Intense Correlation Between Brain Infarction and Protein-Conjugated Acrolein

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Background and Purpose—We recently found that increases in plasma levels of protein-conjugated acrolein and polyamine oxidases, enzymes that produce acrolein, are good markers for stroke. The aim of this study was to determine whether the level of protein-conjugated acrolein is increased and levels of spermine and spermidine, the substrates of acrolein production, are decreased at the locus of infarction.

Methods—A unilateral infarction was induced in mouse brain by photoinduction after injection of Rose Bengal. The volume of the infarction was analyzed using the public domain National Institutes of Health image program. The level of protein-conjugated acrolein at the locus of infarction and in plasma was measured by Western blotting and enzyme-linked immunoabsorbent assay, respectively. The levels of polyamines at the locus of infarction and in plasma were measured by high-performance liquid chromatography.

Results—The level of protein-conjugated acrolein was greatly increased, and levels of spermine and spermidine were decreased at the locus of infarction at 24 hours after the induction of stroke. The size of infarction was significantly decreased by N-acetylcysteine, a scavenger of acrolein. It was also found that the increases in the protein-conjugated acrolein, polyamines, and polyamine oxidases in plasma were observed after the induction of stroke.

Conclusions—The results indicate that the induction of infarction is well correlated with the increase in protein-conjugated acrolein at the locus of infarction and in plasma. (Stroke. 2009;40:3356-3361.)

Key Words: acrolein ■ brain infarction ■ neuroprotective agents ■ polyamines ■ risk factors

Brain stroke is a serious pathology. However, there is a lack of good biomarkers for the early phase of stroke. We recently found that increased levels of protein-conjugated acrolein (PC-Acro) and the enzymes responsible for its production, polyamine oxidases (spermine oxidase and acetylpolyamine oxidase), are good biomarkers for human stroke. We also found that measurement of PC-Acro together with interleukin-6 and C-reactive protein makes it possible to identify small infarctions, that is, silent brain infarction, with high sensitivity (89%) and specificity (91%). This tool for early identification of stroke may help in the application of suitable therapy to delay or reduce aggravation of stroke.

It is thought that cell damage is mainly caused by reactive oxygen species (ROS) such as superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (•OH). However, when we compared the toxicity of acrolein (CH$_2$=CHCHO) and ROS, we found that acrolein was more toxic than H$_2$O$_2$ and slightly more toxic than •OH in cell culture systems. Furthermore, acrolein is thought to be produced by lipid peroxidation, but we found that it was more effectively produced from 2 polyamines (spermine and spermidine), which are abundant and essential for cell growth in eukaryotic cells. Acrolein is spontaneously formed from 3-acetamidopropanal (CH$_3$CONH[CH$_2$]$_2$CHO) produced from spermine by spermine oxidase (SMO) and acetylpolyamine oxidase (AcPAO). In this report, we examined whether acrolein is indeed produced from spermine and spermidine at the locus of infarction using photo-induced thrombosis model mice and found that PC-Acro is increased together with a decrease in spermine and spermidine during infarction.

Materials and Methods

Photochemically Induced Thrombosis Model Mice
All animal experiments were approved by the Institutional Animal Care and Use Committee of Chiba University and carried out according to the Guidelines for Animal Research of Chiba University. Male C57BL/6 mice (7-week-old) were purchased from Japan SLC Inc (Hamamatsu, Japan). Eight-week-old mice weighing 22 to 26 g were anesthetized with inhalation of 3% isoflurane (Abbott

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Japan). Anesthesia was continued with 1.5% isoflurane during operation, and body temperature was kept at 37°C with a heating pad. The thrombotic occlusion of the middle cerebral artery was induced by the photochemical reaction: an incision was made between the left orbit and the external auditory canal, and the temporalis muscle was detached from dura mater to expose the proximal section of the middle cerebral artery. Immediately after intravenous injection of photosensitizer, Rose Bengal (20 mg/kg), through a jugular vein, green light (wavelength: 540 nm) emitted from a xenon lamp (Hamamatsu Photonics Japan) illuminated the middle cerebral artery for 10 minutes. After middle cerebral artery occlusion, incised skin was restored. At 24 hours after the induction of photochemically induced thrombosis (PIT) stroke, the brain was removed and sectioned into 2-mm thick coronal slices. Each slice was incubated with 5% triphenyltetrazolium chloride solution at 37°C for 30 minutes. Volume of infarction was analyzed on a Macintosh computer using the National Institutes of Health image program. Where indicated, N-acetylcysteine (250 mg/kg) in phosphate-buffered saline was injected intraperitoneally at 0, 3, and 6 hours after induction of infarction. Experiments were performed using 10 mice in each group.

### Measurement of PC-Acro and Polyamines

Brain tissues at the locus of infarction of the PIT model mice and at the same locus of control mice were homogenized using an Ultra-Turrax homogenizer in 0.5 mL of buffer A containing 10 mmol/L Tris/HCl (pH 7.5), 1 mmol/L dithiothreitol, 10% glycerol, 0.2 mmol/L EDTA, and 0.02 mmol/L FUT-175 (6-amino-2-naphthyl-4-guanidinobenzoate), a protease inhibitor. Total proteins (20 μg) were stained with Coomassie Brilliant Blue R-250 after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the level of PC-Acro was measured by Western blotting using 20 μg protein of tissue homogenate and polyclonal antibody against bovine serum albumin-conjugated acrolein (MoBiTec Germany). The level of albumin was similarly measured using antibody against mouse albumin (Bethyl USA). PC-Acro, in which acrolein is converted to N-(3-formyl-3,4-dehydropiperidino-lysine) (FDP-lys) in plasma was measured by the method of Uchida et al using the ACR-LYSINE ADDUCT ELISA system (NOF Corporation) and 0.05 mL plasma. After the reaction was terminated, absorbance at 450 nm was measured by a microplate reader, Bio-Rad Model 550. Polyamines in brain tissues and plasma were extracted with 5% trichloroacetic acid and measured by high-performance liquid chromatography as previously described. Protein was determined by the method of Lowry et al.

### Assays for SSAT, AcPAO, and SMO

SSAT activity in the homogenate was measured as described using 10 μmol/L [1-14C]acetyl-CoA, 3 mmol/L spermidine, and 140 μg protein in a total volume of 0.1 mL. After incubation at 37°C for 10 minutes, radioactivity bound to a cellulose phosphate disc was measured. AcPAO and SMO in the homogenate were measured as described using 0.2 mmol/L acetylspermine or spermine as substrate and 20 μg protein in a total volume of 0.1 mL. After incubation at 37°C for 4 hours (AcPAO) and 20 hours (SMO), polyamines were extracted with 5% trichloroacetic acid and the level of spermidine in the supernatant was measured by high-performance liquid chromatography as described previously.
linearly under these experimental conditions. AcPAO and SMO in plasma were measured by incubating the reaction mixture at 37°C for 48 hours. The reaction mixture (0.1 mL) contained 0.08 mL of plasma. Where indicated, 1 mmol/L aminoguanidine, an inhibitor of diamine oxidase, or 0.25 mmol/L MDL72527, N,N'-bis(2,3-butenadienyl)-1,4-butanediamine, an inhibitor of polyamine oxidases, were added to the reaction mixture.

Statistics
Values are indicated as means ± SE. Data of control and PIT groups were analyzed by Student t test, and a statistical difference was shown by probability values.

Results
Increase in PC-Acro and Decrease in Spermine and Spermidine at the Locus of Infarction
To determine whether acrolein is produced at the locus of brain infarction, a unilateral infarction was induced in mouse brain by photoinduction after injection of Rose Bengal. The volume of the infarction was determined by staining 2-mm thick coronal slices with triphenyltetrazolium. This stains the viable brain tissue red, whereas infarct tissue remains unstained. Under our experimental conditions, the average volume of infarction at 24 hours after photoinduction was 23 mm³ (Figure 1A). Acrolein is formed from spermine and spermidine by SMO, SSAT, and AcPAO (Figure 1B). Because free acrolein is rapidly converted to PC-Acro through its interaction with lysine side chains in proteins (Figure 1C), PC-Acro at the locus of brain infarction was measured by Western blotting using an antibody against FDP-Lys. Ac-Acro (68 kDa) at the locus of the brain infarction was 28-fold higher than that at the same locus of control mice (Figure 1D). The protein was also stained with antibody against albumin (Figure 1C), suggesting that most of PC-Acro is albumin.

We next measured the level of polyamines at the locus of brain infarction in comparison with the level at the same locus of control mice to confirm that acrolein is mainly produced from spermine and spermidine. As shown in Figure 2A, the levels of both spermine and spermidine decreased significantly after the infarction, whereas the level of putrescine increased. The results strongly suggest that acrolein is produced during the conversion of spermine to spermidine and spermidine to putrescine. Enzymatic activities involved in the production of acrolein (SMO, SSAT, and AcPAO) slightly increased after the infarction (Figure 2B). However, the activities were enough to cause the decrease in spermine and spermidine after infarction shown in Figure 2A. The activity of diamine oxidase, which also produces acrolein, was very low in the brain (data not shown), and the size of infarction decreased significantly when 100 mg/kg of MDL72527, an inhibitor of polyamine oxidases, was added. These data support the idea that acrolein is produced from spermine and spermidine by polyamine oxidases.

It has been shown that PC-Acro is increased in plasma together with acrolein-producing enzymes after infarction. Thus, PC-Acro in plasma was measured, and it was found that PC-Acro was significantly higher in PIT model mice than in control mice (Figure 3A). A significant increase in putrescine and spermidine in plasma was also observed in PIT model mice (Figure 3B). SMO and AcPAO activities, which are sensitive to MDL72527, was significantly higher in the plasma of PIT model mice than control mice (Figure 3C). Diamine oxidase, which is able to produce acrolein from polyamines and is sensitive to aminoguanidine, is found in plasma of mice. However, diamine oxidase activity was nearly equal in plasma of both control and PIT mice (Figure 3C). These results suggest that PC-Acro, polyamines, and polyamine oxidases were released from the locus of infarction.

Decrease in PC-Acro and Increase in Polyamines by N-Acetylcysteine at the Locus of Infarction
We found that N-acetylcysteine is a strong acrolein scavenger rather than a ROS scavenger, although it is often considered as a scavenger of ROS, that is, N-acetylcysteine greatly reduced the inhibition of cell growth induced by acrolein, slightly reduced it when induced by ·OH, but not by H₂O₂ in a cell-culture system. Thus, the effects of N-acetylcysteine on brain infarction were examined to confirm the correlation between brain infarction and PC-Acro. At 24 hours after induction of infarction, the average volume of infarction decreased from 23 mm³ to 16 mm³ (Figure 4A), PC-Acro at the locus of infarction greatly decreased (Figure 4B), and polyamine content increased significantly (Figure 4C) by the injection of N-acetylcysteine. Furthermore, PC-Acro in plasma slightly decreased (Figure 4D). The polyamine contents in control mice did not change significantly by the injection of N-acetylcysteine (Figures 2A and 4C). These results, taken together, suggest that acrolein is effectively scavenged by N-acetylcysteine.

Discussion
In this study, we found a striking correlation between brain infarction and the level of PC-Acro at the locus of...
infarction. It has been reported that free acrolein is increased in Alzheimer disease brain, and that another unsaturated aldehyde, 4-hydroxy-2-nonenal (HNE) produced from unsaturated fatty acids at 4 hours after oxidative injury, is correlated with infarct volume at 48 hours, although HNE is less toxic than acrolein. There is also a report that aldehyde dehydrogenase, an acrolein and HNE degrading enzyme, functions in protection against oxidative stress. It has been also reported that hydrogen acts as a therapeutic antioxidant of mice. Under these conditions, the decrease in HNE by hydrogen was more distinct than that in oxidized guanine (8-OH-G), which is produced by ROS. Thus, not only ROS, but also unsaturated aldehydes such as acrolein and HNE should be taken into consideration as the factors of cell damage such as brain infarction. These phenomena are probably general among mammals because the data are obtained with mice, rats, and humans.

Figure 3. Increase in PC-Acro, polyamines, and polyamine oxidases in plasma by brain infarction. PC-Acro (A), polyamine content (B), and polyamine oxidases (C) in plasma of 10 mice in each group (control and infarct) were measured as described in “Materials and Methods.” Polyamine oxidase activities in plasma were shown as MDL72527 and aminoguanidine-sensitive activities. Data are shown as mean±SE. *P<0.05; **P<0.01 compared with control mice.

Figure 4. Effect of N-acetylcysteine, an acrolein scavenger, on the size of brain infarction (A), the levels of PC-Acro (B) and polyamines (C) at the locus of infarction, and the level of PC-Acro in plasma (D). Experiments were performed using 10 mice in each group as described in “Materials and Methods.” Saline and NAC, phosphate-buffered saline, or N-acetylcysteine was injected to PIT mice; sham (NAC), N-acetylcysteine was injected to control mice. Data are shown as mean±SE. *P<0.05; **P<0.01; ***P<0.001 compared with control mice.
It has been reported that acrolein can be produced from membrane phospholipids, although the major aldehydes produced during lipid peroxidation are malondialdehyde and HNE.\textsuperscript{25} However, acrolein production from arachidonic acid during lipid peroxidation was very low.\textsuperscript{1} There are reports that 3-aminopropanal, which automatically produces acrolein, is generated from spermine and is strongly involved in cell damage during ischemia in rats.\textsuperscript{26} Accordingly, data obtained thus far strongly suggest that acrolein is mainly produced from spermine and spermidine.

There is a report that SSAT and SMO are induced during kidney ischemia–reperfusion injury in rats.\textsuperscript{27} However, the activity of SSAT, AcPAO, and SMO at the locus of infarction only slightly increased compared with the activity at the same locus of control mice. Some of SMO and AcPAO was released from the locus of infarction to blood during ischemia (Figure 3C). Thus, total AcPAO and SMO may also be induced at the locus of infarction under our experimental conditions.

It is salient to note that the major acrolein-conjugated protein was albumin. Albumin conjugated with acrolein may easily accumulate at the locus of infarction because of its reduced solubility compared with unconjugated albumin. Recently, it has been reported that ischemia-modified albumin levels are high in cerebrovascular diseases.\textsuperscript{28} This albumin is probably conjugated with acrolein.

The size of infarction was decreased by N-acetylcysteine. Although N-acetylcysteine is considered as a scavenger of ROS,\textsuperscript{29} our results indicate that N-acetylcysteine is probably conjugated with acrolein.\textsuperscript{1,2,18,30} The difference of polyamines in plasma between PIT model mice and patients with stroke is that spermidine increased and spermine did not decrease in PIT model mice. This is probably explained as follows: (1) SMO activity in mice brain is high, so that spermidine increased; and (2) the relative size of infarction is big in PIT model mice, so that significant amount of spermine is released from the locus of infarction due to tissue damage.

The results, taken together, support an idea that acrolein is one of the major cellular toxic compounds and mainly produced from polyamine oxidation, and its toxicity is relieved by N-acetylcysteine. Accordingly, severity of renal failure and cerebral stroke is correlated well with the level of protein-conjugated acrolein.\textsuperscript{1,2,18,30}

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### Disclosures

None.

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