Cerebral Blood Flow Restoration and Reperfusion Injury After Ultraviolet Laser–Facilitated Middle Cerebral Artery Recanalization in Rat Thrombotic Stroke

Brant D. Watson, PhD; Ricardo Prado, MD; Alexander Veloso, BS; J-P. Brunschwig, BS; W. Dalton Dietrich, PhD

Background and Purpose—A reversible model of focal thrombotic stroke was developed in the rat and examined for histological evidence of reperfusion injury after clinically relevant times of recanalization.

Methods—The distal middle cerebral artery of 28 male Sprague-Dawley rats was occluded by 562-nm laser-driven photothrombosis for 0.5, 2, and 3 hours or permanently (each n = 7) and was recanalized by 355-nm UV laser irradiation. Occlusive material was examined by transmission electron microscopy. Cortical cerebral blood flow was monitored by laser-Doppler flowmetry. Brain infarcts were examined histologically at 3 days.

Results—After occlusion, cortical cerebral blood flow was reduced to 33±4% of baseline for all groups and was restored to 82±9%, 75±3%, and 93±7% of baseline for the 0.5-, 2-, and 3-hour groups, respectively, following recanalization after 29±8, 38±20, and 70±33 minutes of UV laser irradiation. The thrombotic occlusion contained compactly aggregated platelets but no fibrin, with length (1.2 to 1.8 mm) proportional to the ischemic period. During recanalization, microchannels containing erythrocytes and scattered leukocytes and bordered by intact disaggregated platelets infiltrated the thrombus. Infarct volumes (mm3) at 3 days were 12±3, 75±10, and 38±2 for the permanent case and 8±4, 24±3, and 30±9 for the 0.5-, 2-, and 3-hour cases, respectively, thus demonstrating reperfusion injury histologically in the latter 2 groups. No hemorrhage was seen.

Conclusions—UV laser–facilitated dissolution of a conventionally refractory platelet thrombus provides a novel and effective method for restoring blood flow without hemorrhagic complications during thrombotic stroke. This was the first observation of histologically confirmed reperfusion injury in such a model. (Stroke. 2002;33:428-434.)

Key Words: laser ■ platelet aggregation ■ reperfusion injury ■ stroke, ischemic ■ thrombolysis ■ rats

A nimal models of thrombotic or thromboembolic stroke are becoming increasingly important in basic and preclinical studies of prospective therapies, especially those concerned with thrombolytic1 and, more recently, antiplatelet regimens.5 Oclusive platelet thrombi are of particular concern because they contain little or no fibrin3 and thus are extremely resistant to thrombolytic drugs such as the tissue plasminogen activators approved for acute stroke therapy.3–7 The prevention or removal of refractory platelet thrombi has attracted much interest, as shown by the increasing utilization of arginine-glycine-aspartic acid antagonists2,3,8–10 and thrombin inhibitors11 to inhibit or destabilize intraplatelet fibrinogen cross-links between platelet glycoprotein (GP) IIb-IIIa membrane receptors. Experimentally, such a fibrin-free white thrombus can be produced photochemically in arteries in vivo by a process termed laser-driven photothrombosis.12 Although we often have used this technique to occlude the distal middle cerebral artery (MCA) to simulate thrombotic stroke in the rat,13–15 studies of the consequences of reperfusion after clot lysis in these models have been very few, requiring special conditions. For example, “soft” platelet thrombi transiently occluding an MCA segment can be dissolved hemodynamically after local vasodilation with nimodipine.16

A search for a more focal method of vasodilation revealed that UV light exposure from a filtered arc lamp could relax preconstricted aortic rings prepared in vitro17 and also arteries in vivo by irradiation with pulsed 351-nm XeF18 or 248-nm KrF19 UV lasers. The mechanisms of dilation are attributed to photoacoustic alteration of smooth muscle contractile proteins by shock waves generated during erythrocyte vaporization at high laser-energy density20 or photolytic release of nitric oxide at low energy density,19 respectively. Accordingly, we examined the capability of a 355-nm pulsed, low energy-density UV laser to dilate the patent rat MCA and then to dilate and recanalize the occluded MCA after photo-
thrombosis, concurrent with laser-Doppler measurements of cortical cerebral blood flow (CBF). Full recanalization was complicated by extensive deposits of occlusive material (examined by transmission electron microscopy [TEM]) adjacent to the primary platelet thrombus. Finally, we examined the distal MCA territory 3 days afterward for histological evidence of cerebral reperfusion injury.

Materials and Methods

Surgical Preparation

All animal surgical and maintenance procedures were approved by the Animal Care and Use Committee of the University of Miami in accordance with US Public Health Service regulations. Briefly, male Sprague-Dawley rats (n=34) weighing 250 to 350 g were anesthetized with 2.5% halothane in a 70%/30% mixture of nitrous oxide and oxygen and were maintained on 1% halothane by means of mechanical ventilation. The left distal MCA was exposed via a 3-mm burr hole with the dura intact, essentially as previously described,13,14 with head and body temperatures maintained at 36 °C. A second burr hole was created between the coronal and 37 °C. A second burr hole was created between the coronal and

<table>
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<th>TABLE 1. Physiological Data</th>
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MAP indicates mean arterial pressure. Values are mean±SD. Arterial blood gases in the postocclusion period are averages encompassing times of ischemia and reperfusion.

Distal MCA Photothermobic Occlusion Facilitated by Dye Laser Irradiation

Laser-driven photothermosis of the distal MCA was administered as described previously,13 but only a single site ranging from 150 to 250 μm in diameter over all groups was irradiated to the point of occlusion, while all other arteries remained patent. Occlusion was generated within 3 to 5 minutes by the interaction of the 562-nm dye laser beam at powers of 3.5 to 6.5 mW interacting with the intravenously injected photosensitizing dye rose bengal (20 mg/kg) and was accompanied by prominent vasoconstriction and blanching of the distal MCA distally12 and somewhat proximally as well. The lengths of the blanched segments were minimized by using lower irradiation intensities (approximately 130 mW/mm²) than those used previously15 and (in the proximal case) by inducing the primary thrombus at a point distal to a well-perfused G1 branch.

Distal MCA Recanalization Facilitated by UV Laser Irradiation

A Q-switched, frequency-tripled neodymium:yttrium-aluminum-garnet (Nd:YAG) laser (Minilase II, New Wave Research) producing 5-ns pulses at a rate of 20 Hz and a UV wavelength of 355 nm was used to irradiate the distal MCA. Laser power was measured with a detector head (model 380101) interfaced to a power meter (model 365, Scientech). The UV laser beam was spatially and optically filtered, expanded by a ×10 beam telescope (model BXUV-4.0-10X, CVI Laser Corp) and then focused by a 25-cm focal length BK7 glass plano-convex lens onto the distal MCA, encompassing its diameter. Diameter. Dilation of the patent distal MCA with the UV laser beam was examined quantitatively in a closed cranial (fused silica) window preparation (n=7).21 Recanalization of an occluded distal MCA was begun by low-intensity UV laser irradiation (approximately 20 mW/mm²) immediately proximal to the occlusion. This induced dilation of the adjacent thrombosed segment, and thin rivulets of fresh blood were observed to infiltrate the thrombus rapidly. The UV intensity was then increased until the infiltrating blood columns coalesced, indicating disappearance of thrombosed material and recanalization of the segment. The UV beam was then advanced distally (by approximately half the segment diameter), and the process was repeated until complete recanalization was observed visually and verified by normalization of cortical CBF. The duration of irradiation and laser power were then recorded. The primary thrombus segment was the most vasoconstricted and thus required more UV intensity and time. Exposure of dural vessels to UV irradiation was minimized to avoid hemorrhage.

Light Microscopic Analysis of Infarct Volume

Animals that underwent distal MCA occlusion were perfusion-fixed at 3 days in FAM (40% formaldehyde, glycerol, and methanol, 1:1:9 by volume), and their brains were embedded in paraffin from which 10-μm-thick sections were obtained at 25-μm intervals and stained by hematoxylin and eosin, as described previously.14 For calculating infarct volume, histological sections at 8 coronal levels were digitized at ×1 power by means of a CCD camera (Xillix Technologies Corp), or microscopic images were digitized by means of a Sony CCD color video camera, and data were recorded with an MCID image analysis system (Imaging Research, Inc). Total infarct volume was derived by numerical integration of sequential infarct areas according to Simpson’s rule, with correction for edema.22 Areas bordering the infarct epicenter were evaluated for incomplete infarction (a mixture of normal and necrotic neurons)23 at ×400 magnification.

TEM Analysis

Animals were transcardially perfused beginning with a 2-minute flush of isotonic saline delivered at a pressure equal to the rat’s mean arterial blood pressure, followed by 500 mL of fixative solution composed of 2% glutaraldehyde and 100 mmol/L sucrose in 0.05 mol/L NaPO₃ buffer. The brain was removed and placed overnight in fresh fixative chilled to 4°C. The desired distal MCA segment was carefully removed from the surface of the brain, placed into a 1% solution of OsO₃ in 0.1 mol/L NaPO₃ buffer, and stored overnight at 4°C. The segment was then prepared for TEM essentially as
described previously and was examined and photographed in a TEM (model CM-10, Philips Research Laboratories) operated at 60 or 80 kV.

**Statistical Analysis**
Differences in infarct volume and CBF among the treatment groups were determined by ANOVA followed by Newman-Keuls test. All data are expressed as mean ± SD.

**Results**

**Physiological Variables**
Physiological variables are shown in Table 1. No significant changes were observed throughout the experiment.

**Response of the Patent Distal MCA to UV Laser Irradiation**
In the closed cranial window preparation, the patent distal MCA in the initial group of 7 rats was exposed for 2 minutes to 355-nm pulsed UV laser irradiation; a typical response over time is shown in Figure 1. Dilations with respect to baseline of 64%, 64% and 58% were observed after 5 seconds, 1 minute, and 2 minutes, respectively, and were essentially unchanged for 30 minutes afterward.

**Response of the Occluded Distal MCA to 355-nm UV Laser Irradiation**
Recanalization of occluded segments aged for 0.5 to 3 hours was facilitated by 355-nm UV laser irradiation as described in Materials and Methods and was observed in every treated rat. Figure 3 depicts UV treatment of a thrombotic occlusion aged for 3 hours. The original diameter of the entire segment was recovered, but some mural thrombotic material appeared intermittently up to 2 hours after UV treatment. The average UV beam intensity at the onset of recanalization was 48 ± 32 mW/mm² (n=21). The irradiation time required for recanalization was proportionate to the age of the thrombus (r=0.96, P<0.001; Table 2).
TABLE 2. Distal MCA Responses to Thrombotic Occlusion and Subsequent Recanalization

<table>
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<tr>
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<th>Occlusion</th>
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<td></td>
<td>Permanent</td>
<td>0.5 h</td>
<td>2 h</td>
<td>3 h</td>
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<tr>
<td>Postocclusion</td>
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<tr>
<td>diameter, % of</td>
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<tr>
<td>baseline</td>
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<tr>
<td>Primary occlusion</td>
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<tr>
<td>length, μm</td>
<td>220±60†</td>
<td>193±46†</td>
<td>163±29†</td>
<td>230±30†</td>
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<tr>
<td>Final occlusion</td>
<td></td>
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<tr>
<td>length, mm</td>
<td>1.6±0.3</td>
<td>1.1±0.2§</td>
<td>1.3±0.2§</td>
<td>1.9±0.5</td>
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<tr>
<td>Recanalization</td>
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<tr>
<td>time for primary</td>
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<tr>
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<td>6±3</td>
<td>15±5</td>
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<td>29±8</td>
<td>38±20</td>
<td>70±33</td>
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<td>Post–UV-treated</td>
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<tr>
<td>diameter, % of</td>
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<tr>
<td>baseline</td>
<td>119±25*</td>
<td>122±23*</td>
<td>115±21*</td>
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Values are mean±SD.
*P<0.05 vs baseline diameter.
†Initially photothrombosed (constricted) segment, different from final occlusion lengths (P<0.05).
‡P<0.05 vs permanent occlusion.
§P<0.05 vs 3-h group.

The process of recanalization was examined structurally in another distal MCA segment after an occlusive thrombus was aged for 0.5 hour (Figure 4A) and then underwent UV treatment until blood appeared to infiltrate its proximal half (Figure 4B) and surrounded the thrombus concomitant with distal MCA dilation (Figure 4C). More interesting, however, was the penetration of blood directly into the thrombus owing to the formation of multiple internal channels congruent with and even beyond the UV beam location (Figure 4C). Under TEM, the borders of the proximal infiltrating channels are composed of platelets that appear to have separated cleanly from their cross-channel neighbors (Figures 5A and 5C). In distal channels, however, the borders may be diffuse owing to mechanical damage to platelet membranes (Figure 5B). The channels thus comprise a complicated network filled with erythrocytes, plasma, and occasional leukocytes. No degradation of the infiltrated erythrocytes under 355-nm UV irradiation was seen, thus indicating the absence of destructive photocoagulative effects, but vacuolation that had developed in smooth muscle cells in the primary photothrombosed segment was exacerbated (not shown). No vacuolation was seen in UV laser–irradiated patent segments.

Response of Cortical CBF to Occlusion and Subsequent Recanalization

The time histories of cortical CBF response to the permanent occlusion experiment and to the experiments based on occlusion for 0.5, 2, and 3 hours followed by recanalization are shown in Figure 6. Laser-Doppler cortical CBF was reduced to 30±3% of baseline for permanent occlusion and 36±4%, 37±2%, and 35±2% of baseline for the 0.5-, 2-, and 3-hour groups, respectively. The ischemic periods were followed by time intervals representing the average times for UV treatment for each group (Table 2) until resumption of cortical CBF to near-normal levels, amounting to 82±9%, 75±3%, and 93±7% of baseline for the 0.5-, 2-, and 3-hour groups, respectively, during 2 hours of observation after full recanalization.

Note that the true ischemic interval is the sum of the predesignated occlusion time, plus the time taken to recanalize the total occlusion length (Figure 6 and Table 2): approximately 1 hour for the 0.5-hour occlusion group, 2 hours 40 minutes for the 2-hour group, and 4 hours 10 minutes for the 3-hour group. Recovery of flow at the later times in a thrombotic animal model of stroke is quite unusual but is consonant with the time scale for stroke patients undergoing acute thrombolytic therapy.

Reperfusion Injury in the Distal MCA Territory

Brain infarcts in the 4 groups at 3 days appeared only in the ipsilateral hemisphere, were confined to the dorsolateral cerebral cortex, and displayed typical cellular alterations consistent with ischemic necrosis. Infarct volumes were 12±3 mm³ for the permanent cases and 8±4, 24±3, and 30±9 mm³ for the cases in which recanalization was begun after 0.5, 2, and 3 hours of thrombosis-induced ischemia (Figure 7). Because the infarct volumes for the 2- and 3-hour
cases far exceeded that for the permanently occluded cases \((P<0.001)\), reperfusion injury is thus demonstrated. No hemorrhages were observed. In addition, incomplete infarction\(^\text{13}\) was observed sporadically at the periphery of the infarct epicenter as a small percentage of its area. For the permanent, 0.5-, 2-, and 3-hour groups, this was 4.3%, 0.45%, 4.0%, and 1.5%, respectively. The frequency of occurrence in each group was 5 of 7, 1 of 7, 4 of 7, and 3 of 7, respectively.

**Discussion**

These studies have revealed that (1) a cerebral artery occluded by an extensive, fibrin-free platelet thrombus can be recanalized by a novel laser method\(^\text{15}\) that instigates the formation of multiple, progressively enlarging channels in the thrombus interior and (2) CBF can be restored to nearly normal levels at clinically relevant times, only to engender reperfusion injury. This occlusive platelet thrombus is intracerebral to conventional lytic methods, but the UV laser method overcomes these limitations and thus provides an unusual and effective means to restore blood flow during thrombotic stroke. Direct UV irradiation of the stabilized platelet mass is evidently unnecessary, because the intrathrombus channels can still form distal to that point (Figure 4C). It may also be possible to recanalize cerebral arterial branches indirectly owing to the diffusible nature of the dilation effect (Figure 1). Finally, because cerebral ischemia in this model is induced by occlusive thrombosis only, collateral pathways are not compromised by concurrent mechanical ligature (eg, of the common carotid arteries) and are thus available as pharmacological conduits. This model of reversible thrombotic stroke is therefore reduced to its most essential aspects.
rhage was 10%. Recently, a percutaneously transmitted high energy-density dye laser beam has been used clinically to disintegrate brain artery occlusions photoacoustically. With similar optical fiber technology, percutaneous UV laser irradiation at much lower energy densities may possibly augment or substitute for intra-arterial thrombolytic interventions. Finally, although platelet arginine-glycine–aspartic acid inhibitors have augmented thrombolytic therapy clinically and have suppressed platelet accumulation in ischemic microvessels in baboons, they cannot be used in the rat because a conformational change in the rat platelet GPIIb-IIIa receptor after binding of the inhibitor drug allows fibrinogen to bind as well. The UV laser method nonetheless facilitates the dissociation of intraplatelet binding forces, with apparently endogenous factors or their derivatives that are insensitive to this limitation.

However, despite near-complete restoration of cortical CBF in the reversible thrombotic stroke groups, histological infarct volumes at 3 days after reperfusion at the relatively late absolute times of 3 to 4 hours were enhanced by 2- to 2.5-fold compared with the permanently occluded group. The chief criterion for reperfusion injury, ie, conversion of tissue from a reversible to an irreversible state of injury, is thus fulfilled. Such direct evidence for reperfusion injury in thrombotic stroke has not been reported previously. Possible potentiators of thrombotic reperfusion injury include platelet secretions that evidently damage the blood-brain barrier downstream and presage severe morphological changes in neurons, astrocytes, and endothelium. The participation of acute inflammation is indicated by the intense platelet aggregation distal to the primary thrombus, which likely elicits a similar response in the ischemic microvasculature and also by the acute association of leukocytes with the recanalizing thrombus (Figure 5A). Previously, reperfusion injury was demonstrated in the brains of rats mechanically recirculated between 2 and 5 hours after dual-ligature occlusion (MCA and ipsilateral common carotid artery) as approximately 3-fold enhancement of infarct volume at 1 day compared with the permanently occluded group. Our cortical CBF results (Figure 5) indicate that reperfusion injury is potentiated by intermediate, but not severe, levels of ischemia in cerebral tissue and by delayed cortical CBF restoration and blood-brain barrier damage. Identification of the processes contributing to reperfusion injury in our reversible model of thrombotic stroke now appears feasible, as does study of their eventual mitigation.
Acknowledgments

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References


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