Neuroprotective Role of Transgenic PAF-Acetylhydrolase II in Mouse Models of Focal Cerebral Ischemia

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Background and Purpose—Platelet-activating factor (PAF) and oxidized unsaturated free fatty acids have been postulated to aggravate neuronal damage in the postischemic brain. Type II PAF-acetylhydrolase (PAF-AH II) not only terminates signals by PAF by its PAF-hydrolyzing activity but also protects cells against oxidative stress. We examined whether PAF-AH II can rescue cerebral neurons against ischemic insults.

Methods—Transgenic mice overexpressing human PAF-AH II in neurons were generated and enzyme expressions were examined biochemically and histochemically. The mice were subjected to 60 minutes of transient middle cerebral artery occlusion followed by reperfusion for 24 hours. The infarction and apoptosis were estimated by TTC staining and fluorescence TUNEL staining, respectively.

Results—Overexpression of PAF-AH II was found in brains of transgenic mice by Western blot and enzymatic activity analyses. In immunohistochemistry, human PAF-AH II expression was found throughout the central nervous system, especially in neurons of neocortex, hippocampus, and basal ganglia. The neurological deficit scores, cerebral edema index, and relative infarction volume were all significantly (P<0.05) lower in transgenic mice (1.30±0.72, 1.12±0.04, and 14.0±7.7%, respectively) than in wild-type mice (2.56±0.93, 1.23±0.12, and 31.9±9.7%, respectively). Percentages of apoptotic cells were also significantly (P<0.001) lower in transgenic mice (cortex, 5.2±3.3%; hippocampus, 3.4±7.0%) than in wild-type mice (cortex, 41.1±16.9%; hippocampus, 58.9±15.3%).

Conclusions—These results indicate that PAF-AH II exerts strong neuroprotective effects against ischemic injury and suggest a possibility for clinical use of this enzyme in cerebral ischemia. (Stroke. 2007;38:1063-1068.)

Key Words: apoptosis • focal ischemia • neuroprotection • PAF-acetylhydrolase • transgenic mice

The platelet-activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) was originally isolated from immunoglobulin E-stimulated basophils as a potent lipid mediator.1,2 PAF is involved in many pathological processes, such as allergy, inflammation, shock, trauma, and ischemia.3 PAF is produced not only by basophils but also by a variety of cells, including neuronal cells.4 The role of PAF in ischemic injury in the central nervous system (CNS) has been initially proposed based on the results showing that selective PAF receptor antagonists prevented ischemia-induced CNS damage in animal models.5 Until now, many researchers have reported that PAF contributes to the aggravation of neuronal damage in the postischemic brain as well as in the posttraumatic brain.6 The acetyl group at the sn-2 position of glycerol backbone in the PAF molecule is essential for its biological activity. PAF is rapidly inactivated by deacylation reaction both in vivo and in vitro. This deacylation reaction is catalyzed by PAF-acetylhydrolase (PAF-AH).7 Until now, 3 types of PAF-AH, intracellular type I, type II, and plasma-type PAF-AHs, were found in mammals. Type I PAF-AH is a trimeric enzyme consisting of 2 catalytic subunits and a regulatory subunit.7 The regulatory subunit of type I PAF-AH is a product of the LIS1 gene, which is the causative gene for Miller–Dieker lissencephaly.8 Type II PAF-AH is a monomeric enzyme of 40 kDa that has significant homology to plasma-type PAF-AH.9 Both monomeric enzymes hydrolyze not only PAF but also short-chain phospholipid and oxidized...
fragment of polyunsaturated fatty acid at the sn-2 position and, by doing so, terminate signals by PAF and attenuate the toxicity induced by oxidized PAF-like lipids.\textsuperscript{10}

In fact, we previously reported that the transfection of the plasma-type PAF-AH gene attenuates glutamate-induced apoptosis in cultured rat cortical neurons.\textsuperscript{11} We also reported that the overexpression of PAF-AH II in Chinese hamster ovary K1 cells suppresses the tert-butylhydroperoxide-induced apoptotic cell death, most likely by its antioxidant effects.\textsuperscript{12} These findings indicate that the overexpression of PAF-AH II in neurons may protect CNS neurons against ischemic damage by terminating signals by PAF and oxidized PAF-like lipids. In the present study, we generated transgenic (TG) mice overexpressing human PAF-AH II in CNS neurons and investigated the effect of PAF-AH II on the size and histology of the infarct.

Materials and Methods

Generation of PAF-AH II Transgenic Mice

The 4.50-kbp EcoRI-XhoI genomic DNA fragment of Thy-1.2 gene\textsuperscript{13} was cloned from mouse embryonic stem cell DNA library. The 1.2-kbp human PAF-AH II cDNA\textsuperscript{9} (nucleotide number 188-1406; Genbank accession No. NM000437) was ligated between Thy-1.2 promoter and SV40 polyadenylation signal sequence. The resultant hybrid gene (7.3 kbp), separated from the pBlueScript SK- (Stratagene) by EcoRI, was microinjected into the male pronuclei of fertilized eggs from BDF1 females. The identification of TG mice was performed by polymerase chain reaction on mouse genomic DNA; the sequences of the primers for transgene amplification were 5'-ACCGGTAAGCTTGAGCCGTGATGAAATG-3' and 5'-GGGCTCTAGACTTTACAAATGGCCAGT-3'. Polymerase chain reaction was performed in thermal cycler (BioRad) for 35 cycles with the following parameters: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 90 seconds. The polymerase chain reaction products were resolved on 1.5% agarose gel and visualized by ethidium bromide staining. Heterozygous TG mice were mated to C57/black 6j mice >3 times and analyzed. All animals were maintained and subjected for analyses in the mouse room at the Department of Biochemistry, University of Toyama Faculty of Medicine, Toyama, Japan. The procedures were approved by the Institutional Animal Care and Use Committee guidelines at University of Toyama.

Western Blot Analysis and Enzyme Assays

The cerebrum, cerebellum, and spinal cord were homogenized with a potter-type homogenizer in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 250 mmol/L sucrose, pepstatin A (5 \(\mu\)g/mL), antipain (5 \(\mu\)g/mL), leupeptin (5 \(\mu\)g/mL), and 0.25 mmol/L PMSF. Western blotting for PAF-AH II was performed as described\textsuperscript{14} on 40 \(\mu\)g protein using mouse anti-human PAF-AH II monoclonal antibody (1:500) that had been established previously.\textsuperscript{15} Enzymatic activities of PAF-AH were determined as described\textsuperscript{16} using 100 000g supernatant fraction of the homogenates.

Immunohistochemistry and Detection of Apoptosis

Mice were perfused with cold phosphate-buffered saline under anesthesia with sodium pentobarbital (intraperitoneal injection, 50 mg/kg body weight). After immersion fixation with 4% paraformaldehyde overnight, tissues were embedded in paraffin, and 10-\(\mu\)m-thick tissue coronal sections were glass-mounted. Immunohistochemistry was performed by using an amino acid polymer detection system (Histofine Mouse Stain Kit; Nichirei). The sections were incubated at 4°C overnight with mouse monoclonal antibody to human PAF-AH II\textsuperscript{16} (1:100). After washing, the immunoreactive product was visualized by diaminobenzidine solution and counterstained with hematoxylin. For detection of apoptosis, paraffin-embedded sections from mice killed at 24 hours after reperfusion were examined for TUNEL reactivity, using the DeadEnd Fluorometric TUNEL System (Promega) according to manufacturer’s recommendation. The final sections were observed with a fluorescence microscope (AX80; Olympus) and the green fluorescence positive cells were judged to be apoptotic.

Focal Cerebral Ischemia and Laser Doppler Flowmetry

Adult male mice (age, 12 to 24 weeks; weight, 30 to 35 g) were subjected to transient occlusion of the middle cerebral artery (MCA) by an intraluminal suture method as described.\textsuperscript{17} Cerebral blood flow was monitored by laser Doppler flowmetry (ALF21; ADVANCE) throughout the experiment. The probe (ALF probe; ADVANCE) was fixed to the skull overlying the territory supplied by the MCA. An interruption of the cerebral blood flow in the MCA territory was
confirmed by documenting a >80% decline in relative cerebral blood flow. A return to >50% of basal cerebral blood flow within 10 minutes of suture withdrawal confirmed a reperfusion of the MCA territory. Animals that did not meet both ischemic and reperfusion flow criteria were excluded from the study. At 24 hours after reperfusion onset, neurological deficits were scored as described.18

Evaluation of Cerebral Edema and Infarction
The brains were dissected and coronal slices (2 mm in thickness) were prepared from the frozen forebrain by using the mouse Brain Matrix (Braintree Scientific). Brain slices were then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) at 37°C for 15 minutes. The areas of right hemisphere, left hemisphere and infarction were respectively quantitated by an NIH image public domain software (version 1.63) at each slice. The volumes of right hemisphere, left hemisphere, and infarction were calculated according to the slice thickness of 2 mm per slice and the infarct volume was corrected for swelling as described by Doerfler et al.19 A cerebral edema index was generated from the ratio of the left-to-right hemispheric volume. The relative infarction volume was expressed as percentage of the corrected infarct volume in the ipsilateral hemispheric volume. The investigators who performed the image analyses were blinded to the study groups.

Statistical Analyses
Values are expressed as mean±SD. Neurological deficit scores were evaluated for statistical significance by the Mann-Whitney U analysis. The other values were analyzed by a Student t test. Significance was assumed at P=0.05 in all statistical analyses.

Results
To generate the TG mice that express human PAF-AH II, human PAF-AH II cDNA was ligated to the mouse Thy-1.2 promoter (Figure 1A), which drives high expression of the transgene in most neurons in the CNS.13 By microinjection into fertilized eggs, we established 4 independent lines of TG mice for human PAF-AH II. In Western blot analysis using anti-human PAF-AH II monoclonal antibody, 3 lines of TG mice (lines 94, 186, and 190) highly overexpressed human PAF-AH II in the brain (Figure 1B). We next evaluated PAF-AH II expression in cerebrum, cerebellum, and spinal cord in the line 94. The human PAF-AH II was expressed not only in cerebrum but also in cerebellum and spinal cord (Figure 1C). Next, we determined the PAF-AH II activities in the 100 000g supernatant fraction of the brain homogenates. The PAF-AH II activities in the homogenates of the TG mice (lines 94, 186, and 190) were increased compared with the wild-type (WT) mice (Figure 1D), indicating that the transgene-derived PAF-AH II is enzymatically active.

To examine the transgene-derived human PAF-AH II expression in the TG brain, we performed immunohistochemistry using anti-human PAF-AH II monoclonal antibody. In the TG mice, human PAF-AH II immunoreactivities were observed widespread throughout the brain, with higher levels in the cortex, hippocampus, and basal ganglia (Figure 2B). Under higher magnification, immunopositive neurons showed basically diffuse and sometimes granular staining patterns within neuronal cell bodies of the TG mice (Figure 2F, 2G, and 2H). The immunoreactivities were essentially positive in neurons but not in glial cells. In the brain of WT mice, there were very weak immunoreactivities (Figure 2A, 2C, 2D, and 2E).

Reduced Neurological Deficit in Transgenic Mice After Focal Cerebral Ischemia and Reperfusion

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<tr>
<th>Animal Group</th>
<th>Neurological Deficit Score</th>
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<td>0</td>
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<tr>
<td>WT (n=27)</td>
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<td>TG (n=27)</td>
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Twenty-four hours after reperfusion onset, neurological deficits were scored according to the following criteria: 0, normal motor function; 1, flexion of contralateral torso and forelimb upon lifting to the whole animal by the tail; 2, circling to the contralateral side but normal posture at rest; 3, leaning to the contralateral side at rest; 4, no spontaneous motor activity. The WT and TG mice were tested for neurological deficits by investigators blinded to the experiment.

*P<0.001 using ANOVA followed by Mann–Whitney U analysis.
Transient focal cerebral ischemia was performed on mice by intraluminal occlusion of the left MCA, as previously described.17 The decrease in cerebral blood flow at the MCA territory was not significantly different between WT and TG mice (data not shown). The neurological deficits were scored at 24 hours after ischemia (Table). The neurological deficit scores were significantly (P<0.001) lower in the TG mice (1.30±0.72, n=27) when compared with their WT littermates (2.56±0.93, n=27). Next, infarct sizes at the level of coronal sections 2, 4, and 6 mm distant from the frontal pole (Figure 3A) were determined by TTC staining. The infarction in the WT mice was large, and almost the entire MCA territory appeared infarcted (Figure 3B, 3C, and 3D). In contrast, the infarction in the TG mice was small and limited to the area corresponding to the striatum (Figure 3, 3E through 3J). As shown in Figure 3K, the edema index was significantly (P<0.05) lower in the TG mice (1.12±0.04, n=7) than in the WT mice (1.23±0.12, n=8). As shown in Figure 3L, the relative infarction volume was also significantly (P<0.01) lower in the TG mice (14.0±7.7%, n=7) than in the WT mice (31.9±9.7%, n=8).

We next evaluated the morphology of neurons by hematoxylin-eosin staining (Figure 4A and 4B). Under higher magnifications, the cortical as well as the hippocampal neurons in the WT mice showed drastic changes, with large irregular areas of spongiosis and neuronal shrinkage (Figure 4C and 4D). In contrast, the neurons in the respective portions were well-preserved in the TG mice (Figure 4E and 4F). Next, we examined the apoptotic reactions at 24 hours after ischemia by fluorescence TUNEL staining. Many TUNEL-positive cells were observed in the cortex and hippocampus of the WT mice.

![Figure 3. Edema index and relative infarction volume in the WT and TG mice after 60-minute MCA occlusion and 24-hour reperfusion. Brains were sectioned with 2 mm thickness (A) and stained with TTC (B through J). Distances from the frontal pole were 2 mm (B, E, and H), 4 mm (C, F, and I), and 6 mm (D, G, and J). Shown are photographs of consecutive sections from one of the WT mice (B, C, and D). Consecutive sections from Tg mouse 1 (E, F, and G) and Tg mouse 2 (H, I, and J). The cerebral edema index (K) as well as the relative infarction volume (L) were significantly lower in the Tg mice (n=7) when compared with WT mice (n=8). *P<0.05 and **P<0.01 compared with WT mice, using Student t test.](http://stroke.ahajournals.org/issue/5/3/1066/S9671959.Figure3.3w.png)
Figure 4. Hematoxylin-eosin staining of ipsilateral coronal sections after 24 hours of reperfusion in the WT mouse (A) and the TG mouse (B). Under higher magnifications, the cortical (C) and hippocampal (D) neurons of WT mice showed large irregular areas of spongiosis and neuronal shrinkage. In contrast, the cortical (E) and hippocampal (F) neurons of TG mice were well preserved. In TUNEL analyses after 24 hours of reperfusion, the TUNEL-positive apoptotic cells showed green fluorescence in the cortex (G) and hippocampus (H) of the WT mouse. TUNEL-positive cells were barely seen in the cortex (I) and hippocampus (J) of the TG mouse. In G through J, cells were counterstained with DAPI (blue).

Discussion

The involvement of PAF in the development of various CNS disorders, such as cerebral ischemia, brain injury, subarachnoid hemorrhage, inflammation, panic disorders, and spinal cord ischemia, has been demonstrated previously. Because selective PAF receptor antagonists ameliorated the brain damage after cerebral infarction, activation of the PAF-PAF receptor signaling system appears to play a critical role in the occurrence and progression of neuronal injury induced by cerebral ischemia. Because previous reports indicate that PAF-AH terminates signals by PAF through its PAF-hydrolyzing activity, overexpression of PAF-AH II in neurons can theoretically have beneficial effects on CNS neurons against ischemic insults. In fact, the plasma-type PAF-AH, an enzyme that has a similar biochemical activity in comparison with PAF-AH II, prevented myocardial ischemia-reperfusion injury, as well as lung reperfusion injury.

In the present study, we have established novel TG mice overexpressing human PAF-AH II in CNS neurons and examined the hypothesis. Western blot analyses and enzymic activity assays confirmed the overexpression of PAF-AH II in CNS (Figure 1), and the immunohistochemistry showed cytosolic staining pattern of human PAF-AH II in neurons of cerebral cortex, hippocampus, and basal ganglia (Figure 2). The amino acid sequence of PAF-AH II does not show any significant similarity to any subunit of intracellular type I PAF-AH, and until now there is no evidence that PAF-AH II is involved in cortical development. In fact, we did not detect structural abnormalities in the cortical layers of the transgenic brain (Figure 2B). After ischemia/reperfusion, the cerebral edema as well as infarction volume were significantly reduced in the TG mice in comparison with the WT mice (Figure 3). The histopathological analyses showed that the neurons of TG mice but not of WT mice were well-preserved (Figure 4). Furthermore, the neurological deficits were significantly reduced in TG mice in comparison with WT mice (Table). These results clearly show that PAF-AH II exerts strong neuroprotective effects against ischemic injury in the CNS and that the silencing of the PAF–PAF receptor signaling system is indeed effective in preventing neuronal injury induced by ischemia.

During cerebral ischemia, neuronal injury is induced by a deprivation of oxygen and glucose. Until now, various mechanisms by which cerebral ischemia induce neuronal damages have been proposed. These include glutamate excitotoxicity, radical-mediated oxidative damage of lipid membranes, energy
failure resulting from an inhibition of energy-producing enzymes such as aconitate, and depletion of nicotinamide-adenine dinucleotide by poly(ADP-ribose) synthase activation.24 Furthermore, reoxygenation by reperfusion provides oxygen as a substrate for enzymatic oxidation reactions and, by doing so, dramatically changes the redox status of postischemic brain. The signaling pathways of programmed cell death are then activated, leading to neuronal death.24 Reactive oxygen species are especially thought to play a central role in the development and aggravation of ischemic damages.

Produced in the course of stroke, reactive oxygen species oxidize unsaturated fatty acyl chains in membrane phospholipids and produce toxic phospholipids hydroperoxides and a variety of oxidized phospholipids, all of which can cause various cell damage, leading to apoptosis and/or necrosis.24 The peroxidized and oxidized phospholipids should therefore be promptly hydrolyzed to prevent neuronal damage. We previously reported that PAF-AH II has the ability to hydrolyze short chain phospholipids and oxidized fragments of polynsaturated fatty acids at the sn-2 position,16 and it protects against oxidative stress when overexpressed in Chinese hamster ovary-K1 cells.13 It is possible that the transgene-derived intracellular PAF-AH II acted as a scavenger of oxidized phospholipids in neurons that were exposed to ischemic insults, thereby reducing neuronal injury after focal cerebral ischemia. In fact, high levels of PAF-AH enzymatic activity were maintained in the postischemic brains (data not shown). However, further experiments are needed to clarify this point.

It should be noted that PAF-AH II was overexpressed not only in the brain but also in the spinal cord (Figure 1C). Until now, mouse models of spinal cord diseases such as spinal cord injury,25 spinal cord ischemia,26 and amyotrophic lateral sclerosis27 have been developed. By applying these disease models to our transgenic mice, it is possible to study the effect of overexpressed PAF-AH II against various spinal cord diseases.

The present results, which clearly showed the protective effects of PAF-AH II overexpression in neurons, indicate that the delivery of recombinant PAF-AH II to postischemic neurons has a therapeutic significance to prevent neuronal death after transient ischemia, and such experiments are now underway in our laboratory.

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Disclosures
None.

References
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