Original Contribution

Protective Effects of Brain Infarction by *N*-Acetylcysteine Derivatives

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Background and Purpose—We recently found that acrolein (CH_2=CH-CHO) is more strongly involved in brain infarction compared with reactive oxygen species. In this study, we looked for acrolein scavengers with less side effects.

Methods—Photochemically induced thrombosis model mice were prepared by injection of Rose Bengal. Effects of *N*-acetylcysteine (NAC) derivatives on brain infarction were evaluated using the public domain National Institutes of Health image program.

Results—NAC, NAC ethyl ester, and NAC benzyl ester (150 mg/kg) were administered intraperitoneally at the time of induction of ischemia, or these NAC derivatives (50 mg/kg) were administered 3× at 24-h intervals before induction of ischemia and 1 more administration at the time of induction of ischemia. The size of brain infarction decreased in the order NAC benzyl ester>NAC ethyl ester>NAC in both experimental conditions. Detoxification of acrolein occurred through conjugation of acrolein with glutathione, which was catalyzed by glutathione *S*-transferases, rather than direct conjugation between acrolein and NAC derivatives. The level of glutathione *S*-transferases at the locus of brain infarction was in the order of administration of NAC benzyl ester>NAC ethyl ester>NAC>no NAC derivatives, suggesting that NAC derivatives stabilize glutathione *S*-transferases.

Conclusions—The results indicate that detoxification of acrolein by NAC derivatives is caused through glutathione conjugation with acrolein catalyzed by glutathione *S*-transferases, which can be stabilized by NAC derivatives. This is a new concept of acrolein detoxification by NAC derivatives.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2018;49:00-00. DOI: 10.1161/STROKEAHA.118.021755.)**

Key Words: acetylcysteine ■ acrolein ■ brain glutathione transferase ■ infarction ■ mice

Although brain stroke is a serious disease, there is a lack of reliable biomarkers for the early phase of stroke. It is thought that cell damage is mainly caused by reactive oxygen species consisting of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide, and hydroxyl radical.[1] However, we found that acrolein (CH_2=CH-CHO) produced mainly from spermine ($NH_2[CH_2]_3NH[CH_2]_4NH[CH_2]_3NH_2$)—one of the polyamines, which is essential for cell growth and viability,[2,3] is more toxic than reactive oxygen species.[4–6] Acrolein is spontaneously formed from 3-aminopropanal ($NH_2[CH_2]_2CHO$) produced from spermine by SMO (spermine oxidase) and less effectively from 3-acetamidopropanal ($CH_3CONH[CH_2]_2CHO$) produced from spermine and spermidine ($NH_2[CH_2]_3NH[CH_2]_4NH_2$) by SSAT (spermidine/spermine N^1-acetyltransferase) and AcPAO (acetylpolyamine oxidase).[7,8]

Accordingly, we studied whether acrolein is a biomarker for brain stroke. We found that increased levels of protein-conjugated acrolein (PC-Acro) and the enzymes responsible for its production, polyamine oxidases (SMO and AcPAO), are good biomarkers for human stroke.[9] 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 (84%) and specificity (84%).[10] This tool for early identification of stroke may help in the application of suitable therapy to delay or reduce aggravation of stroke. In addition, we confirmed that

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acrolein scavengers, such as *N*-acetylcysteine (NAC)[11] and *N*-benzylhydroxylamine,[12] decreased the size of brain infarction using photochemically induced thrombosis (PIT) mice.

This time, we searched for acrolein scavengers with low side effects to maintain the quality of life of elderly people. We found that NAC ethyl ester (NACEt) and NAC benzyl ester (NACBn) are good candidates to maintain quality of life of the elderly. The mechanism of the decrease of brain infarction by NAC, NACEt, and NACBn was also studied in more detail.

Materials and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. The Checklist of Methodological and Reporting Aspects for Articles is available in the online-only Data Supplement.

PIT Model Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chiba University and performed according to the Guidelines for Animal Research of Chiba University. PIT model mice were prepared using 8-week-old male C57BL/6 mice (22–26 g) as described previously.[11] Animals were randomized to treatment groups ahead of time. Mice were anesthetized with 4% isoflurane and then maintained on 1.5% isoflurane using a small-animal anesthesia system. For induction of ischemia, immediately after intravenous injection of photosensitizer Rose Bengal (20 mg/kg), through a jugular vein, green light (wave length, 540 nm) emitted from a xenon lamp (Hamamatsu Photonics, Japan) illuminated the middle cerebral artery for 10 minutes. NAC derivatives were administered intraperitoneally. At 24 hours after the induction of PIT stroke, the brains were 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, which was not stained with triphenyltetrazolium, was analyzed on a Macintosh computer using the National Institutes of Health image program with treatment concealment. The efficacy of NAC derivatives was calculated as the volume of decreased infarction per mmol of compounds administrated per kg of mouse body weight. A total of 80 mice (10 in each treatment group) were submitted to the PIT surgery. No mouse presented signs of paresis, convulsion, remarkable weight loss, or any symptoms, and all mice were submitted to the further analysis.

Measurement of Protein, Polyamines, Glutathione, and PC-Acro

Brain tissue was washed and homogenized using an Ultra-Turrax homogenizer (Janke & Kunkel KG) in 0.5 mL of buffer A containing 10 mmol/L Hepes-KOH, pH 7.5, 1 mmol/L dithiothreitol, 10% glycerol, 0.2 mmol/L EDTA, and 0.02 mmol/L FUT-175 (6-amidino-2-naphthyl-4-guanidinobenzoate)—a protease inhibitor. Protein was measured by the method of Lowry et al,[13] with a BCA (bicinchonic acid) protein assay kit (Nacalai Tesque, Inc) after 5% trichloroacetic acid precipitation of homogenized brain tissue using BSA as a standard. Polyamines in the 5% trichloroacetic acid-soluble fraction were separated on a Hitachi high-performance liquid chromatography system on which a TSK gel Polyaminepak column (4.6 by 50 mm, Tosoh corporation) heated to 50°C was mounted.[14] Detection of polyamines was by fluorescence intensity after the column effluent at 50°C with an o-phthalaldehyde solution containing 0.06% o-phthalaldehyde, 0.4 mol/L boric buffer (pH 10.4), 0.1% Brij-35, and 37 mmol/L 2-mercaptoetahnol.[14] Glutathione was measured using total glutathione assay kit (Northwest Life Science Specialities, LLC) according to the manufacturer's instructions. The level of PC-Acro was measured by Western blotting[15] as described previously,[11] using antibody against FDP-lysine [N^{ε}-(3-formyl-3,4-dehydropiperidino-lysine)].[16]

Synthesis of NAC Derivatives

NAC was purchased from Sigma Aldrich. NACEt was synthesized according to the method published previously.[17] NACBn was synthesized as follows. First, *O*-benzyl *N,N′*-diisopropylisourea was synthesized by mixing 19.3 mmol benzylalcohol/2 mL and 19.5 mmol *N,N′*-diisopropylcarbodiimide/3 mL in the presence of $CuCl_2$ (0.42 mmol) and purified by Al_2O_3 open chromatography (solvent system: 30% ethylacetate/70% n-hexane). The yield of *O*-benzyl *N,N′*-diisopropylisourea was 4.25 g. Then, NAC (1.33 g, 8.15 mmol) in 5 mL tetrahydrofuran was mixed with *O*-benzyl *N,N′*-diisopropylisourea (1.92 g, 8.18 mmol) and stirred for 1.5 hours at room temperature. The reaction mixture was filtered through Celite 545 (Kanto Chemical Co, Inc) and evaporated. Crude product was purified by SiO_2 flash chromatography (solvent system: 30% ethylacetate/70% n-hexane). The NACBn in the solvent was further purified by recrystallization through the addition of n-hexane. Yield of NACBn was 1.21 g.

Measurement of the Levels of GST-π mRNA and Protein in Neuro2a Cells

Mouse neuroblastoma Neuro2a cells (10^6 cells) were cultured in 2 mL of DMEM supplemented with 50 U/mL streptomycin, 100 U/mL penicillin G, and 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2 for 2 days with 100 µmol/L each of NAC, NACEt, or NACBn in the presence or absence of 10 µmol/L acrolein. Levels of mRNA of GST-π and β-actin were measured using SuperPrep Cell Lysis and RT Kit (TOYOBO) according to the manufacturer's protocol using primer sets of GSTP-F (5′-TTTTGAGACCCTGCTGTCC-3′) and GSTP-R (5′-TTATTAGTGCTGGGAAAACGGG-3′) for GST-π and actin-F (5′-CAGGTCATCACTATTGGCAACCAGCGGTTC-3′) and actin-R (5′-GGAGCCAGAGCAGTAATCTCCTTCTGCATC-3′) for β-actin. The levels of GST-π, -θ, and -µ were measured by Western blotting[15] using their antibodies (Abcam) and 30 µg protein of brain tissue homogenate. As a control, the level of β-actin was measured using its antibody (Santacruz).

Measurement of the Level of Free Acrolein in the Presence of Glutathione or GST-π

Acrolein was measured according to the method of Alarcon.[18] The reaction mixture (0.3 mL) containing 67 mmol/L Na^+-phosphate buffer, pH 7.5, 0.5 mmol/L glutathione, and 10 µM acrolein was incubated at 37°C for 20, 40, 60, and 120 seconds. Where indicated, 3 µg GST-π (Alpha Diagnostic International, TX) was added to the reaction mixture. At each interval, 50 µL of the reaction mixture was taken out and mixed with the equal volume of the solution containing 92 mmol/L m-aminophenol, 172 mmol/L hydroxylamine hydrochloride, and 3 mol/L HCl. After the mixture was boiled for 10 minutes, acrolein content was determined by high-performance liquid chromatography according to the method of Bohnenstengel et al,[19] using 80 µL supernatant after centrifugation. Fluorescence of 7-hydroxyquinoline (acrolein derivative) was measured at an excitation wavelength of 358 nm and an emission wavelength of 510 nm.

Estimation of Stability of GST-π in the Presence of Acrolein or NAC Ethyl Ester

A hundred nanograms of GST-π protein were incubated with acrolein, NAC, or both of them derivatives at 37°C for 15 hours in 20 µL PBS. GST-π and its acrolein-conjugated level (PC-Acro) were determined by Western blotting[15] using antibodies against GST-π and FDP-lysine[16] as mentioned above.

Statistics

Values are indicated as means±SD. Normality was assessed by the D'Agostino and Pearson omnibus normality test. The significance of difference between 2 groups was analyzed by Student *t* test. One-way ANOVA followed by Dunnett post hoc test was used to assess the significance of the difference in groups treated with NAC derivatives of normally distributed variables. The Kruskal–Wallis test was used for

non-normally distributed variables. For comparison of GST levels in brain tissue, the significance was estimated using Friedman repeated measures of analysis of ranks. The statistical calculations were conducted using a GraphPad Prism program.

Results

Protection and Prevention of Brain Infarction by NAC Derivatives

We have shown previously that NAC decreased the size of brain infarction using thrombosis model mice.[11] However, it may be difficult to deliver NAC to the brain because it is hydrophilic. Thus, 2 kinds of NAC derivatives (NAC ethyl ester [NACEt] and NAC benzyl ester [NACBn]) were synthesized (Figure 1A), and the effects of these derivatives together with NAC on brain infarction were evaluated by simultaneous administration during brain ischemia or by preadministration before ischemia. Dose administration during brain ischemia was 150 mg/kg, 1×, and that before brain ischemia was 50 mg/kg, 4×, with 24-hour intervals (Figure 1B and 1C). In the case of simultaneous administration, 3 kinds of NAC derivatives reduced the volume of brain infarction in the order NACBn>NACEt>NAC. In the case of preadministration, only NACEt and NACBn significantly reduced the volume of brain infarction. To clarify the efficacy of NAC derivatives, the strength of these compounds to decrease the size of infarction was calculated as the volume of decreased infarction per millimole of compounds administrated per kilogram of mouse body weight and expressed as efficacy in Figure 1B and 1C. NACBn showed the strongest efficacy on the infarction volume in both single and preventive administrations. Single administration of NAC derivatives at lower dose (75 mg/kg) reduced the infarct volumes at almost the same extent of 150 mg/kg administration

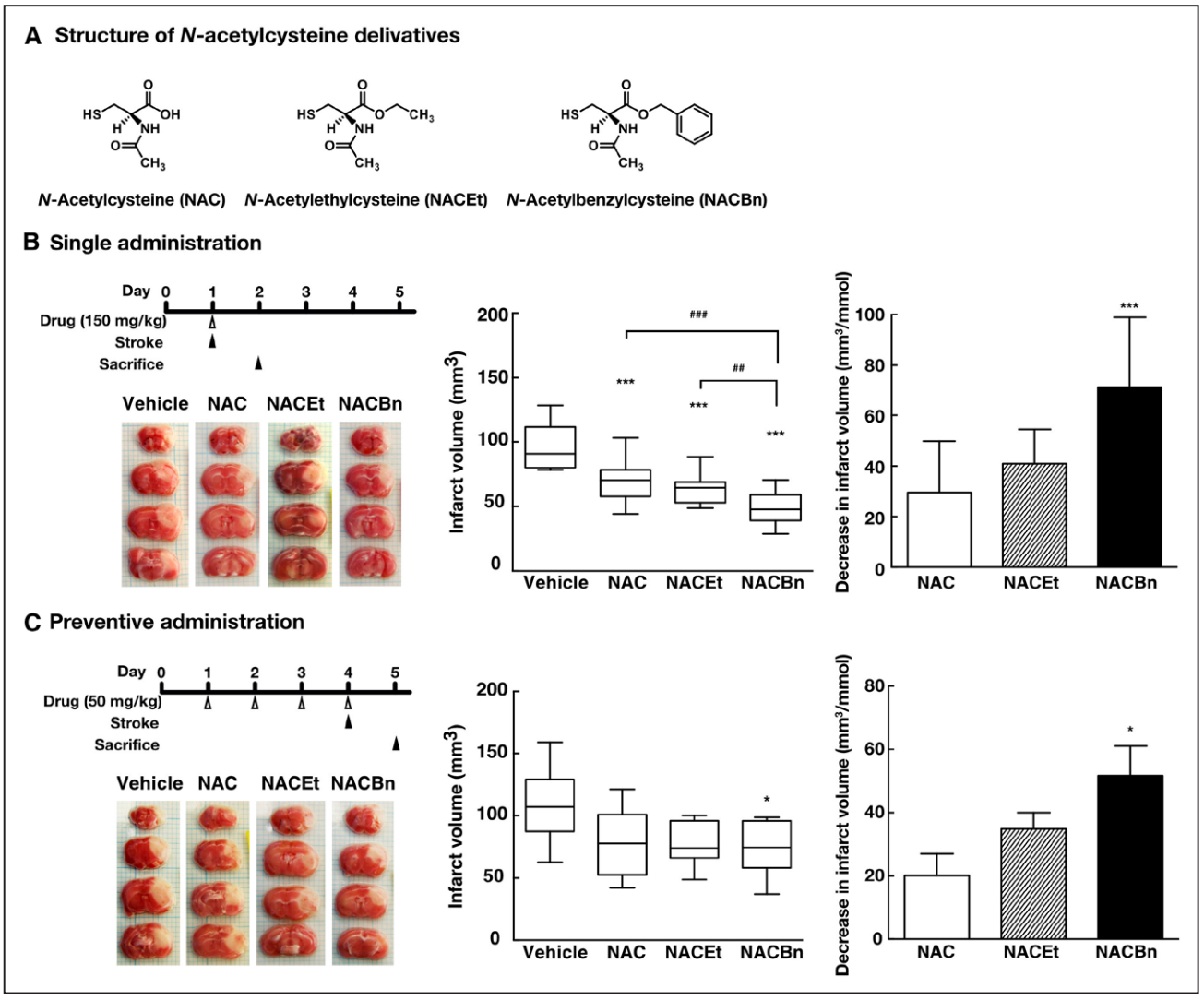

Figure 1. Preventive effects of *N*-acetylcysteine (NAC) derivatives on brain infarction. Infarction volume of photochemically induced thrombosis model mice was measured as described in Materials and Methods. **A**, Structures of NAC derivatives are shown. **B**, NAC derivatives (150 mg/kg) were administered intraperitoneally to mice right after the operation of infarction. **C**, NAC derivatives (50 mg/kg) were preinjected 4× at 24-hour intervals before the operation of infarction. Experiments were performed using 10 mice in each group. Infarct volume and efficacy were measured and calculated as described in Materials and Methods. Horizontal line within the box indicates median, the bottom and the top of the boxes indicate the 25th and 75th percentiles, and the whiskers (vertical lines) indicate the 5th and 95th percentiles. **P*<0.05. ****P*<0.001, against vehicle. ##*P*<0.01, NAC ethyl ester (NACEt) against NAC benzyl ester (NACBn). ###*P*<0.001, NAC against NACBn.

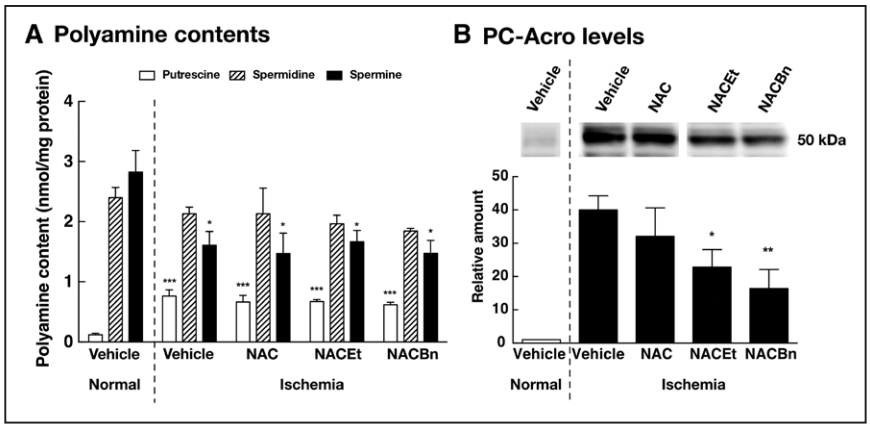

Figure 2. Effect of preadministration of *N*-acetylcysteine (NAC) derivatives on polyamine contents (**A**) and the level of protein-conjugated acrolein (PC-Acro; **B**) at the locus of normal and ischemic brain. Polyamine contents and the level of PC-Acro were measured as described in Materials and Methods using 10 mice in each group. Data are shown as mean±SD. NACBn indicates NAC benzyl ester; and NACEt, NAC ethyl ester. **P*<0.05, ***P*<0.01, ****P*<0.001 against normal.

(data not shown). Essentially, the same results were obtained when the brains were sectioned into 1-mm-thick coronal slices instead of 2-mm thick. The results suggest that NACEt and NACBn could be taken up and be kept in the brain more effectively than was NAC and that these 2 derivatives may become candidates as a preventive medicine for brain damage because acrolein is a major toxic compound during brain infarction.[4,5]

Mechanism of Acrolein Detoxification by NAC Derivatives

First, effects of preadministration of NAC derivatives (50 mg/kg, 4×) on the levels of polyamines and PC-Acro were evaluated. As shown in Figure 2, the decrease in spermine and spermidine at the locus of ischemia was not recovered by the NAC derivatives, but the level of PC-Acro at 50 kDa protein—one of the major proteins conjugated with acrolein—decreased in the order NACBn>NACEt>NAC. In parallel, the level of glutathione was measured because acrolein is primarily detoxified by glutathione. As shown in Figure 3, the glutathione level was significantly decreased by NACEt and NACBn, and slightly by NAC, suggesting that NAC derivatives stimulate conjugation between acrolein and glutathione. The results indicate that NACEt and NACBn contribute more to prevention of brain infarction than NAC because these 2 NAC derivatives accumulate in brain more effectively than NAC.

It has been reported that acrolein conjugation with glutathione is catalyzed by glutathione-*S*-transferases (GSTs),[20] and major GSTs in brain are GST-π, -θ, and -μ.[21,22] Accordingly, the levels of GST-π, -θ, and -μ were estimated by Western blotting. When brain tissue was damaged by ischemia, the levels of GSTs decreased to ≈50% of normal tissue (Figure 4). However, when NAC and its derivatives were administered, the levels of GSTs were recovered in the order NACBn>NACEt>NAC. The protein levels of 3 GSTs existing in brain were nearly equal (Figure 4). Then, it was examined whether GST-π really stimulates acrolein–glutathione conjugation using purified GST-π. As shown in Figure 5, the level of free acrolein decreased in the presence of glutathione, indicating that conjugation between acrolein and glutathione occurs nonenzymatically. The addition of GST-π decreased free acrolein more rapidly, confirming that GST-π catalyzes acrolein–glutathione conjugation. The half-life ($T_{1/2}$) of the rate of elimination of acrolein at 37°C under our experimental conditions was 376±24.7 seconds in the absence of glutathione, 362±26.9 seconds in the absence of glutathione and GST-π, 23.7±0.58 seconds in the presence of glutathione, and 10.4±1.83 seconds in the presence of glutathione and GST-π, respectively. Accordingly, the rate of elimination of acrolein becomes ≈15-fold faster in the presence of glutathione and 35-fold faster in the presence of both glutathione and GST-π.

Then, the mechanism of increase in the level of GST-π by NAC derivatives was studied. As shown in Figure 6A, the level of GST-π mRNA in Neuro2a cells cultured at 37°C for 48 hours was nearly equal in the presence and absence of acrolein and NAC derivatives, indicating that the increase in GST-π is at the post-transcriptional level. So, stability of purified GST-π in PBS was evaluated in the presence of acrolein and NAC derivatives at 37°C for 15 hours. As shown in Figure 6B, GST-π was conjugated with acrolein and was polymerized, but its conjugation and polymerization by acrolein was inhibited by NAC and its derivatives. These results suggest that NAC derivatives disturb the conjugation between acrolein and the active site of GST-π, and then active GST-π is involved in the acrolein detoxification by catalyzing the conjugation between free acrolein and glutathione.

Discussion

Because the global population of elderly people is increasing in the world, it is important to maintain quality of life of the elderly. If it is possible to protect or delay the aggravation of

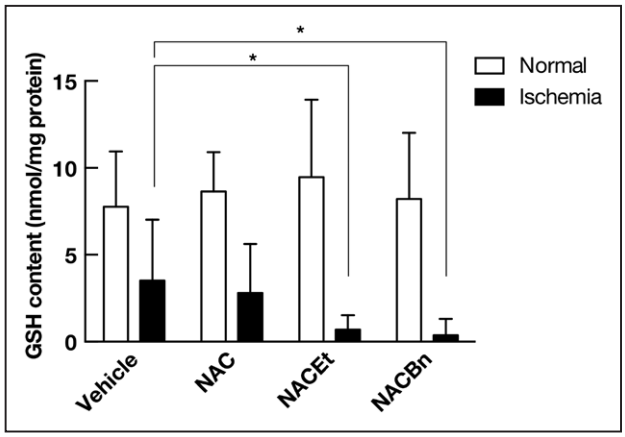

Figure 3. Effect of preadministration of *N*-acetylcysteine (NAC) derivatives on glutathione (GSH) content. GSH content at the locus of normal and ischemic brain was measured as described in Materials and Methods using 10 mice in each group. Data are shown as mean±SD. NACBn indicates NAC benzyl ester; and NACEt, NAC ethyl ester. **P*<0.05.

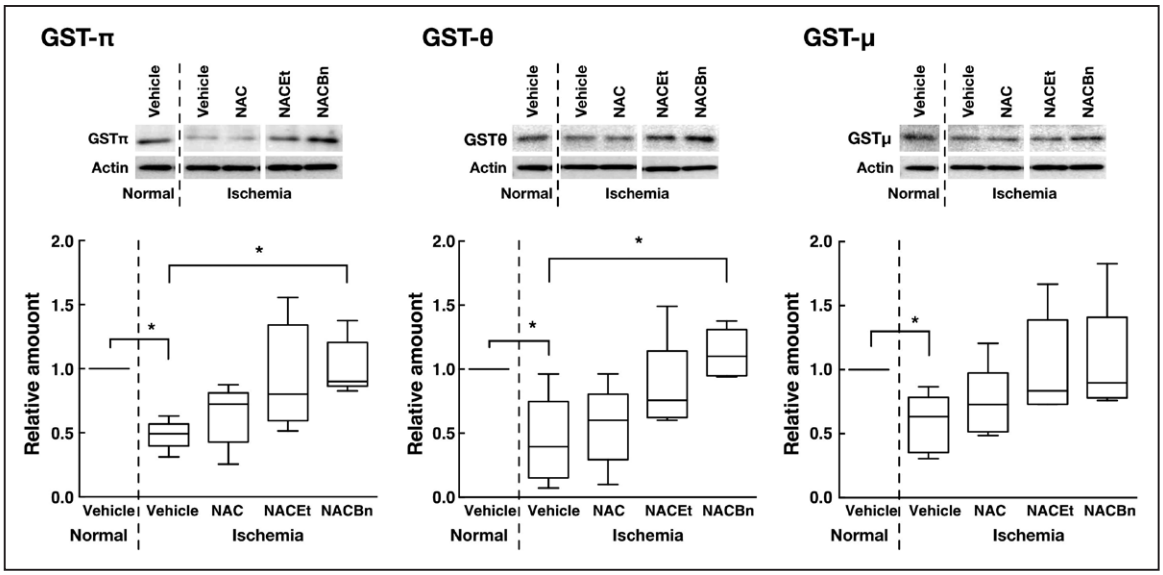

Figure 4. Effect of preadministration of *N*-acetylcysteine (NAC) derivatives on the levels of GSTs. Levels of GSTs at the locus of brain ischemia were measured by Western blotting[15] in comparison with that at the normal brain as described in Materials and Methods using 5 mice in each group. A typical pattern of Western blotting was also shown. The amount of protein used for measurement of GST and β-actin was 30 and 5 μg, respectively. Horizontal line within the box indicates median, the bottom and the top of the boxes indicate the 25th and 75th percentiles, and the whiskers (vertical lines) indicate the 5th and 95th percentiles. NACBn indicates NAC benzyl ester; and NACEt, NAC ethyl ester. *P<0.05.

brain infarction and dementia, it could greatly contribute to the maintenance of quality of life of the elderly. We have previously reported that a toxic compound, acrolein, becomes a good biomarker for brain infarction[11] and dementia.[23] Thus, we looked for compounds that decrease the level of acrolein during brain damage and do not have severe side effects. We recently found that NAC decreases the size of brain infarction.[11] Because NAC does not have severe side effects, we looked for protective effects of NAC derivatives through 4× administrations at 24-hour intervals and found that NACEt and NACBn have protective effects (Figure 1C). It may be feasible that these 2 compounds could be drunk every day for several months after checking the side effects of NAC derivatives. It has been also reported that NAC is effective for Alzheimer disease and Parkinson disease.[24]

The mode of action of NAC derivatives was also unique. It was hypothesized that NAC derivatives directly conjugate with acrolein instead of glutathione. However, NAC derivatives increased the levels of 3 kinds of GSTs (π, θ, and μ) during brain infarction, by stabilizing these proteins although we have not yet studied the mechanism of stabilization of GSTs by NAC derivatives in detail. GST-π stimulated the conjugation of acrolein with glutathione >2-fold (Figure 5). So, the stabilization of GSTs plays important roles for detoxification of acrolein. It was also noted that significant differences in PC-Acro levels in NAC derivatives treated groups (Figure 2), whereas the reduction of infarct volume was similar in these groups (Figure 1). The results suggest that NAC derivatives act by multiple mechanisms besides acrolein detoxification. We are now studying the mechanism of stabilization of GSTs by NAC derivatives, as well as the destination of acrolein–glutathione complex. We found that a major acrolein–glutathione derivative in urine, 3-hydroxypropyl mercapturic acid, decreases after stroke,[25] suggesting that detoxification ability against acrolein decreases in patients with stroke in parallel with the decrease in glutathione. The results also suggest that the level of glutathione is important to maintain the ability of GSTs.

In case of stroke in rats, it has been reported that HIF1 (hypoxia-inducible factor 1) contributes to NAC protection in stroke.[26] However, in our PIT model mice, the level of HIF1 did not change significantly (data not shown). Because the levels of GST mRNAs did not change under our experimental conditions (Figure 6A), involvement of HIF1—one

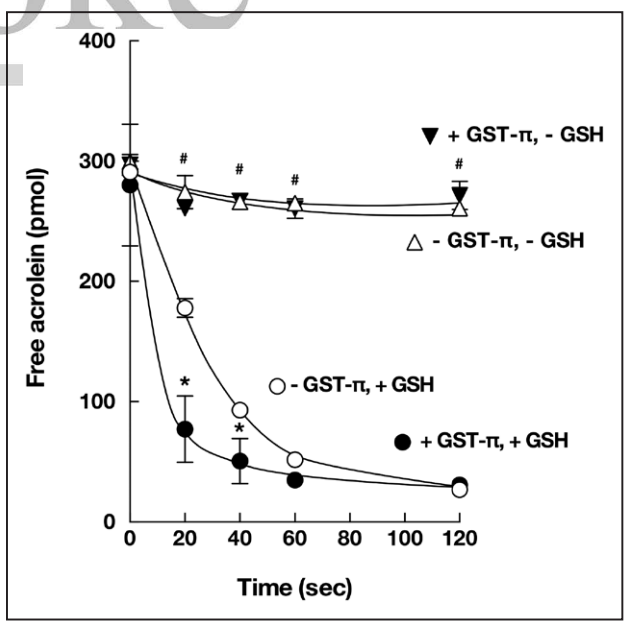

Figure 5. Stimulation of acrolein metabolism by glutathione (GSH) and GST-π. The level of free acrolein was measured by high-performance liquid chromatography after incubation with GSH, GST-π, or both of them as described in Materials and Methods using 3 samples in each group. Δ, –GST-π, –GSH; ▼, +GST-π, –GSH; ○, –GST-π, +GSH; ●, +GST-π, +GSH. #P<0.01 against ○; *P<0.05 against ○.

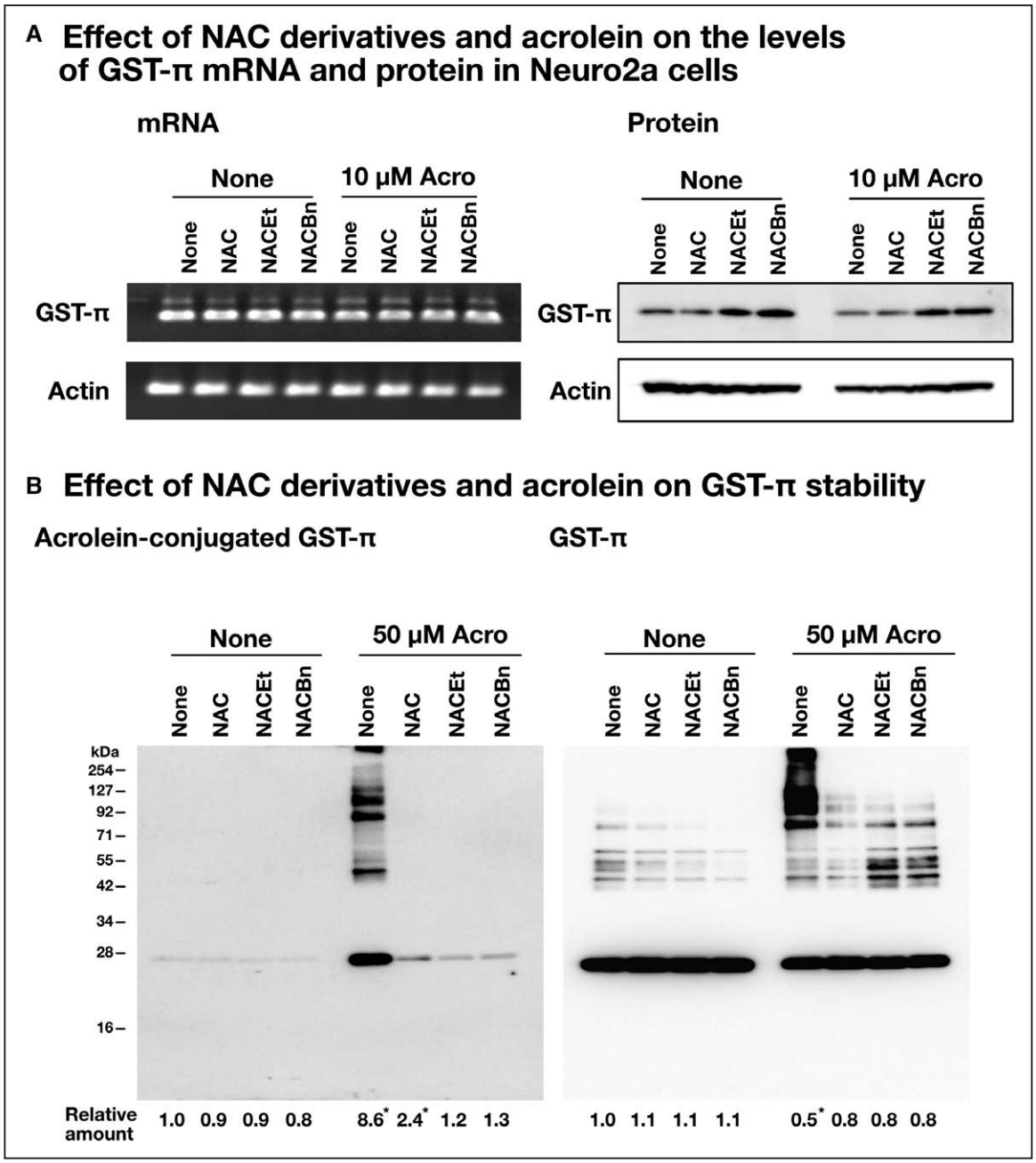

Figure 6. The level of GST-π in Neuro2a cells cultured under various conditions (**A**) and that of acrolein-conjugated GST-π incubated with acrolein or *N*-acetylcysteine (NAC) derivatives (**B**). **A**, The levels of GST-π mRNA and GST-π protein in Neuro2a cells cultured under various conditions at 37°C for 48 hours were measured as described in Materials and Methods. Levels of β-actin mRNA and β-actin protein were measured as control. The concentration of NAC derivatives used was 100 μM. **B**, Purified GST-π (100 ng) was incubated in the presence of acrolein (50 μM), NAC derivatives (50 μM), or both of them at 37°C for 15 hours. The level of GST-π (**right**) and its acrolein-conjugated GST-π (**left**) was determined by Western blotting. Experiments were repeated 3×, and essentially, the same results were obtained. NACBn indicates NAC benzyl ester; and NACEt, NAC ethyl ester. *P<0.05 against control.

of the transcription factors for GST mRNAs—may be small, although its reason is not clear.

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Disclosures

None.

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Protective Effects of Brain Infarction by *N*-Acetylcysteine Derivatives

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Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☒ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☐ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☐ An overall study timeline is provided.
Inclusion and exclusion criteria	☒ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☐ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☒ Type and methods of randomization have been described. ☐ Methods used for allocation concealment have been reported.
Blinding	☐ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☒ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☒ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☐ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☐ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☒ Statistical methods used have been reported. ☐ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☒ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☐ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☐ Statements on funding and conflicts of interests have been provided.