Penumbral Microcirculatory Changes Associated With Peri-infarct Depolarizations in the Rat

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Background and Purpose—This study was designed to investigate the influence of peri-infarct depolarization elicited by occlusion of the middle cerebral artery on the dynamics of the microcirculation.

Methods—The microcirculation in the frontoparietal cortex of 9 rats was visualized in real time through a closed cranial window with the use of laser-scanning confocal fluorescence microscopy combined with intravenous fluorescein isothiocyanate (FITC)—dextran and FITC-labeled erythrocytes. The direct current potential/electrocorticogram was continuously monitored. Intraluminal focal ischemia was induced for 2 hours in 6 rats anesthetized with halothane and mechanically ventilated. Reperfusion was monitored for 1 hour. Three rats underwent sham operation. Brains were removed 24 hours after occlusion and processed for histology.

Results—In control conditions, the velocity of fluorescent erythrocytes through capillaries was 0.51±0.19 mm/s (mean±SD), and the diameter of the arterioles studied was 33±12 μm. Under ischemia, erythrocyte velocity through capillaries was significantly decreased to 0.33±0.14 mm/s, while arteriole diameter did not change significantly. During spontaneous peri-infarct depolarizations, arteriole diameter was significantly increased (119±23% of baseline), while capillary erythrocyte velocity was further decreased by 14±34%. The direction of arteriolar blood flow episodically and transiently reversed during approximately half of the peri-infarct depolarizations. The decrease in capillary erythrocyte velocity was more pronounced (23±37%) in these cases. After reperfusion, the microcirculatory variables rapidly returned to baseline. All rats in the ischemic group had infarcts 24 hours after occlusion.

Conclusions—Peri-infarct depolarization has an adverse influence on penumbral microcirculation, reducing capillary perfusion by erythrocytes, despite dilatation of arterioles. These findings suggest that a steal phenomenon contributes to the deleterious effect of these depolarizations. (Stroke. 2002;33:606-612.)

Key Words: cerebral ischemia, focal ■ microcirculation ■ microscopy, fluorescence ■ penumbra ■ rats

Transient peri-infarct depolarizations (PIDs) occur intermittently in the penumbra during the first few hours after occlusion of the middle cerebral artery (MCAO).1,2 Studies in which the number of depolarizations was intentionally decreased3–4 or increased5 indicate that PIDs have a deleterious effect on the extent of ischemic injury since there is a positive correlation between the number or the total duration of depolarizing events and the infarct volume. However, the mechanism by which PIDs exert this deleterious influence remains poorly understood.

Although there are several types of transient PID, most of them are akin to spreading depression.6,7 Spreading depression can be experimentally elicited by applying K+ or glutamate to the neocortex,8,9 while PIDs (we will here use the term PIDs for spreading depression–like depolarizations) are probably due to the spread of increased extracellular K+ or glutamate from the core of the lesion. Both phenomena result in a transient loss of transmembrane ionic gradients, silencing electrocorticographic activity and causing a negative shift of the direct current (DC) potential, which spreads over the whole cortex at 3 to 5 mm/min.10

Recovery from the depolarized state requires the activation of ion pumps, with an increase in metabolic activity and a rise in oxygen demand.11 In normal tissue with adequate regulation of blood flow, the increased ATP requirement is offset by hyperperfusion,10 so that spreading depression does not cause any neuronal damage.12

The deleterious effect of PIDs in underperfused tissue may be due to a lack of a compensatory increase in blood supply.13 This would lead to progressive ATP depletion and an inability to restore ionic gradients. PIDs would then turn into terminal depolarizations. This hypothesis is supported by several findings. First, PIDs are associated with an increase in blood flow, which is proportional to the residual blood flow.14 The rise in blood flow during PID is of the same order of magnitude as that obtained after spreading depression in

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physiological circulatory conditions but is greatly reduced\textsuperscript{2,14} or even absent\textsuperscript{15} when the residual blood flow is severely decreased. Second, a transient decrease in tissue Po\textsubscript{2} has been reported to accompany the passage of PIDs.\textsuperscript{15} Third, the duration of PIDs is prolonged at low flow rates\textsuperscript{16,17} and increases with the duration of ischemia.\textsuperscript{2}

However, other data do not support the aforementioned hypothesis. The prolongation of PIDs when blood flow is severely reduced is not due to compromised repolarization but rather their nature of anoxic depolarization.\textsuperscript{7} In addition, lowering oxygen availability during experimentally induced spreading depression increases the duration of depolarization but leads to neither terminal depolarization nor brain damage, even after a cumulative depolarization time of up to 70 minutes.\textsuperscript{18} Therefore, the energy depletion that causes the PID-associated terminal depolarization, resulting in expansion of the infarct core, does not seem to be linked to the high energy requirement of repolarization but to another indirect effect of PID, which remains to be clarified.

A “steal phenomenon”\textsuperscript{14} could account for these discordant data. This concept is based on the fact that a wave of PID induces an increase in blood flow only in those tissues in which perfusion is not severely reduced, ie, in the periphery of the ischemic penumbra. In these areas, close to the source of collateral supply via the anastomotic network, the arteriolar vasodilatation would recruit arterial blood formerly destined to severely hypoperfused regions, resulting in a further decrease in blood flow close to the ischemic focus. The blood supply in these areas would then drop below the membrane failure threshold, and anoxic depolarization would occur.

The parenchymal microcirculation can be directly monitored in real time by laser-scanning confocal fluorescence microscopy.\textsuperscript{19} This technique is appropriate for testing our hypothesis and could also provide a general picture of the dynamic microvascular consequences of focal cerebral ischemia, which is still lacking, as noted by del Zoppo.\textsuperscript{20}

This study was therefore performed to directly assess the dynamic changes in the microcirculation induced by focal ischemia and reperfusion in the penumbral zone and to determine the influence of PIDs on the penumbral microcirculation.

Materials and Methods
Experiments were performed under permit No. 02934 from the French Ministry of Agriculture. Nine adult male Sprague-Dawley rats (weight, 270 to 320 g) were used in this study.

Experimental Protocol

Preparation
Anesthesia was induced with 4% halothane in 30% O\textsubscript{2}/70% N\textsubscript{2}O and maintained throughout the preparation with 1.5% to 2% halothane. Rats were intubated and artificially ventilated. Arterial and venous catheters were placed in the femoral vessels. Rectal temperature was kept close to 37°C throughout the experiment with a feedback-controlled heating pad connected to a rectal probe.

Rats were placed in a stereotaxic apparatus to prepare a closed cranial window above the right frontoparietal cortex (posterior, 1.5 mm to bregma; lateral, 5 mm to the midline; Figure 1). The bone was thinned with a saline-cooled dental drill and carefully removed over an area of 4 mm\textsuperscript{2}. The dura mater was reflected, and a 150-μm-thick quartz microscope coverglass was sealed to the bone with dental cement (Palafem, Kuzler).

A right frontotral craniotomy (diameter 2 mm) was also performed 2 mm rostral to the bregma and 2 mm lateral to the sagittal suture (Figure 1). The dura mater was left intact. An Ag/AgCl wire (diameter 0.07 mm, uncoated tip 1.5 mm) was placed between the calvarium and the meningeal surface to record the potential and the electrocorticogram. An Ag/AgCl disk electrode was inserted under the skin of the neck as a reference electrode.

The right carotid arterial tree was isolated, and a cylinder of melted adhesive (length 2 mm, diameter 0.38 mm) attached to a nylon thread (0.22 mm in diameter) was advanced from the lumen of the external carotid artery (ECA) into the internal carotid artery (ICA) 5 mm after the external skull base. The portion of the thread remaining outside the ECA was inserted into a catheter, which was then secured to the ECA stump with a suture. This system\textsuperscript{21} enabled us to remotely occlude and reperfuse the middle cerebral artery (MCA) under the confocal microscope by advancing the nylon thread a further 4 mm or by subsequent withdrawal.

Experiment
The rat was placed under the confocal microscope, and its head was secured in a custom-built stereotaxic device closely fitting the confocal microscope stage. The halothane concentration was reduced to 0.8% to 1%. Blood was regularly sampled to check arterial gases and pH (Corning). The hematocrit was measured before and at different times after the tracers were injected.

Fluorescein-isothiocyanate (FITC)–dextran (molecular weight=70 000; 2.5 mg/mL) in 0.9% NaCl was injected (0.5 mL) intravenously to visualize microvessels and delineate their lumen. FITC-labeled erythrocytes (previously prepared in vitro\textsuperscript{19}) were injected via the same route at a tracer dose (≤2%). The whole cranial window was systematically explored with a ×10 objective. The most appropriate area of investigation (presence at least of 1 pial arteriole and a dense capillary network) was then chosen with a ×20 objective. The entire experiment was then performed with this ×20 objective. Intraparenchymal capillaries were visualized by changing the focus from the pial arterioles to a maximal depth of 200 μm beneath the surface of the brain. Images were video recorded.

Sequences were recorded during a control period, at the time of MCAO, for the following 2 hours, and for 1 hour after the thread was removed. Short video recordings (3 minutes each, focusing on arterioles for 1 minute and on capillaries for 2 minutes) were used to avoid continuous laser illumination. Video recordings were made every 20 minutes and during each PID.

Laser-Scanning Confocal Fluorescence Microscopy
The method is described in detail by Seylaz et al.\textsuperscript{19} Briefly, a confocal laser-scanning unit (Viewscan, BioRad) attached to a microscope (Optiphot-2, Nikon) was used to explore the rat cortical microcirculation. The light source was an argon-krypton laser (λ=488 nm), and an appropriate filter was used for microscopy of fluorescein. The fluorescent tracers were injected intravenously, and images of the microvascular network at various depths were recorded.

Figure 1. Location of closed cranial window and electrode.
at video speed (50/s) with an SIT camera (Hamamatsu) and a PAL-VHS video recorder (Panasonic).

**Histopathological Evaluation**
Twenty-four hours after ischemia, rats were deeply anesthetized with halothane, and their brains were removed, frozen for 1 minute in isopentane at −40°C, and stored at −80°C. Coronal section (15 μm) of the brains were cut with a cryostat at 500-μm intervals and stained with cresyl violet. An image analysis system (Biocom) was used to measure the areas of the cortical and striatal infarcted as well as noninfarcted tissues. The volume of the infarct was then calculated. It was corrected for edema with the following formula: corrected infarct volume = measured infarct volume [(1−(ipsilateral−contralateral hemispheric volume)/total infarct volume).

**Image Analysis**
Images were digitized and analyzed offline to determine the diameter of the arterioles and the velocity of fluorescently labeled erythrocytes through the capillaries. Segments (2 to 4) from 1 arteriole tree per rat were selected. Their internal diameter was automatically measured on digitized images with the use of custom-built Optimas software at each recording period. Measurements of all segments of the same arteriole at the same time were averaged because changes in diameter were similar in all parts of the same arteriole.

The erythrocyte velocity was measured through 3 to 5 capillaries per rat at approximately the same times as the arteriole diameters were measured. The velocity was measured over capillary segments 70 to 130 μm long. We calculated the average arteriole diameters and erythrocyte velocities in capillaries for each recording sequence in each rat. The recording sequences were classified as baseline (before MCAO), MCAO (during MCAO except during a PID), PID (during MCAO while a PID occurred), and reperfusion, and these categories were used as “sequence category” factor levels in ANOVAs. Mean arteriole diameter for each recording sequence was expressed as a percentage of the baseline value. Mean erythrocyte velocity through capillaries is expressed as an absolute value (mm/s) or as a percentage of the mean value of MCAO sequences.

The determination of blood flow reversal through arterioles was done by visually inspecting the video recordings. The flow direction was easily identified because the velocity of fluorescent erythrocytes slowed considerably before blood flowed in the opposite direction.

**Statistical Analysis**
Statistical analyses were performed by ANOVA with the use of Statview software (Abacus Concepts) followed by Fisher protected least significant difference post hoc tests, when appropriate. Values are given as mean±SD, and P<0.05 was accepted as significant.

**Results**

**Basal Conditions**
The mean diameter of the arterioles investigated was 33±12 μm in ischemic rats and 25±15 μm in sham-operated rats. The mean velocity of labeled erythrocytes through capillaries was 0.51±0.19 mm/s in ischemic rats and 0.51±0.25 mm/s in sham-operated rats. Some arterioles showed spontaneous motility. The physiological variables are shown in the Table. These values were within the expected ranges for halothane- anesthetized artificially ventilated rats. The hematocrit was not significantly modified by the injection of the tracers.

**MCA Occlusion**
None of the physiological parameters was modified by the advancement of the thread through the ICA (Table). There was no significant change in the microvascular variables in sham-operated rats at any time during the experiment. No statistically significant increase in the average arteriole diameter occurred when the thread was advanced up to the MCA branch point (Figure 2), although vasodilatation was the most common finding for the whole ischemic period (Figure 3). The velocity of erythrocytes through capillaries was significantly reduced to 0.33±0.14 mm/s (Figure 4). The efficacy of MCAO was checked 24 hours later by histology. In contrast to basal conditions, fluorescent erythrocytes were clearly evident in both arterioles and venules, indicating a marked reduction in their flow rate. Nonfluorescent cells appeared as black round masses that rolled and sludged on the vessel walls (Figure 5). Blood flow was sluggish in most venules, but established thrombi were never observed. Blood flow transiently stopped or reversed direction in arterioles in 15.4% of the MCAO recordings. The duration of these events ranged from 2 to 300 seconds.

| Physiological Parameters of MCA-occluded Rats at Various Times of the Experiment |
|---------------------------------|---------------------------------|
| **Basal Conditions**            | **1-h MCAO**                    | **30-min Reperfusion** |
| PaO₂, mm Hg                     | 144.0±33.7                      | 168.3±51.5             | 149.9±39.5 |
| PacO₂, mm Hg                    | 38.7±3.8                        | 41.8±10.7              | 35.6±1.8 |
| pH                              | 7.46±0.03                       | 7.43±0.09              | 7.46±0.03 |
| MABP, mm Hg                     | 78.5±6.2                        | 75.3±8.0               | 68.3±6.0 |
| Body temperature, °C            | 36.2±2.4                        | 37.0±0.4               | 36.8±0.1 |

MABP indicates mean arterial blood pressure.

**Figure 2.** Mean changes in arteriole diameter, expressed as percentage of baseline value (control), after MCAO, during PID, and after reperfusion (Reperf). *Significant difference vs all other values (P<0.05).
We were unable to see an opening of the blood-brain barrier by fluorescein leakage across arteriolar walls, except transiently at 1 arteriolar branching in 1 rat.

A geometric comparison of the whole network under basal conditions and during ischemia indicated a marked horizontal displacement (by 85 ± 12 μm) of the capillary bed with respect to the surface microvessels toward the midline in 4 rats (those exhibiting a cortical infarct 24 hours later) (Figure 6). The shift started between 30 and 60 minutes of occlusion and was in the same direction each time, revealing longer parts of the penetrating microvessels than under control conditions. This shift never reversed.

**Transient PIDs**

There were no PIDs in any of the sham-operated rats. Transient PIDs occurred in all the rats subjected to MCAO over the 2 hours of MCAO. Their mean number was 4.5 ± 3.0 per rat, and their mean duration was 2.0 ± 1.0 minutes.

PIDs caused a significant transient increase in arteriole diameter (119 ± 23% of baseline) compared with that at baseline or with that after MCAO (107 ± 18% of baseline) (Figures 2 and 7). At the same time, erythrocyte velocity through capillaries was transiently decreased to 86 ± 34% of the mean value after MCAO, indicating that PIDs enhanced ischemic conditions. In addition, blood flow in arterioles stopped and reversed frequently during PIDs (Figure 8). These changes occurred in 43% of the PID recordings. Their duration ranged from 30 to 120 seconds. Statistical comparison of microvascular changes during PIDs “with” and “without” circulatory disturbances revealed similar increases in arteriole diameter in both cases (data not shown). In contrast, the velocity of erythrocytes through capillaries was significantly more reduced during the microcirculatory disturbances, when it was 77 ± 37% of its mean value under MCAO, than in the absence of microcirculatory disturbances, when it was 94 ± 30% of its mean value under MCAO (Figure 9).

**Microcirculation Under Reperfusion**

The blood flow through arterioles was visibly increased when the thread was removed, but there was no significant change in the mean diameter of arterioles (Figure 2). The velocity of labeled erythrocytes through capillaries returned to its baseline value of 0.50 ± 0.24 mm/s (Figure 4). There was no capillary recruitment (ie, newly perfused capillaries) for either plasma or erythrocytes. Sluggish blood flow persisted in some venules, and an increasing number of round black masses (possibly activated monocytes as shown in pilot experiments with rhodamine 6G labeling) were rolling and fixing.

**Histopathology**

No lesion was visible in the brains of sham-operated rats (n=3) 24 hours after the thread was placed through the ICA, whereas all rats subjected to MCAO (n=6) had an infarct. Occlusion produced infarcts in both the cortex and subcortex in 4 rats (cortex, 107.5 ± 11.3 mm³; subcortex, 19.5 ± 7.7 mm³) and in the
subcortex only in 2 rats (18.9±1.3 mm³). Edema accompanied all cortical infarcts.

**Discussion**

This study reports the first direct visualization in real time of the plasma and erythrocytes flowing through small-caliber pial arterioles (approximately 35 μm) and intraparenchymal capillaries within the penumbral zone after MCAO and reperfusion. Although the area explored was in the outer part of the penumbra, the moderate microcirculatory changes occurring within this area are assumed to reflect those occurring in the inner penumbral zone, with some attenuation.

The major findings of the present study on PIDs are that MCAO alone fails to induce a homogeneous response of arterioles in terms of changes in diameter, but there is a systematic decrease in erythrocyte capillary perfusion. However, the spontaneous transient waves of depolarization cause arterioles to dilate, resulting in a further decrease in erythrocyte capillary perfusion. Finally, the decrease in erythrocyte velocity was more pronounced when there were local reversals of blood flow in arterioles during PIDs than when blood flow direction did not change.

Whatever the mechanism of the transient dilatation associated with PID, the striking feature of our present investigation is that it is associated with a decrease in capillary erythrocyte velocity. The reduced capillary perfusion by erythrocytes under spreading depression–like depolarizations could be due to an active constriction of the intraparenchymal precapillary arterioles that are in proximity to the greatest

**Reversal of blood flow in an arteriole**
A phenomenon first described in the brain in response to ischemia is peri-infarct depolarization (PIDs), the erythrocyte capillary circulation was heterogeneous and unpredictable in terms of both speed and trajectory. PIDs may also have had a restricted transit through the capillary network with respect to the pial arteriole and venule diameter measured after forebrain ischemia and is probably due to the residual blood flow in focal ischemic conditions. The decrease in capillary erythrocyte velocity during MCAO indicates the penumbral location of the field under investigation. The reversals of blood flow in arterioles demonstrated that arteriolar anastomoses were functional. However, collateral flow did not prevent the decrease in parenchymal capillary perfusion, although they impeded a complete arrest. Swelling of astrocytic endfeet and/or venous thrombosis may be partly responsible for the reduced erythrocyte flow through capillaries, in addition to the reduced perfusion pressure. Polymorphonuclear leukocytes may also have had a restricted transit through the capillary segments of microvascular beds. However, the rapid return to basal levels of the microvascular parameters after reperfusion indicates that only reversible events are involved, despite the ongoing process of infarction. No hyperemic reaction occurred when the thread was removed, as found with other methods after 2 hours of focal ischemia. This is in contrast to the increase in erythrocyte velocity and arteriole diameter measured after forebrain ischemia and is probably due to the residual blood flow in focal ischemic conditions.

In summary, this is the first dynamic investigation of the ischemic penumbra microcirculation to indicate that the propagation of transient PIDs may contribute to the development of an infarct, at least in part via a decrease in capillary perfusion by erythrocytes. We postulate that such depolarizations are a key pathological event in the hemodynamics of focal ischemia, even in moderately ischemic tissue with an efficient collateral blood flow. It is difficult to assess the clinical relevance of the brief enhancement of ischemic conditions by PIDs in the penumbra, since the occurrence of PIDs in humans has not been studied during stroke. However, the consequences of repeated PIDs on cell energy can be additive in these experimental conditions and can irreversibly influence adjacent regions more critically affected.

An alternative explanation is that there is an intracerebral steal, a phenomenon first described in the brain in response to hypercapnia in focal ischemic conditions. This proposal is supported by the frequent changes in the direction of blood flow through arterioles during the propagation of PIDs and a further reduction in erythrocyte velocity through capillaries. These observations suggest that there is a link between the changes in flow direction in arterioles and the reduction in capillary perfusion. These changes in blood flow direction likely reflect focal changes in arteriolar resistance due to the dilatation associated with PID. The arteriole dilatation does not lead to changes in blood flow when the wave of PID is close to the ischemic focus, since perfusion pressure is very low. However, when the wave reaches the periphery of the MCA territory, where perfusion pressure may be close to normal, blood flow in this area increases. Since total blood flow in the MCA territory is limited by the diameter of anastomoses, any focal increase in blood flow in this territory is likely to be accompanied by a decrease in perfusion in other parts of the same arterial territory. Strong et al. showed that stimulation of a cortical area within the MCA territory after MCAO caused an increase in blood flow in the stimulated area, with a decrease in blood flow in an adjacent region. It is difficult to assess the clinical relevance of the brief enhancement of ischemic conditions by PIDs in the penumbra, since the occurrence of PIDs in humans has not been studied during stroke. However, the consequences of repeated PIDs on cell energy can be additive in these experimental conditions and can irreversibly influence adjacent regions more critically affected.

Concerning basal and ischemic conditions (excluding PIDs), the erythrocyte capillary circulation was heterogeneous and unpredictable in terms of both speed and trajectory. Capillary recruitment, for both plasma and erythrocyte flow, did not occur during reperfusion, as we also found for the hyperemic phase after global ischemia. The decrease in capillary erythrocyte velocity during MCAO indicates the penumbral location of the field under investigation. The reversals of blood flow in arterioles demonstrate that arteriolar anastomoses were functional. However, collateral flow did not prevent the decrease in parenchymal capillary perfusion, although they impeded a complete arrest. Swelling of astrocytic endfeet and/or venous thrombosis may be partly responsible for the reduced erythrocyte flow through capillaries, in addition to the reduced perfusion pressure. Polymorphonuclear leukocytes may also have had a restricted transit through the capillary segments of microvascular beds. However, the rapid return to basal levels of the microvascular parameters after reperfusion indicates that only reversible events are involved, despite the ongoing process of infarction. No hyperemic reaction occurred when the thread was removed, as found with other methods after 2 hours of focal ischemia. This is in contrast to the increase in erythrocyte velocity and arteriole diameter measured after forebrain ischemia and is probably due to the residual blood flow in focal ischemic conditions.

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