Combination Treatment With N-Acetyl-Seryl-Aspartyl-Lysyl-Proline and Tissue Plasminogen Activator Provides Potent Neuroprotection in Rats After Stroke

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Background and Purpose—N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), an endogenously produced circulating peptide in humans and rodents, exerts anti-inflammatory and cardioprotective activities in various cardiovascular diseases.

Methods—The present study evaluated the neuroprotective effect of AcSDKP alone and in combination with thrombolytic therapy in a rat model of embolic focal cerebral ischemia.

Results—We found that treatment with AcSDKP alone at 1 hour or the combination treatment with AcSDKP and tissue plasminogen activator (tPA) at 4 hours after stroke onset substantially increased AcSDKP levels in plasma and cerebrospinal fluid and robustly reduced infarct volume and neurological deficits, without increasing the incidence of brain hemorrhage compared with ischemic rats treated with saline, AcSDKP alone at 4 hours, and tPA alone at 4 hours. Moreover, the combination treatment considerably reduced the density of nuclear transcription factor-κB (NF-κB), transforming growth factor β (TGF-β), and plasminogen activator inhibitor-1 (PAI-1) positive cerebral blood vessels in the ischemic brain, all of which were associated with reduced microvascular fibrin extravasation and platelet accumulation compared with tPA monotherapy. In vitro, AcSDKP blocked fibrin-elevated TGF-β1, PAI-1, and NF-κB proteins in primary human brain microvascular endothelial cells.

Conclusions—Our data indicate that AcSDKP passes the blood–brain barrier, and that treatment of acute stroke with AcSDKP either alone at 1 hour or in combination with tPA at 4 hours of the onset of stroke is effective to reduce ischemic cell damage in a rat model of embolic stroke. Inactivation of TGF-β and NF-κB signaling by AcSDKP in the neurovascular unit may underlie the neuroprotective effect of AcSDKP. (Stroke. 2014;45:1108-1114.)

Key Words: capillary permeability ■ ischemia ■ stroke

Stroke is a leading cause of death and disability worldwide. However, tissue plasminogen activator (tPA), the only Food and Drug Administration–approved treatment for acute stroke is constrained by its narrow therapeutic window and potential adverse side effects of brain hemorrhage. It becomes increasingly recognized that the perturbation of the neurovascular unit, a structure and functional interdependent microvascular and parenchyma network, after stroke leads to neurovascular disruption. AcSDKP either alone at 1 hour or in combination with tPA at 4 hours of the onset of stroke is effective to reduce ischemic cell damage in a rat model of embolic stroke. Inactivation of TGF-β and NF-κB signaling by AcSDKP in the neurovascular unit may underlie the neuroprotective effect of AcSDKP. (Stroke. 2014;45:1108-1114.)

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1108
cardiovascular events without apparent reduction of blood pressure. These data imply that an elevation of plasma AcSDKP levels may contribute to the neuroprotective effect of ACE inhibitors on stroke. However, the effect of AcSDKP on acute stroke has not been investigated. In the present study, using a rat model of embolic middle cerebral artery occlusion (MCAO), we examined the neuroprotective effect of AcSDKP on acute stroke. Our data showed that treatment of acute stroke with AcSDKP alone or in combination with tPAs substantially reduced neurovascular damage and improved neurological outcome.

Materials and Methods

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. All outcome measures were performed by observers blinded to the treatments.

Animal Model

Male Wistar rats weighing 350 to 400 g (Charles River Laboratories) were subjected to embolic middle MCAO, as previously described (see online-only Data Supplement for detailed Methods).11

Experimental Protocols

To examine the effect of AcSDKP on acute stroke, AcSDKP at a dose of 0.8 mg/kg per day was administered daily for 3 days starting 1 or 4 hours after MCAO intra-arterially for 2 hours followed by a subcutaneous infusion with an osmotic pump (Alzet model 1003D) for 3 days (n=7/group). The dose of AcSDKP was selected based on our published studies showing that AcSDKP at a dose of 0.8 mg/kg per day exerts potent cardiovascular protective actions in experimental hypertension in the rats. To achieve rapid and broad distribution of AcSDKP in the brain, intra-arterial injection was initially chosen. Ischemic rats subjected to the same volume of saline infusion (n=12) with the identical protocol described above starting 1 hour after stroke onset were used as a control group. To examine whether combination of AcSDKP with tPA is effective for acute stroke, ischemic rats subjected to AcSDKP (n=13) or identical volume of saline treatment (n=12) with the same infusion method described above were cotreated with tPA (10 mg/kg) intravenously starting 4 hours after MCAO. To examine whether blockage of AcSDKP with neutralizing monoclonal antibody specifically against AcSDKP (mAb-Ac) abolishes the effects of the combination treatment with AcSDKP and tPA on stroke, mAb-Ac or a control antibody against rat immunoglobulin G (IgG) (0.4 mg/kg, Sigma) was given intraperitoneally (LP) 4 and 48 hours after MCAO in rats subjected to the combination treatment with AcSDKP and tPA (n=6/group).

AcSDKP Enzyme Immunoassay

Plasma and cerebrospinal fluid (CSF) samples were obtained 24 hours after initiation of AcSDKP administration. AcSDKP levels in plasma and CSF were measured using a commercially available enzyme immunoassay kit accordingly to manufacturer instructions (Cayman Chemicals).

Behavioral Tests

Longa’s 5-point scale was used for acute assessment of neurological deficit 30 minutes after MCAO.22 Rats with a score between 1 and 3 were randomized into treatment groups. To detect sensorimotor deficits, a battery of behavioral tests including adhesive removal test, foot-fault test,13 and modified neurological severity score (mNSS)14 was performed 1 and 7 days after onset of MCAO by an observer blinded to the treatments (see online-only Data Supplement for detailed Methods).

MRI Measurements

MRI measurements were performed using a 7 Tesla Agilent MRI/MRS system (Santa Clara, CA) in rats subjected to tPA monotherapy and the combination treatment of AcSDKP and tPA initiated at 4 hours after MCAO (n=10/group), T2 weighted image and diffusion-weighted images were performed 1, 72, and 144 hours after MCAO. Areas of ischemic damage were calculated from MRI parameters of T2 map using threshold values of 2 standard deviations above the corresponding contralateral nonischemic hemisphere.15

Histopathologic Analysis

Rats were euthanized 7 days after MCAO, and infarct volume was measured on hematoxylin and eosin–stained coronal sections using the microcomputer imaging device system (Imaging Research Inc), as previously described.16 Gross hemorrhage, defined as blood evident to the unaided eye on the hematoxylin and eosin–stained coronal sections, was evaluated on 7 hematoxylin and eosin–stained coronal sections for each animal. Data are presented as the percentage of gross hemorrhage in each experimental group.

Immunohistochemistry Analysis

Additional rats subjected to tPA monotherapy (n=4), the combination treatment with AcSDKP and tPA (n=6), and saline (n=4) at 4 hours after stroke onset were euthanized 24 hours after initiation of treatment. The brains were removed, and consecutive frozen coronal sections (8 μm) at bregma −0.4 to −1.4 mm were obtained for immunohistochemical analysis (see online-only Data Supplement for detailed Methods).

Primary Human Cerebral Endothelial Cell Culture

Primary human brain microvascular endothelial cells (HBECs) obtained from Applied Cell Biology Research Institute (Kirkland, WA) were cultured in endothelial cell growth media according to the manufacturer protocols. To examine directly whether AcSDKP blocks fibrin-induced activated nuclear transcription factor-κB (NF-κB; p65),16 plasminogen activator inhibitor-1 (PAI-1), and transforming growth factor β 1 (TGF-β1) expression in cerebral endothelial cells, AcSDKP (1 nM) and fibrin (1.5 μg/mL; Sigma) were simultaneously added into the cultured HBECs culture for 6 hours. The HBECs treated with fibrin in the absence of AcSDKP was used as a control. The HBECs were collected at 6 hours after treatment, and the protein levels of p65, PAI-1, and TGF-β1 were determined by Western blot analysis.

Statistic Analysis

Data are presented as the mean±SE. One-way ANOVA was used to compare multiple-group values (ie, measurements of lesion volume, immunohistochemical data, MRI measurements, and AcSDKP levels). If the main effect of treatment group was significant at P<0.05, then all pairwise comparisons between treatment groups were tested. Adjustments for multiple comparisons were made using Hochberg’s method. Fisher exact test was used to test the gross hemorrhagic rates among the groups.

Results

Neuroprotective Effect of AcSDKP

Before treatment, all ischemic rats exhibited neurological deficits measured by the Longa 5-point scale (1.9±0.1 for AcSDKP at 1 hour, 2.0±0.0 for AcSDKP at 4 hours, 1.9±0.1 for saline; P>0.05). However, ischemic rats treated with AcSDKP starting at 1 hour, but not 4 hours, exhibited significant (P<0.05)
improvement of neurological function measured by the adhesive removal test at days 1 and 7, and by foot-fault and mNSS tests at day 7 compared with ischemic rats treated with saline (Figure 1). Histopathologic analysis revealed that treatment of ischemia with AcSDKP initiated at 1 hour, but not 4 hours, significantly (*P* < 0.05) reduced infarct volume compared with the saline treatment 7 days after MCAO (Figure 1). There was no statistically significant difference in the incidence of gross hemorrhage between saline (1 out 8, 25%) and AcSDKP at 1 hour (1 out of 7, 28%) groups. These data indicate that AcSDKP treatment initiated at 1 hour after onset of stroke exerts a neuroprotective effect.

Using this model, we previously demonstrated that tPA administered 4 hours after MCAO did not have the neuroprotective effect.11 We thus examined whether AcSDKP in combination with tPA can reduce ischemic neuronal damage. Consistent with our previous findings, monotherapy of tPA at 4 hours did not significantly improve and reduce neurological function at day 7 compared with the saline treatment (Figure 1).

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**Figure 1.** Infarct volume and neurological functional outcome. A shows the effects of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) alone and in combination with tissue plasminogen activator (tPA) on infarct volume assessed 7 days after middle cerebral artery occlusion (MCAO). B, C, and D show the neurological functional outcome measured by adhesive removal test, foot-fault test, and neurological severity score at 1 (black bar) and 7 (open bar) days after stroke onset, respectively. mAb-Ac indicates anti-AcSDKP monoclonal antibody; and Rat-IgG, rat immunoglobulin G.

**Figure 2.** MRI measurement. A and B show T2 weighted MRI at 1, 72, and 144 hours after middle cerebral artery occlusion (MCAO) of a representative rat treated with tissue plasminogen activator (tPA; A) and the combination of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) and tPA (B) 4 hours after stroke. C and D are hematoxylin and eosin (H&E)–stained sections obtained from the same representative rats 7 days after MCAO. Bar graph shows quantitative data of T2 values.
outcome and infarct volume, respectively, 7 days after MCAO. However, the combination therapy considerably improved neurological outcome and substantially reduced infarct volume compared with monotherapy of AcSDKP and tPA initiated at 4 hours and saline groups (Figure 1). The combination therapy did not significantly increase the incidence of gross hemorrhage (1 out of 7, 14%) compared with AcSDKP alone (1 out of 7, 14%) and tPA alone (3 out 8, 38%).

To examine the specificity of AcSDKP therapeutic effect, a neutralizing antibody against AcSDKP was administered along with AcSDKP and tPA 4 hours after MCAO. The behavioral benefits of the combination treatment of AcSDKP and tPA were completely abolished by this neutralizing antibody compared with ischemic rats treated with a control antibody against rat IgG (Figure 1). These data suggest that AcSDKP likely contributes to the observed therapeutic effect of the combination treatment.

MRI measurements were performed to assess evolution noninvasively of the ischemic lesion in rats treated with the combination therapy of AcSDKP and tPA or monotherapy of tPA, starting at 4 hours of MCAO. MRI analysis revealed that at 1 hour of MCAO, all rats exhibited ischemic lesion within the territory supplied by the middle cerebral artery as measured by diffusion-weighted imaging (Figure 2). Ischemic lesion in rat treated with tPA alone increased during 144 hours (Figure 2). In contrast, ischemic rats treated with the combination of AcSDKP and tPA yielded a significantly reduced lesion over the same period compared with tPA alone (Figure 2). Together, these data indicate that the combination therapy has the neuroprotective effect on acute stroke.

To examine whether AcSDKP passes the blood–brain barrier (BBB), we measured AcSDKP levels in plasma and CSF by means of enzyme immunoassay. Normal or ischemic rats treated with AcSDKP alone, or the combination of AcSDKP and tPA at 4 hours after MCAO, exhibited significant elevation of AcSDKP levels in plasma and CSF 24 hours after the initiated treatment (Figure 3). The neutralizing antibody against AcSDKP completely suppressed augmentation of AcSDKP levels in rats subjected to the combination treatment (Figure 3). These data indicate that AcSDKP can pass the BBB.

**Effects of Treatments on Thrombosis and Vascular Permeability**

In addition to its thrombolytic effect, tPA induces an impairment of the BBB, especially when tPA is given beyond a therapeutic window.\(^{11,17}\) We, thus, examined the effect of the
adjuvant treatment with AcSDKP and tPA on BBB integrity and cerebral microvascular patency. Monotherapy of tPA 4 hours after MCAO significantly increased thrombosis in cerebral microvessels and BBB leakage measured by microvascular platelet accumulation and parenchymal fibrin deposition, respectively, compared with the saline treatment (Figure 4). However, the adjuvant treatment with AcSDKP completely blocked microvascular thrombosis and BBB leakage induced by tPA and stroke (Figure 4). These data suggest that improvement of microvascular patency and integrity by AcSDKP likely results in reduction of infarct volume and consequently leads to a decrease of neurological deficits.

Effects of Treatments on TGF-β1, NF-κB, and PAI-1 Expression

We previously demonstrated that AcSDKP inactivated the TGF-β1/phospho-Smad 2 (p-Smad 2) signaling in vascular fibrosis, observed in the experimental hypertension and myocardial infarction.7,18 Our immunohistochemistry data showed that stroke induced TGF-β1 and p-Smad 2/3 immunoreactive vessels 24 hours after MCAO (Figure 5). Compared with the saline treatment, the combination of AcSDKP and tPA significantly reduced the densities of TGF-β1 and p-Smad 2/3 immunoreactive vessels, whereas tPA monotherapy failed to reduce the densities of TGF-β1 and p-Smad 2/3 immunoreactive vessels (Figure 5). In addition, the combination treatment resulted in a significant decrease in TGF-β1 immunoreactive vessels, and a trend toward reduction in p-Smad 2/3 immunoreactive vessels compared with the tPA monotherapy (Figure 5).

Activation of TGF-β1 upregulates PAI-1.19 Double immunostaining showed that stroke considerably induced PAI-1 immunoreactive vessels, and monotherapy with tPA further increased the PAI-1 immunoreactive vessel density compared with saline-treated rats 24 hours after MCAO (Figure 5). However, adjuvant treatment with AcSDKP completely restrained the densities of PAI-1 immunoreactive vessels evoked by stroke and tPA (Figure 5).

NF-κB modulates cerebral vascular patency and integrity.20 We found that stroke substantially induced the active form of NF-κB, p65 immunoreactivity in vessels within the ipsilateral hemisphere, and monotherapy of tPA augmented p65 immunoreactive vessels 24 hours after MCAO, which was completely suppressed by the combination therapy of AcSDKP and tPA (Figure 5). Collectively, these in vivo data suggest that AcSDKP suppresses TGF-β1/p-Smad 2/3 and NF-κB signals in cerebral vessels triggered by stroke and tPA.

To examine whether AcSDKP acts directly on cerebral endothelial cells, HBECs were treated with fibrin in the

Figure 5. Cerebrovascular transforming growth factor β (TGF-β1), p65, and plasminogen activator inhibitor 1 (PAI-1) expression. A shows immunoreactivity of TGF-β1 in a representative normal, ischemic rats treated with tissue plasminogen activator (tPA) monotherapy, and ischemic rats treated with the combination of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) and tPA. B shows colocalization of p-Smad 2/3 (red) with TGF-β1 immunoreactive vessels (green) in a representative ischemic rat treated with saline. C shows double immunofluorescent staining of PAI-1 (green) with endothelial barrier antigen (EBA, red) from a representative normal rat, and in ischemic rats treated with saline, tPA, or AcSDKP+tPA. D shows p65 immunoreactivities from a representative normal rat, and in ischemic rats treated with saline, tPA, or AcSDKP+tPA. Bar graphs show the quantitative data of TGF-β1, p-Smad 2/3, PAI-1, and p65 immunoreactive vessels in the ischemic brain, respectively. Bars=50 μm.
The present study, for the first time, demonstrates that administration of AcSDKP initiated 1 hour after MCAO or combination of AcSDKP and tPA given 4 hours after stroke onset substantially reduced infarct volume and neurological deficits. Improved cerebral microvascular patency and integrity by AcSDKP likely contribute to the observed neuroprotective effect.

Treatment of acute stroke requires rapid restitution of cerebral blood flow in the ischemic cerebral microvascular bed, preserving BBB integrity, and minimizing ischemic cell death.1 Thrombolysis with tPA restores blood flow through its desirable fibrinolytic action. However, delayed tPA treatment may adversely exacerbate stroke-induced neurovascular dysfunction via aggravating the prothrombotic cascade, inflammatory response, and BBB disruption.23 The present study demonstrated that AcSDKP potently blocked secondary thrombus formation and BBB leakage, which leads to neuroprotective effects when treatment is initiated acutely (1 hour) after stroke onset. More importantly, we found that adjuvant treatment with AcSDKP completely blocked tPA-aggravated secondary thrombus formation and BBB leakage, and concomitantly blocked the lesion expansion in the ischemic rats. Elevation of plasma AcSDKP level has been observed in patients treated with ACE inhibitors, which is well known to maintain the hemostatic balance of fibrinolytic and procoagulant factors, and to attenuate inflammatory and fibrotic responses in patients with cardiovascular disease and stroke.21,22 Collectively, these data suggest that AcSDKP has therapeutic potential for treatment of patients with acute stroke.

After ischemic insult, the cerebral endothelial cells rapidly converted into a prothrombotic and proinflammatory state, which leads to a neurovascular dysfunction that aggravates the progression of ischemic brain damage.1 In the present study, we found that stroke-induced neurovascular disruption was associated with upregulation of cerebrovascular TGF-β1, PAI-1, and NF-κB expression, all of which play important roles in regulating thrombosis and inflammatory responses after stroke. TGF-β1 is a multipotent cytokine involved in cell growth, matrix protein synthesis, and inflammation. The activation of TGF-β1 induces the expression of PAI-1, a major inhibitor of fibrinolysis derived from endothelial cells, and modulates vascular barrier function via promoting matrix metalloprotease production.19 In addition, the activation of NF-κB signaling evokes the transcription of proinflammatory genes, which has a direct effect on modulating neurovascular homeostasis after stroke.20 Thus, the activation of cerebrovascular TGF-β1 and NF-κB signaling by stroke likely trigger secondary thrombosis, inflammation, and BBB disruption. In the present study, we found that administration of AcSDKP substantially reduced cerebrovascular TGF-β1 and NF-κB signals elicited by stroke and tPA, which were closely associated with decreases of thrombosis and BBB leakage. Moreover, our in vitro data indicate that AcSDKP suppresses TGF-β1 and NF-κB signals in cerebral endothelial cells, which are consistent with published studies showing that AcSDKP treatment protects against renal and cardiac damage via inhibiting TGF-β1 and NF-κB–mediated inflammatory responses and fibrosis.21 Therefore, suppression of cerebrovascular TGF-β1 and NF-κB signals by AcSDKP may be one of the mechanisms underlying the neuroprotective effects of AcSDKP on acute stroke.

Alternatively, AcSDKP may confer the neuroprotection by directly acting on parenchymal neural cells. In the present study, we found that the CSF and plasma AcSDKP remain at basal levels 24 hours after stroke onset, suggesting that stroke does not affect endogenous AcSDKP level. However, systemic administration of AcSDKP was accompanied with the elevation of CSF AcSDKP level in the ischemic rats, indicating that AcSDKP can readily cross the BBB. Although the biological role of endogenous and exogenous AcSDKP in the central nervous system is not clear, neuroprotective and neurorestorative activities of Tβ4, the putative precursor of AcSDKP, have been previously reported.22,23 In vitro, the application of Tβ4 protects against excitotoxicity-induced neuronal death.24 In experimental stroke and traumatic brain injury (TBI), Tβ4 treatment leads...
to neuroprotection, neurorestoration, and improvement of neu-
rofunctional recovery.25 These findings raise a possibility
that AcSDKP acts on multiple cellular targets within the
neurovascular unit, and subsequently exerts beneficial effects
in the treatment of experimental stroke. Additional studies are
warranted to investigate the effects of exogenous AcSDKP on
neuronal damage and the biological functions of endogenous
AcSDKP in the brain.

Several characteristics of AcSDKP indicate that this tet-
rapeptide is a promising neuroprotective agent for ischemic stroke, and therefore merits further preclinical
development and evaluation. First, as a naturally occurring
peptide, the pharmacokinetics and metabolism of AcSDKP
have been well established, and there is no apparent toxicity
in rodents. Second, although the BBB poses significant chal-
lenes to the permeation of neuroprotective agents, AcSDKP
can readily cross the BBB in the intact and ischemic brain.
More importantly, with the identification of multiple molecu-
lar mechanisms and mediators on the pathogenesis of stroke,
the multitargeted effects of AcSDKP on the neurovascular unit
could achieve optimized therapeutic efficacy. Furthermore,
with increased use of thrombolytic therapy in patients with
acute stroke, the safety and efficacy of the combination treat-
ment observed in the present study should encourage further
efforts to develop clinical trials of AcSDKP in combination
with tPA for the treatment of acute stroke.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Supplemental Data:

Animal model: Rats were subjected to embolic middle cerebral artery occlusion (MCAO). Briefly, the right common carotid arteries (CCA), the right external carotid artery (ECA) and the internal carotid artery (ICA) were exposed via a midline incision. A modified PE-50 catheter containing a single fibrin rich clot was gently advanced from the ECA into the ICA until its tip positioned at the origin of MCA. The clot was then injected through the catheter. The catheter was withdrawn immediately after injection.

Behavioral tests:

Longa’s five point scale was used for acute assessment of neurological deficit 30 minutes after MCAO. 0 = no deficit, 1 = failure to extend the left forepaw fully, 2 = circling to the left, 3 = falling to the left side, and 4 = unable to walk spontaneously. Based on our experience, rats with a score 0 generally do not have an ischemic lesion, whereas rats with scores 1 to 3 usually have an ischemic lesion within the territory supplied by the middle cerebral artery. Ischemic rats with a score greater than 3 generally have a lethal stroke, and thus, were excluded from the present study. Thus, rats with a score between 1 and 3 were randomized into treatment groups.

A battery of behavioral tests to detect sensorimotor deficits was performed 1 and 7 days after onset of MCAO by an observer blinded to the treatments.

Adhesive removal test: An adhesive removal test was employed to measure somatosensory deficits. Briefly, 2 pieces of adhesive-backed paper dots (113.1 mm²) were placed on the wrist of each forelimb. The mean time (seconds) required to remove
stimuli from the left limb was recorded. All rats were given three trials per testing day with a **cut-off time** of 120 seconds.

**Foot-fault test:** A modified foot-fault test was employed to measure forelimb placement dysfunction. The total number of steps (movement of each forelimb) that the rat used to cross the grid and the total numbers of foot faults for left forelimb were recorded.

**Modified neurological severity score (mNSS):** Rats were tested for motor, sensory, reflex, and balance dysfunctions with the mNSS. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

**Immunohistochemistry analysis:** To detect fibrin/fibrinogen accumulation, double immunofluorescent staining was performed with anti-fibrinogen/fibrin mAb (1:1000, Accurate Chemical & Scientific) and anti-endothelial barrier antigen mAb (EBA, Covance; 1:1000). For evaluation of microvascular plasminogen activator inhibitor 1 (PAI-1), double immunofluorescent staining was performed with anti-PAI-1 mAb (1:500, Santa Cruz) and anti-EBA mAb. To examine platelet accumulation, a polyclonal antibody against rat thrombocyte (Inter-Cell Technologies) was used at a titer of 1:4000. For the detection of activated NF-κB, an anti-p65 mAb (RelA, Chemicon), was used at a titer of 1:150. For evaluation of transforming growth factor • 1 (TGF•1) expression, a polyclonal anti-TGF•1 (Santa Cruz) was used at a titer of 1:500. For quantification, the numbers of immunoreactive vessels were counted throughout the territory supplied by the right MCA and data are presented as the density of immunoreactive vessels relative to the imaged area (mm²).