries, such as the pulmonary, renal, and especially basilar artery, which appears to be the most sensitive to phorbol.

Protein kinase C has been implicated as the modulator of contraction and basal myogenic tone in vascular smooth muscle and as the regulator of arterial caliber in pial and other cerebral resistance vessels. These results suggest a direct vasoconstrictor effect of PMA on the cerebral vasculature, which may be sufficient to explain the early and profound decrease in ICBF.

In contrast to the early effects of PMA, a later sustained reduction in ICBF was observed in normal rats after PMA injection, compared with neutropenic rats that showed at least a partial recovery of ICBF at 90 and 120 minutes. This suggests that the sustained decrease in ICBF may be mediated in part by neutrophils. Although leukocyte accumulation in the brain was not documented in this study, in rats infused with PMA, WBC recovery occurred at 90 and 120 minutes, which could represent systemic demargination and recirculation of neutrophils. This could result in the delivery of additional neutrophils to the already compromised cerebral circulation, causing leukocyte trapping and maintenance of the ICBF reduction. A similar time course was noted in a sheep model of thrombin-induced pulmonary vascular injury, in which neutrophil activation resulted in increased pulmonary artery pressure and decreased pulmonary blood flow for at least 5 hours. Neutrophil depletion normalized pulmonary artery pressure and blood flow at 180 and 240 minutes after the thrombin challenge. These results suggest that in the setting of compromised blood flow, neutrophil activation may contribute to further impairment of perfusion and conversely, that neutrophil depletion might enable more rapid recovery of blood flow. Further studies are required to demonstrate the distribution of leukocytes within the cerebral circulation after their activation.

Platelet activation and aggregation caused by carotid artery injection of either arachidonic acid or adenosine diphosphate are known to cause endothelial damage and stroke. PMA causes platelet activation and aggregation in a dose and time course similar to that for leukocytes. fMLP can also stimulate platelet aggregation indirectly via a leukocyte-mediated effect. However, since PMA but not fMLP affected CBF in this study, it seems unlikely that platelets were the primary mediator.

Our observation that fMLP had no effect on ICBF was also observed in monkeys. Faraci et al injected 10^-7 mol/L fMLP as a bolus and also observed no effect on ICBF in normal monkeys. Our dose was similar to that used by Iseki and Ripley, who observed a profound although transient effect on circulating leukocytes. The transient and insignificant decrease in WBC and ANC induced by fMLP compared with PMA may be important. Whereas PMA resulted in a sustained decrease in the number of circulating leukocytes, fMLP effects were short lived, which may be responsible for its lack of CBF effects. A longer interaction between leukocytes and the vascular endothelium may be required for the adverse rheological effects of leukocyte activation to become apparent. We cannot rule out the possibility that a larger dose of fMLP may have an effect on CBF in our model.

An altered endothelial surface or preexisting sluggish blood flow may be an important prerequisite for the production of flow-reducing effects by activated leukocytes. Damaged endothelium is a common finding in
ischemia and reperfusion.14,17 Faraci et al59 showed that although fMLP had no effect on CBF in normal monkeys, in atherosclerotic monkeys fMLP increased cerebral arterial resistance and decreased choroid plexus blood flow. They speculated that in the presence of stenosis or partial obstruction by atherosclerotic plaques, the activation of leukocytes and the release of vasoactive products may contribute to cerebral ischemia. In our model the carotid infusion of PMA may have produced a similar, prerequisite arterial narrowing that allowed neutrophil activation to contribute to the sustained decrease in CBF. Slight gush flow may have augmented leukocyte adhesion to the endothelium that was produced by specific upregulation of leukocyte adhesion receptors on leukocytes and endothelial cells after PMA administration.55 Neutrophils have been shown to obstruct the cerebral microvasculature after ischemia and reperfusion.54 Recent studies in ischemia and shock models have demonstrated beneficial effects of inhibiting leukocyte adherence with monoclonal antibodies directed against leukocyte cell-surface receptors.55-58

In summary, carotid artery infusion of PMA caused a significant and sustained decrease in CBF in normal rats. The early decrease in ICBF was not related to leukocyte activation but may have been due to protein kinase C activation in platelets or directly in the cerebral vasculature. The sustained decrease in ICBF, however, was attenuated in neutrophil-depleted rats, suggesting that activated neutrophils contribute to the sustained decrease in ICBF in areas with already compromised flow. The possible role of neutrophils in delayed ICBF reduction after PMA administration should be further clarified by additional studies documenting neutrophil accumulation in the cerebral circulation. In addition, the contribution of neutrophils to disturbances in CBF in the presence of pathologically altered states, such as ischemia, stroke, and trauma, merits further investigation.

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References
It is an attractive prospect to be able to study a process or function in vivo with the precision and control that is achievable in tissue culture but with the preservation of the manifold influences of the internal milieu that are impossible to faithfully recreate under artificial conditions. The purpose of this study was to investigate in vivo the effect on cerebral blood flow (measured by the hydrogen clearance technique) of activating leukocytes with chemotaxins. A number of difficulties are encountered in the design and conduct of such studies, however. It is, for instance, difficult to isolate the effect of an intervention on the process or function of interest. The selection of phorbol myristate acetate (PMA), an activator of the ubiquitous intracellular signaling molecule, protein kinase C (PKC), as one of the leukocyte stimulants is a case in point. A great many effects could be expected to follow upon the activation of PKC in many different cells and tissues and some of these effects could potentially be misleading.1

Two major inferences have been drawn from this study. First, the early decrease in local cerebral blood flow (ICBF) due to PKC activation by PMA involved a direct effect on vascular smooth muscle cells and did not depend on platelet and leukocyte activation. Second, activated neutrophils do contribute to the sustained late phase of decreased ICBF noted after PMA administration. Several refinements in design might have placed these inferences on firmer ground, and the authors have acknowledged most of them in their article. A dose-response design might have revealed that the present single doses of chemotaxins are suboptimal or supraoptimal. Direct measurement of leukocyte accumulation in the brain by any of several available techniques would have provided evidence for or against the major conclusions in the study, and measurement of adhesion molecule expression on leukocytes and endothelium could have provided desirable mechanistic detail.

We can expect further work in this interesting area from this productive laboratory.

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Reference
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