Leukocyte-Induced Acute Endothelial Dysfunction in Middle Cerebral Artery in Rabbits
Response to Aggregating Platelets

S.E. Akopov, MD, PhD; R. Sercombe, PhD; J. Seylaz, PhD

Background and Purpose
Recent evidence suggests a possible role for leukocytes in angiospastic reactions of large cerebral arteries. This study examined the effect of activation of endogenous circulating leukocytes on endothelium-dependent relaxation in the middle cerebral artery in rabbits.

Methods
Leukocytes were activated by rapid injection of either 40 μg/kg phorbol 12-myristate 13-acetate, or 0.2 mg/kg N-formyl-methionyl-leucylphenylalanine into the left carotid artery. Control rabbits received an equal volume of vehicle. Concentration-dependent isometric tension responses of the left and right middle cerebral artery to the dilators acetylcholine, ADP, sodium nitroprusside, or calcium ionophore (A23187), as well as to aggregating platelets, were compared in vitro in control animals and in animals killed 10 minutes after the injection of leukocyte activators in normal and leukocyte-depleted rabbits.

Results
In the control animals there was no significant difference in the reactivity of the left and right middle cerebral arteries. The injection of the leukocyte activators led to enhanced contractile responses to aggregating platelets and a significant reduction in the endothelium-dependent relaxation in response to acetylcholine, ADP, and A23187 in the left middle cerebral artery (the injected side), whereas the effect of an endothelium-independent dilator sodium nitroprusside remained unchanged. In leukocyte-depleted rabbits the injection of either of the leukocyte activators used did not induce significant changes in the reactivity of the left middle cerebral artery.

Conclusions
Intravascular leukocyte activation appears to induce an acute disturbance of the endothelium-dependent relaxation. Under these conditions, platelet activation might result in marked angiospastic reactions of large cerebral arteries. (Stroke. 1994;25:2246-2252.)

Key Words: endothelium • leukocytes • platelet aggregation • vasospasm

There is a body of evidence that suggests that polymorphonuclear leukocytes (PMNs) and monocytes are implicated in the development of myocardial and cerebral ischemia. Recent evidence suggests a possible role for leukocytes in angiospastic reactions of large cerebral arteries. This study examined the effect of activation of endogenous circulating leukocytes on endothelium-dependent relaxation in the middle cerebral artery in rabbits.

Materials and Methods
Experiments were performed on 49 Fauve de Bourgogne rabbits weighing 2.3 to 2.8 kg that were anesthetized with ketamine (10 mg/kg IV) and chloral hydrate (200 mg/kg IV). The trachea was cannulated, and the animals were ventilated with room air. A catheter was placed in a femoral artery for continuous recording of blood pressure. The left lingual artery was exposed and cannulated with a small polyethylene catheter, and the external carotid and thyroid arteries were ligated at their origin for selective injection of leukocyte activators.
into the cerebral circulation. Body temperature was maintained at 37°C to 38°C with a heating pad.

Two main activators of PMNs and monocytes were used. The first one, 12β-epithelin-13-ethoxycacetate (PMA), was dissolved in dimethyl sulfoxide with further dilution in isotonic phosphate buffer immediately before use. It was injected into the left common carotid artery over 15 seconds in a dose of 40 μg/kg, 0.2 mL/kg. This procedure of PMA injection has been repeatedly shown to produce simultaneous platelet and leukocyte intravascular activation and aggregation as microemboli in rabbits. N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) was similarly dissolved and injected in a dose of 0.2 mg/kg, as a bolus of 0.1 mL/kg, at which it has been shown to produce leukocyte activation and aggregation with marked effects on vessel tone in rabbits.20 Previous studies comparing injection and infusion of both PMA and fMLP indicated that maximum responses in vessels injected with PMA were obtained immediately after the injection of the activator.

Evaluation of changes in large cerebral vessel reactivity was performed on the middle cerebral artery (MCA) because microemboli from a carotid source mostly enter the ipsilateral MCA; only a small proportion of them were observed in the territory of the contralateral anterior cerebral artery and none in the contralateral MCA. Therefore, comparison of the activity of the ipsilateral and contralateral MCA provides a means of evaluating the microembolic effects while avoiding the problem of the interindividual heterogeneity of the animals.

Because leukocyte activation by both PMA and fMLP reaches a maximum very fast and is practically terminated after 10 minutes of passage of activated blood cells through large vessels, we determined the reactivity of the vessels from rabbits killed 10 minutes after injection of the activators. After decapitation, the brain was removed and placed in a physiological solution as previously described.22,23 Three-millimeter segments of MCA were removed under a surgical microscope and placed in 5-mL organ baths containing a solution of the following millimolar composition: 126 NaCl, 5 KC1, 1.2 NaH2PO4, 1.3 MgCl2, 15 NaHCO3, 2.5 CaCl2, and 5.5 glucose; the solution was gassed with 4% CO2/96% O2 at a pH of 7.3 to 7.4. The segments, mounted on L-shaped holders, were allowed to equilibrate for 90 minutes at 37°C before the isometric tension measurements were begun. Bath solution was changed every 15 minutes. The resting tension was maintained at approximately 500 mg. Segments from the left, right, and control side (the right MCA) were studied in parallel. The number of rings used for vessel precontraction in our experiments was determined using ANOVA. All calculations were performed by means of SPSS PC+ statistical software. A probability value less than .05 was considered statistically significant.

**Results**

There were no significant differences among any of the groups of rabbits studied regarding the amplitude of the MCA contraction evoked by either KC1 (100 mmol/L) or histamine (10−5 mol/L), the latter being used for vessel precontraction in our experiments.

Relaxation of precontracted MCA segments in response to an endothelium-independent dilator, sodium nitroprusside, was similar in the left and right MCAs of control animals and after PMA or fMLP injection (Fig 1). In control animals, ACh (receptor-mediated endothelium-dependent dilator) and A23187 (non-receptor-mediated endothelium-dependent dilator) caused comparable relaxation of the left and right MCA (Figs 2 and 3). However, after either PMA or fMLP injection, the concentration-response curves of response to these agonists in the left MCA (injected side) were shifted to the right, and the magnitude of their effects was significantly reduced, especially in PMA-injected animals (Figs 2 and 3). In the right MCA, ACh and A23187 produced concentration-dependent dilatation similar to that in control animals.

In our conditions, ADP induced only moderate concentration-dependent relaxation even in control rabbits (Fig 4). This relaxation was of comparable magnitude in the left and right MCAs of control animals and in the right MCA of PMA- or fMLP-injected animals. In the left MCA of PMA- and fMLP-injected rabbits, the
FIG 1. Concentration-response curves show dilator effects of sodium nitroprusside on the middle cerebral artery (MCA) of control rabbits (A, n=8) and after 4β-phorbol 12β-myristate 13α-acetate (B, n=8) and N-formyl-methionyl-leucylphenylalanine (C, n=5) injection into the left carotid artery. Open circles indicate right MCA; closed circles, left MCA. Values are mean±SEM and are expressed as percent of the maximal relaxation induced by 3x10⁻⁴ mol/L papaverine.

effect of ADP was drastically reduced, in some cases to almost total disappearance (Fig 4).

After the treatment with meclorothamine, the number of circulating leukocytes was significantly reduced to 12% to 18% of baseline value (Table). The differential count of the leukocytes remained unchanged, and the platelet count was also unaffected by meclorothamine (Table). Investigation of MCA reactivity to endothelium-dependent agonists showed that, after leukocyte depletion, the difference in the concentration-response curves between the left and right MCAs almost totally disappeared in both PMA- and fMLP-injected rabbits (Fig 5). In non-precontracted segments of the MCA from control animals, as well as precontracted segments of the right MCA, aggregating platelets caused comparable contractions of the left and right MCA (Fig 6). After both PMA and fMLP injection, platelets induced similar contractions of non-precontracted segments of the right MCA, whereas significantly greater contractions were evoked in segments of the left (injected) MCA (Fig 6). In segments of the right MCA (n=3) from PMA- or fMLP-injected animals precontracted with histamine, aggregating platelets induced further constriction of 7.7±2.9% in PMA-injected animals and 5.4±2.2% in fMLP-injected animals (P<.05 compared with the right MCA).

Discussion

Intravascular leukocyte activation is likely to produce regional circulatory disorders. Numerous investigations have shown that intravascular injection of PMA produces strong leukocyte-mediated microcirculatory disturbances, depression of blood flow and tissue metabolism, and edema formation in the lung,5-7,14 the heart,3-5,27-29 and the brain.19-30 Similarly, fMLP injection also produces vasoconstriction and microcirculatory disorders in these organs.3,6,30 Supporting these findings, the present study demonstrates for the first time that intravascular leukocyte activation by either PMA or fMLP injection into the carotid artery markedly suppresses the endothelium-dependent relaxation in the MCA.
It can be speculated that, after the injection of PMA or fMLP into the left common carotid artery, the passage of activated leukocytes through the dependent MCA caused leukocyte-endothelium interactions to occur, resulting in acute failure of vascular relaxation. Such alterations in vasoreactivity, induced in vivo in the present experiments, seem similar to those induced in vitro in other investigations.13-15 Very similar changes in MCA reactivity were produced by the two leukocyte activators, supporting the hypothesis that the effects observed were mediated via intravascular activation of leukocytes. Both PMA and fMLP activate PMNs and monocytes, producing intravascular aggregation, adhesion to the endothelium, and release of oxidants, proteases, and lipid metabolites.2 fMLP is a more selective leukocyte agonist, whereas PMA can also activate platelets in vivo. However, both activators also have binding sites on the endothelium and smooth muscle cells, the role of which is poorly understood.3 Thus, in principle, our data also could be the result of direct effects of the activators on the vessel wall.

In general, control experiments in published work have shown that intra-arterial injection of these activators in the absence of leukocytes does not induce any action on the vascular bed,4,19,27-29 and no in vitro effects have been observed on endothelium-dependent reactions in concentrations that activate leukocytes.31 However, in some experiments using relatively high concentrations of the activators in vitro, fMLP produced vessel relaxation and nitric oxide release, and PMA has depressed endothelium-dependent reactions.32-33 Such phenomena are most unlikely to be significant in our experiments. Nevertheless, we performed a special investigation of PMA and fMLP effects on the MCA reactivity after intracarotid injection in leukocyte-depleted animals. The data obtained clearly show that, after the decrease in the leukocyte count by 80% to 90% of baseline value, neither PMA nor fMLP induced significant changes in MCA responses to endothelium-dependent dilators. Thus, the effects of these agents on the endothelium-dependent responses observed in this study may be considered to be mediated via intravascular activation of leukocytes. It is not, however, unlikely that some other factors are involved in the leukocyte-induced endothelial dysfunction, especially when leukocyte activation was provoked by the less selective agonist PMA. Our data show that the PMA-induced reduction in the vascular responses to all endothelium-dependent dilators is more marked than that induced by fMLP injection. Although the exact mechanisms are not clear, this difference might be associated with simultaneous activation of leukocytes and platelets after intracarotid injection of PMA. Presumably, activated platelets can elicit additional PMN activation and adhesion to the vessel wall, since such an interaction of these cells has been documented by many in vitro investigations.13,34
Hematologic Values After Leukocyte Depletion with 1.5 mg/kg IV Meclorethamine

<table>
<thead>
<tr>
<th>Hematologic Values</th>
<th>Group 4 (n=7)</th>
<th>Group 5 (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocyte count, $\times 10^3$</td>
<td>9.0±0.9</td>
<td>9.1±1.2</td>
</tr>
<tr>
<td>Differential leukocyte count, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMNs</td>
<td>43.4±6.9</td>
<td>40.7±5.8</td>
</tr>
<tr>
<td>Monocytes</td>
<td>5.2±1.6</td>
<td>7.0±1.5</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>41.8±7.9</td>
<td>48.4±6.8</td>
</tr>
<tr>
<td>Platelet count, $\times 10^3$</td>
<td>335±24</td>
<td>312±19</td>
</tr>
</tbody>
</table>

PMNs indicates polymorphonuclear leukocytes.

Furthermore, it has been shown that PMA can modify the state of the endothelial cells, resulting in enhanced adhesion of PMNs with more marked generation of superoxide anions.35

The mechanisms of the leukocyte-mediated alterations of the MCA reactivity are not clear. They appear to be associated with a selective depression of endothelium-dependent relaxation without alterations in the smooth muscle cell reactivity because only vasodilatory responses to the endothelium-dependent dilators ACh, ADP, and A23187 were blunted; sodium nitroprusside, an endothelium-independent dilator, produced the same relaxation of the MCA in control rabbits and rabbits injected with PMA or fMLP. Under our conditions, intravascular leukocyte activation evoked a similar reduction of responses to receptor-mediated (ACh, ADP) and non-receptor-mediated (A23187) dilators, indicating that the endothelial dysfunction observed was not associated with disturbances of receptor activation in the endothelial cells. Apparently, the dysfunction might be induced by a direct action on the production and/or release of vasodilators, particularly prostacyclin and endothelium-derived relaxing factor (EDRF), in the endothelium. Such effects could be associated with the release of free radicals from activated leukocytes, which have been demonstrated to impair the production and accelerate the deactivation of EDRFs.1-2,12 Interestingly, the same variant of the endothelial dysfunction has been observed after ischemia-reperfusion.24,36 The endothelial dysfunction caused in vivo is stable, and it may be persistently observed after removal of vessel segments and their preparation for testing the effects of agonists.24,36 The similarity in character of such dysfunction caused by ischemia-reperfusion (see Reference 36

![Graphs](http://stroke.ahajournals.org/)

**Fig 5.** Bar graphs show dilation effects of acetylcholine and A23187 on the middle cerebral artery (MCA) after 4β-phorbol 12β-myristate 13α-acetate (PMA; n=7) and N-formyl-methionyl-leucylphenylalanine (fMLP; n=5) injection into the left carotid artery in leukocyte-depleted rabbits. Open bars indicate right MCA; closed bars, left MCA. Values are mean±SEM and are expressed as percent of the maximal relaxation induced by 3×10⁻⁴ mol/L papaverine.

**Fig 6.** Bar graph shows constrictor effects of aggregating platelets (75,000 cells per microliter) on the non-precontracted middle cerebral artery (MCA) segments of control rabbits (A, n=7) and after 4β-phorbol 12β-myristate 13α-acetate (B, n=6) and N-formyl-methionyl-leucylphenylalanine (C, n=6) injection into the left carotid artery. Open bars indicate right MCA; closed bars, left MCA. Values are mean±SEM and are expressed as percent of the maximal constriction induced by 100 mmol/L KCl.

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for review) and by intravascular activation of leukocytes (the present work) corroborates the hypothesis that leukocytes may be involved in the pathogenesis of the ischemia-reperfusion-induced endothelial dysfunction in cerebral and coronary arteries.36.37

As the intravascular activation of leukocytes causes a severe attenuation of the endothelium-dependent relaxation of the MCA, this phenomenon is likely to favor vasoconstriction of large cerebral arteries. The endothelium-dependent relaxation limits the action of many spasmodenic stimuli and seems to be of particular importance for the prevention of platelet-induced vasoconstrictor reactions. Aggregating platelets release a number of strong vasoconstrictors that can contribute to coronary and cerebral angiospasm.7.38 Under normal conditions, platelet-induced vasoconstriction is prevented by the endothelium-dependent relaxation,29 but endothelial dysfunction due to atherosclerosis35 or ischemia-reperfusion36 promotes an exaggerated constrictor response to aggregating platelets, particularly in large cerebral arteries. Thus, the leukocyte-induced endothelial dysfunction observed in this study could also alter vessel susceptibility to platelet-derived agonists. Our experiments with activated platelets confirm this suggestion, showing significantly increased contractile response in vitro to aggregating platelets in MCAs from rabbits given an intracarotid injection of either PMA or fMLP. In both quiescent and precontracted segments, platelet-induced contraction was almost doubled in the (injected) MCA compared with that of the right (control) MCA.

The increased MCA responses to aggregating platelets after leukocyte activation are also in agreement with the attenuation of the MCA relaxation by ADP observed in the same conditions. In the basilar artery Shimokawa and coworkers40 showed that purinergic mechanisms are responsible for the vasorelaxant component of platelet vasoactivity, so that diminished sensitivity of the MCA to ADP appears to be of particular importance for the exaggerated platelet-induced MCA constriction observed in the present study. It is worth mentioning that in our conditions the ADP-induced vasorelaxation of the rabbit MCA was small even in control animals. Thus, platelet activation may be especially dangerous for promoting cerebral angiospasm in conditions when even the ADP-mediated restriction of platelet contractility is eliminated by endothelial dysfunction. Certainly, the depression of ADP-induced relaxation may not be the sole mechanism of the leukocyte-induced increased susceptibility of the MCA to aggregating platelets. During their interactions, activated leukocytes as well as the endothelial cells and platelets can produce a number of highly active factors, such as platelet-activating factor (PAF), which may favor the spastic vessel reactions (see Reference 41 for review). It seems likely that PAF can, at least partly, elicit process(es) of leukocyte-induced endothelial dysfunction, resulting in augmented vessel responses to aggregating platelets.

Thus, the present study indicates that intravascular leukocyte activation alters the reactivity of the MCA, eliminating the endothelium-dependent mechanism of vessel relaxation in response to various agonists. This phenomenon might favor cerebral vasoconstriction, particularly that induced by aggregating platelets. Certainly, the clinical significance of these findings remains debatable.

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References
Inflammatory processes in acute stroke are being increasingly investigated in laboratories internationally. As a result of this intensive study, numerous mediators and processes are being implicated as participants in acute ischemic brain damage. Investigators are becoming increasingly aware that events at the blood-endothelium interface in ischemia are multifactorial, and efforts to probe the interactions between putative mediators of ischemic and postischemic brain injury are beginning. In this study, activated polymorphonuclear leukocyte (PMN) interaction with endothelium appears to attenuate endothelium-dependent vasodilation and augment platelet-induced vasoconstriction. Other recent examples of interactions include PMN modulation of tumor necrosis factor-α release by macrophages and the capacity of PMN to stimulate release of growth factors from cultured porcine aortic endothelial cells.

Work of this sort sharpens our concepts of the pathophysiology of acute brain ischemia, but it also brings into sharp relief the complexity inherent in ischemic brain damage.

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