HYPOXIC–ISCHEMIC BRAIN INJURY IN NEWBORN RAT : NEUROPROTECTIVE EFFECTS OF ACETAMINOPHEN AND THE INVOLVEMENT OF ACID SPHINGOMYELINASE ACTIVATION

Rena Oguma[1)], Wako Ishida-Nakajima[2)], Masanari Kawamura[3)], Shinobu Miura[4)], Chikako Oyama[1)], Yoko Sato[5)], Hirokazu Arai[1)] and Tsutomu Takahashi[1)]

(received 21 December 2012, accepted 7 January 2013)

[1)]Department of Pediatrics, Akita University Graduate School of Medicine, Akita 010-8543, Japan
[2)]Division of Pediatrics, Akita Municipal Hospital, Akita 010-8543, Japan
[3)]Division of Pediatrics, Hiraka General Hospital, Akita 013-0042
[4)]Division of Yuri General Hospital, Akita 015-8511
[5)]Division of Pediatrics, Akita Red Cross Hospital, Akita 010-1406

Abstract
Acetaminophen is widely used as an analgesic and antipyretic medication. Recently, acetaminophen has been shown as effective against neuronal cell death through its antioxidant and anti-inflammatory properties. In this study, we used the Rice-Vannucci model to examine whether the administration of acetaminophen was protective against hypoxic-ischemic brain injury in immature rat brain. Seven-day-old rat pups that had ligation of the right carotid artery received 20 mg/kg of acetaminophen intraperitoneally immediately before hypoxic exposure of 8% oxygen for 90 min. Compared to controls, acetaminophen treatment group showed decreased macroscopic brain injury scores at 48 h and 168 h after hypoxia-ischemia. Acetaminophen significantly decreased the number of apoptotic and necrotic cells in the cortex, caudate putamen, thalamus, and hippocampus. In addition, the percent brain damage of hypoxia-ischemia, which is another index for brain injury, was improved by the administration of acetaminophen. Our results suggested that acetaminophen inhibited apoptotic and necrotic cell death and played a role in neuroprotection after hypoxia-ischemia in immature rat brain. In addition, we showed that acid sphingomyelinase (ASM), which is as an important enzyme for cellular responses to reactive oxygen species (ROS), might be involved in brain injuries after hypoxie-ischemia. The activation of ASM after hypoxia-ischemia was attenuated by the administration of acetaminophen in our rat model. This attenuation might be caused by the antioxidant property of acetaminophen, supporting the suggestion of its neuroprotective effect on the hypoxic-ischemic brain injury.

Key words : neonate, hypoxic-ischemic brain injury, acetaminophen, acid sphingomyelinase

Correspondence : Tsutomu Takahashi, M.D.
Department of Pediatrics, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
Tel : 018-884-6159
Fax : 018-836-2620
E-mail : tomy@med.akita-u.ac.jp

Introduction

Acetaminophen is widely used as an analgesic and antipyretic medication. Recently, acetaminophen has been shown to be effective against neuronal cell death in *in vivo* and *in vitro* models of neurological disorders through its antioxidant and anti-inflammatory properties. Acetaminophen has been shown to protect hippocampal neurons and PC12 cultures from amyloid β-peptide-induced oxidative stress and reduce the activation of the inflammatory transcription factor NF-κB[1]. In animal models of Parkinson's disease, acetaminophen has been shown to protect dopaminergic neurons *in vitro* from oxidative damage by acute exposure to 6-hydroxydopamine or excess levels of dopamine[2]. Using cerebral cultured neurons that had been exposed to a superoxide-releasing oxidant stressor, acetaminophen was shown to have pro-survival effects on neurons[3]. In addition, that study has shown that acetaminophen increases the levels of expression of the anti-apoptotic protein Bcl-2 in brain neurons. Finally, acetaminophen has been shown to reduce tissue damage, degree of mitochondrial swelling, and loss of mitochondrial membrane potentials in rats with cerebral ischemie-reperfusion-induced injury in a transient global forebrain ischemie model[4]. These results suggested that acetaminophen reduces apoptosis through a mitochondrial-mediated mechanism and that acetaminophen may be a potential hypoxic-ischemic brain damage therapy.

In newborns, hypoxic-ischemic encephalopathy is an injury to the brain that occurs because of hypoxia or ischemic events during the perinatal period. Hypoxic-ischemic encephalopathy may result in death or cause serious impairments in survivors. We have studied hypoxic-ischemic encephalopathy in mature infants with the Rice-Vannucci model, which is a rat model of 7-day-old (P7) rats and which has been used worldwide for the past 10 years[5]. With the rat model, prolonged hypothermia, a calpain inhibitor, and edaravone have been shown to protect newborn rat brains from hypoxia-ischemia[6-8]. Ascorbic acid, which is administered intraventricularly or intraperitoneally, has been demonstrated to decrease hypoxic-ischemic brain injury in newborn rats[9,10]. The protective effects of ascorbic acid on hypoxic-ischemic brain injury might be explained by the facts that neuronal cell damage following hypoxic-ischemic brain injury is partly caused by the production of free radicals and reactive oxygen species (ROS) and that ascorbic acid is a potent antioxidant that scavenges various types of ROS.

Acid sphingomyelinase (ASM) is a lysosomal enzyme that is responsible for the production of ceramide through the hydrolysis of sphingomyelin, the deficiency of which causes Niemann-Pick disease types A and B[11]. Recent findings have revealed an important role of ASM in the initiation of ceramide-induced cell apoptosis, as well as in several diseases, including cardiovascular diseases, diabetes, pulmonary diseases, and neuronal diseases[12]. Some studies have shown that ROS may be one of the factors that cause ASM activation[13,14]. These findings suggest that ASM activation may play a role in hypoxic-ischemic brain injury.

In this study, we examined whether the administration of acetaminophen was protective against hypoxic-ischemic brain injury in immature rat brain. In addition, we studied the involvement of ASM activation in hypoxic-ischemic brain injury with the rat model.

Materials and Methods

Animals

All experiments were performed in accordance with the approved institutional animal care guidelines of Akita University School of Medicine. The experiments were performed on P7 Wister rats ($n=162$) of both sexes. Until the day of surgery, the pups were maintained in cages with their dams in controlled conditions, which consisted of a constant temperature of 22±2°C, a 12-h light/12-h dark cycle, and *ad libitum* access to food and drink. The mean body weight on the day of surgery was 10.6±0.85 g.

Hypoxic-ischemic insult

The pups were separated from their dams and placed in a temperature-controlled incubator that was set to an ambient temperature of 37°C. After each pup was lightly anesthetized with isoflurane (4% induction, 1.5% maintenance), a middle incision was made in the neck. Then,

the right common carotid artery was separated from the vagus and sympathetic nerves and cut with bipolar electrocoagulation forceps. This procedure took approximately 3 min to complete. After 2 h of recovery following the surgical procedure, the pups were exposed to 8% humidified oxygen and nitrogen for 90 min. Just after hypoxic exposure, 20 mg/kg or 50 mg/kg of acetaminophen was injected intraperitoneally, followed by 5 injections of the same dose of acetaminophen at every 12 h. Acetaminophen was dissolved in saline so that the injection volume would be 0.15 ml/animal. An equal volume of saline was injected into vehicle controls. The body weights were measured on P 7, which was just prior to the carotid ligation. Rectal temperatures were measured in each animal with a digital thermometer just before the hypoxia and 30 min after the hypoxia.

Tissue preparation

The pups were divided into the following two groups : the acetaminophen injection group and vehicle controls. They were sacrificed 0 h, 48 h, and 168 h after the hypoxic-ischemic exposure. While the rats were under diethyl-ether anesthesia, the pups were perfused transcardially with phosphate-buffered saline and then 4% paraformaldehyde in 0.1 M phosphate-buffered saline. After brains were embedded in paraffin, 2.5-μm-thick sections were cut and processed for hematoxylin and eoshin (H&E) staining. For western blotting, pups were sacrificed 0 h and 48 h after hypoxic exposure, and samples of the cortex, striatum, thalamus and hippocampus were separated. After homogenizing with lysis buffer, homogenates were centrifuged at 23,500×g for 60 min at 4°C and resulting supernatant was used as cytosol fraction.

Macroscopic evaluation of brain injury

We macroscopically evaluated the brain injuries before brain sampling. While they were under ether anesthesia, the rats were quickly decapitated, and the brains were removed. The degree of brain injury was assessed by evaluating the macroscopic appearance of the brain as previously reported[8] (Fig. 1). In brief, a rating scale for injury was used to rate the appearance of both the hemispheres. This rating scale comprised the following scores from 0 to 4 according to the size of the infarction : 0, no difference between the right hemisphere (ligated side) and left hemisphre ; 1, because of edema, the right hemisphere was larger than the left one, but without infarction ; 2, the site of the infarction in the right hemisphere did not exceed the middle vertical line in the same hemisphere ; 3, the size of the infarction in the right hemisphere exceeded the middle vertical line in the same hemisphere ; and 4, the whole right hemisphere is affected. An investigator who was blinded to the experimental protocol evaluated all brain injuries.

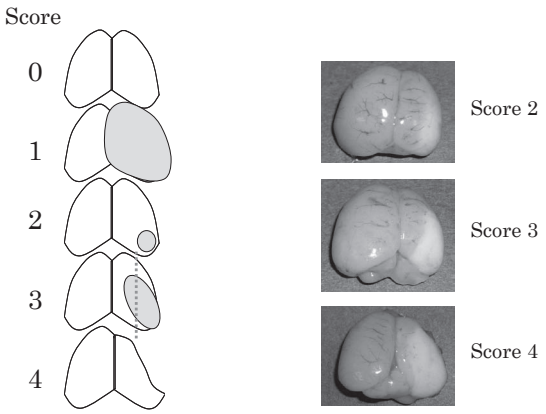

Fig. 1. Macroscopic evaluation of brain injury. A rating scale for injury was used to rate the appearance of both hemispheres. This scale comprised the following scores from 0 to 4 according to the size of the infarction : 0, no difference between the right hemisphere (ligated side) and left hemisphere ; 1, because of edema, the right hemisphere was larger than the left one, but without infarction ; 2, the site of the infarction in the right hemisphere did not exceeds the middle vertical line in the same hemisphere ; 3, the size of the infarction in the right hemisphere exceeded the middle vertical line in the same hemisphere ; and 4, the whole right hemisphere was affected. Three whole brains of rats with (scores of 2, 3, or 4) are shown as examples.

Neuropathological analyses of apoptosis and necrosis

Quantitative analyses of cell death were performed in the frontal cortex, parietal cortex, striatum, thalamus and hippocampus (CA1 and CA3) of the ligated hemisphere as previously described with modification[7]. In brief, an investigator who was blinded to the experimental protocol

examined 2.5-μm-thickness H&E-stained sections (n=4 per group at 0-h, 48-h, and 168-h after hypoxia-ischemia). Cell counts were done at a magnification of 400× with 100-grid squares. Ten fields in each region were evaluated. Five hundred nuclei were counted. We used a previous report as a guide to identify the morphological phenotypes of apoptotic and necrotic cells as follows[15]. The light-microscopic features of apoptotic cells include a regular shape, large and round chromatin clumps, and condensation of the cytoplasm. In contrast, those of necrosis include intense cytoplasmic eosinophilia, dispersed nuclear chromatin, and the loss of nuclear membrane integrity (karyolysis) or nuclear changes alone without eosinophilic cytoplasm. Pyknotic cells were excluded from this count. The scores are presented as mean ± standard error of the difference between 2 means (SED).

Weighing of the hypoxic-ischemic hemisphere

The left (L ; unligated side) and right (R ; ligated side) hemispheres of a pup were separately weighed 48 h or 168 h after hypoxia-ischemia (n=6 each time). Acetaminophen was intraperitoneally administered with the following doses : 0 mg/kg×6, 20 mg/kg×6, or 50 mg/kg×6. The percent brain damage (%) was calculated as (L-R)/L×100.

Acid sphingomyelinase enzyme assay

We used a modification of the method proposed by Chatterjee and Ghosh[16], and assays of cation-independent ASM and Zn^{2+}-dependent secretory ASM were conducted as follows. ^{14}C-sphingomyelin was purchased from Perkin Elmer Inc. (Boston, MA, USA). For the assays of lysosomal ASM and secretory Zn-ASM, the standard 200 μl assay mixture consisted of 100 μl of sample and 50 μl of assay buffer containing 4% Triton X-100 (1.0 M sodium acetate, pH 5.0 ; final concentration of Triton X-100 in the 200 μl assay mix, 1.0 %). When added, the final concentrations of ethylenediamine tetraacetic acid (EDTA) and Zn^{2+} were 0.02 mM and 0.1 mM, respectively. The reaction was initiated by the addition of 50 μl of substrate (20 nmol, ^{14}C-sphingomyelin, 0.08 μCi/20 nmol) in 0.2% taurodeoxycholic acid and then incubated at 37°C for 1 h. The assay was terminated with 100 μl of ice-cold 30% trichloroacetic acid and 200 μl of 2.5% bovine serum albumin. The contents of the tubes were vortexed, allowed to settle for 5 min at room temperature, and then centrifuged for 5 min at 3,000 rpm. Five hundred μl of the supernatant was carefully withdrawn and transferred into glass scintillation vials. The radioactivity was measured directly after mixing the samples with 4.5 ml of Clear-sol II (Nakalai Tesque Inc, Kyoto, Japan) in a liquid scintillation counter (LSC 950, Hitachi Aloka Medical, Lts., Tokyo, Japan). Unless otherwise indicated, the results were given as the means±SED of 3 separate experiments.

Western blotting of ASM

Samples were homogenated in RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing complete™ protease-inhibitor tablets (Roche Diagnostics, Milan, Italy). Homogenates were then sonicated and centrifuged, at 4°C for 60 min at 23,500×g, the debris was discarded, and the supernatants were collected. The protein concentrations of the cell extracts were quantified by BCA protein assay kits according to the manufacturer's recommendations (Thermo Fisher Scientific). Cell lysates were then diluted in an appropriate volume of 1×NuPAGE LDS Sample Buffer that was supplemented with 1× NuPAGE Reducing Agent (Life Technologies Corporation, Grand Island, NY, USA). Sample aliquots were heat-denatured for 10 min at 70°C. Equal amounts of protein (30 μg) were separated by electrophoresis on a 4-12% NuPAGE Bis-Tris Gel System (Life Technologies Corporation), and then transferred to a nitrocellulose membrane according to the manufacturer's instructions. The membrane was blocked in Pierce® Protein-Free T20 (TBS) Blocking Buffer (Thermo Ficher Scientific Inc.) for 1 h at room temperature. Subsequently, the membrane was incubated for 1.5 h at room temperature with a primary rabbit polyclonal anti-ASM antibody (1 : 500) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), and then for 1 h at room temperature with a secondary goat anti-rabbit IgG antibody that was coupled to horseradish peroxidase (1 : 7,500) (Santa Cruz Biotechnology, Inc.). All antibodies were diluted in Blocking Buffer. The signal was visualized with an Immun-Star™ HRP Chemiluminescent Kit (Bio-Rad

Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The membrane was stripped and reprobed with a mouse monoclonal anti-β-actin antibody (Abcam plc, Cambridge, UK), which was used as an internal loading control. Bands were then densitometrically analyzed with a Model GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Inc.).

Statistical analysis

Analyses of the data for body weight, rectal temperature, macroscopic evaluations of brain damage, the quantitative analyses of cell death and western blotting between the acetaminophen injection group and the vehicle controls were analyzed with Microsoft Office Excel 2012 software. Differences with p value less than 0.05 were considered statistically significant. Data are presented as means±standard error of the mean (SEM).

Results

Macroscopic evaluation of brain injury

The body weights at P7 did not significantly differ between the acetaminophen-injection group and vehicle controls (data not shown). Rectal temperatures did not significantly differ just before hypoxia, and 30 min after hypoxia (data not shown). Brain injuries were evaluated macroscopically in all pups (n=6 per time point in each group). No infarctions were found 0 h after hypoxia-ischemia in both groups (data not shown). The scores of the macroscopic brain injuries in the acetaminophen-injection group at 168 h after hypoxia-ischemia were significantly lower than those in the vehicle controls, but there was no significant difference between the 2 groups at 48 h (Fig. 2).

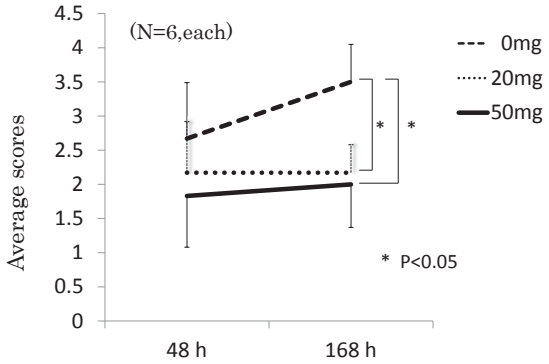

Fig. 2. Macroscopic evaluation of brain injury. The injury ratings were determined from the scale of 0 to 4 according to the macroscopic observations of the appearance of the right (ligated side) hemisphere compared to the left hemisphere. The scores in the acetaminophen-injection group at 168 h after hypoxia-ischemia were significantly lower compared to those of vehicle controls.

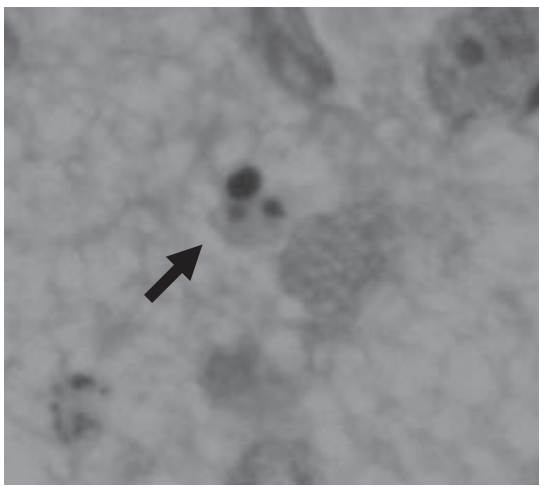

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Fig. 3. Apoptotic cells in hypoxic-ischemic brain injury. Microscopic features of apoptotic cells include a regular shape, large round chromatin clumps, and condensation of cytoplasm. The arrow indicates an apoptotic cell.

Quantitative analysis of cell death

We counted necrotic and apoptotic cells in H&E-stained sections from the hippocampus (CA1 and CA3), caudate putamen, laterodorsal thalamus, frontal cortex, and parietal cortex (Fig. 3 and Fig. 4). The number of apoptotic cells in vehicle controls increased after 48 h after hypoxia-ischemia, which was followed by a reduced number of apoptotic cells at 168 h in all regions. The numbers of apoptotic cells in the acetaminophen-injection group were smaller compared to those of the vehicle controls in all regions (Fig. 5). The numbers of apoptotic cells were significantly different between the vehicle controls and the acetaminophen-injection groups (20 mg/kg and 50 mg/kg) in all regions at 48 h. At 168 h, there

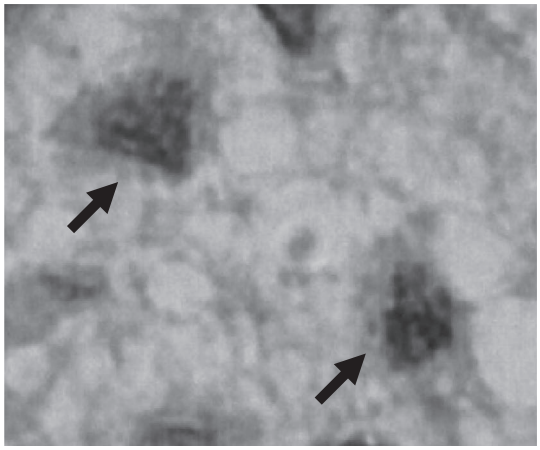

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Fig. 4. Necrotic cells in hypoxic-ischemic brain injury. The features of necrotic cells include intense cytoplasmic eosinophilia, dispersed nuclear chromatin, and the loss of nuclear membrane integrity or nuclear changes alone without eosinophilic cytoplasm. The arrows indicate necrotic cells.

were significant differences between the vehicle controls and the acetaminophen-injection groups (20 mg/kg and 50 mg/kg) in all regions, except for, in the thalamus and parietal cortex where there were no significant differences between the vehicle controls and the acetaminophen-injection groups (20 mg/kg).

The number of necrotic cells gradually increased from 48 h to 168 h after hypoxia-ischemia in all regions of vehicle controls. Significant differences in the numbers were statistically shown between the vehicle controls and the acetaminophen-injection groups (20 mg/kg and 50 mg/kg) in all regions at 48 h and 168 h, except, in the hippocampus (CA3) where there was no significant difference between the vehicle controls and the acetaminophen-injection groups (20 mg/kg) at 48 h (Fig. 6).

Evaluation of brain damage by weighing hypoxic-ischemic hemisphere

The percent brain damages in the brains that were administered acetaminophen were less than those in the

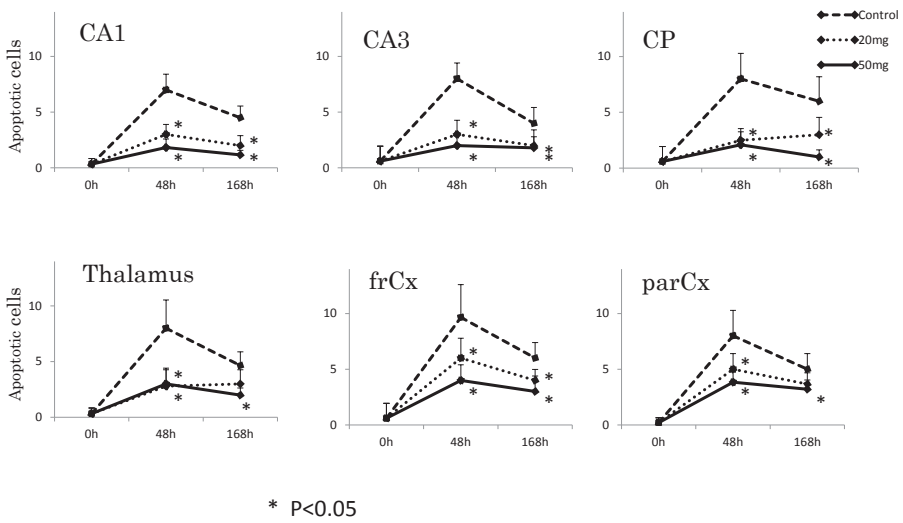

Fig. 5. Apoptotic cells in hypoxic-ischemic brain injury. The numbers of apoptotic cells in the acetaminophen-injection group were smaller compared to those of the vehicle controls in all regions. The numbers of apoptotic cells were significantly different between the vehicle controls and the acetaminophen-injection groups (20 mg/kg and 50 mg/kg) in all regions at 48 h. At 168 h, there were significant differences between vehicle controls and the acetaminophen-injection groups (20 mg/kg and 50 mg/kg) in all regions, except for, in the thalamus and parietal cortex where there were no significant differences between the vehicle controls and the acetaminophen-injection groups (20 g/kg). CA1 and CA3, hippocampal regions ; CP, caudate putamen ; frCx, frontal cortex ; parCx, parietal cortex.

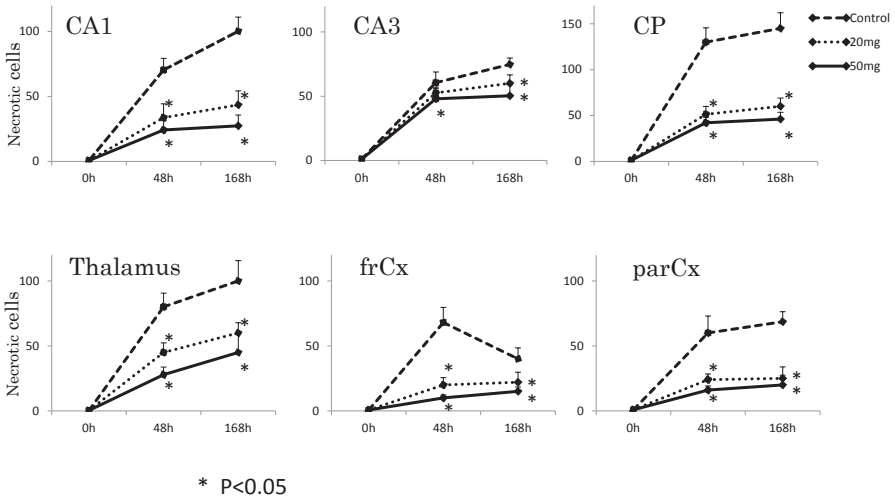

Fig. 6. Necrotic cells in hypoxic-necrotic brain injury. The numbers of necrotic cells gradually increased from 48 h to 168 h after hypoxia-ischemia in all regions of the vehicle controls. Significant differences in the numbers were statistically shown between vehicle controls and the acetaminophen-injection groups (20 mg/kg and 50 mg/kg) in all regions at 48 h and 168 h, except for, in the hippocampus (CA3) where there was no significant difference between the vehicle controls and the acetaminophen-injection groups (20 mg/kg) at 48 h. CA1 and CA3, hippocampal regions ; CP, caudate putamen ; frCx, frontal cortex ; parCx, parietal cortex.

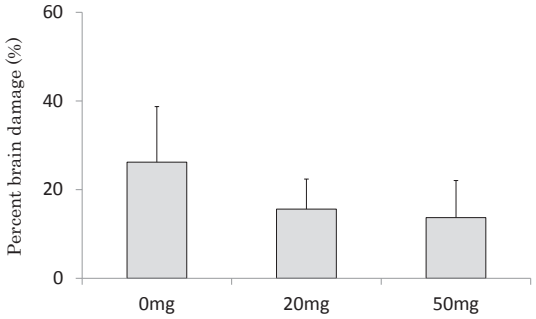

Fig. 7. Percent brain damages in hypoxic-ischemic brain injury. The percent brain damage values for the brains that were administered acetaminophen were less than those values for the brains that were not administered acetaminophen at 48 h and 168 h after hypoxia-ischemia. The differences were not statistically significant.

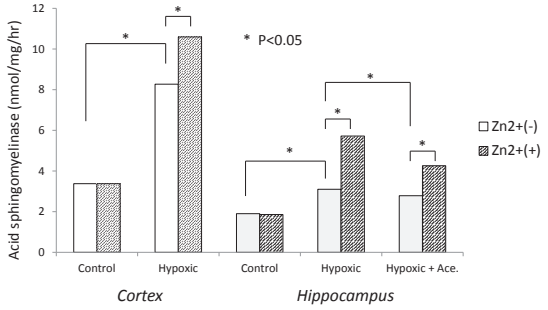

Fig. 8. Acid sphingomyelinase (ASM) activities of brain tissues in hypoxic-ischemic brain injury. In the cerebral cortex, the homogenates of hypoxic-ischemic-injured tissue showed significantly higher ASM activities than those of the contralateral tissues, with positive activation of ASM by the addition of Zn^{2+}. In the hippocampus, the homogenates of hypoxic-ischemic-injured tissue showed significantly higher ASM activity than those of the contralateral tissue, and the homogenates were positively activated by the addition of Zn^{2+}. The administration of acetaminophen significantly reduced the ASM activity that had been activated by hypoxia-ischemia.

brains that were not administered acetaminophen at 48 h and 168 h after hypoxia-ischemia (Fig. 7). However, the differences were not statistically significant.

ASM enzyme assay

The ASM activities of the tissues were determined

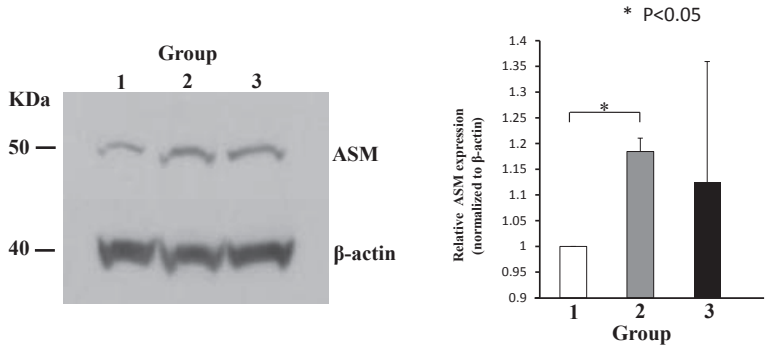

Fig. 9. Western blotting of ASM in hypoxic-ischemic brain injury. ASM was detected as a 50-kDa single band in 3 lysates, and it was co-detected with β-actin as an internal standard. The quantitative analyses of the combined bands showed that the ASM/β-actin ratio was significantly higher for the hypoxic-ischemic and hypoxic-ischemic plus acetaminophen lysates compared to those of controls at 48h after hypoxia-ischemia. The difference between hypoxia-ischemic and hypoxia-ischemic plus acetaminophen lysates was not significant. 1, control ; 2, hypoxic-ischemic ; 3, hypoxic-ischemic plus acetaminophen.

with 2 different buffers (EDTA and Zn^{2+}) for each sample. ASM is known to be activated by Zn^{2+} when ASM is secreted from cells during conditions of oxidative stress or after stimulation with some cytokines. If ASM activity is increased with the addition of Zn^{2+} in the buffer, it shows that the cell has been exposed to some extracellular stimuli. In cerebral cortex, the homogenates of hypoxic-ischemic injured tissue showed higher ASM activities than those of the contralateral tissues, with positive activation of ASM by the addition of Zn^{2+} (Fig. 8). Thus, the increased ASM activity was stimulated by the addition of Zn^{2+}. In the hippocampus, the homogenates of hypoxic-ischemic-injured tissue showed significantly higher ASM activities than those of the contralateral tissue, and it was positively activated by the addition of Zn^{2+} (Fig. 8). Thus, the administration of acetaminophen significantly decreased the ASM activity that was activated by hypoxia-ischemia.

Western blot analysis

The ASM levels of 3 lysates of the control, hypoxic-ischemic, and hypoxic-ischemic plus acetaminophen cerebral tissues were analyzed by western blotting. ASM was detected as a 50-kDa single band in the 3 lysates, and it was co-detected with β-actin, which was used as an internal standard (Fig. 9). The quantitative analyses of the combined bands showed that the ASM/β-actin ratios were significantly higher in the hypoxic-ischemic and hypoxic-ischemic plus acetaminophen lysates compared to that of control at 48 h after hypoxia-ischemia, as shown in Fig. 4. The difference between the hypoxic-ischemic and hypoxic-ischemic plus acetaminophen lysates was not significant.

Discussion

The results of this study showed the neuroprotective effects of acetaminophen in neonatal hypoxic-ischemic rat model. Acetaminophen significantly decreased the macroscopic brain injury score ratings at 168 h after hypoxia-ischemia compared to those of vehicle controls. Quantitative neuropathological analyses demonstrated that the numbers of apoptotic and necrotic cells in the examined brain regions were reduced by the administration of acetaminophen, suggesting that acetaminophen inhibited neuronal cell death in the tissues that were injured by hypoxia-ischemia. This suggestion was supported by the results of the percent brain damage values, which were also reduced by the administration of acetaminophen, although the reduction was not statistically significant.

In our previous studies, ascorbic acid that is administered intraventricularly or intraperitoneally has been shown to decrease hypoxic-ischemic brain injuries in

newborn rats[9,10]. Neuronal cell damage following hypoxic-ischemic brain injury is well-known to be partly caused by the production of free radicals and ROS. The free radicals and ROS that are produced have been shown to be scavenged by ascorbic acid, which is an antioxidant[9,10]. Recent reports have shown that acetaminophen has antioxidant and anti-inflammatory properties that can be protective against neuronal cell death[1-3]. In addition, the neuroprotective properties of acetaminophen have been shown to originate from the reduction of apoptosis through a mitochondrial-mediated mechanisms[4]. Our results showed that acetaminophen might be protective against brain injuries that are induced by hypoxia-ischemia through its antioxidant properties.

In this study, we determined the ASM activities of brain tissues after hypoxia-ischemia. In lysates of cerebral and hippocampal regions that were injured by hypoxia-ischemia, ASM activities were significantly increased compared to those of control tissues. The results of the ASM activity were compatible to the data of the western blotting of ASM (Fig. 8 and Fig. 9). This study showed that the activation of ASM might be caused by the induction of ASM protein in the tissues injured by hypoxia-ischemia. In addition, the ASM enzymes of the lysates were activated by the addition of Zn^{2+}, demonstrating that the ASM enzyme was secreted from the cells that were stimulated by extracellular oxidative stress, ROS, or cytokines. The results suggested that the activation of ASM might be involved in the mechanisms that cause brain injury after hypoxia-ischemia. This study also showed that the administration of acetaminophen reduced the ASM activity that had been activated by hypoxia-ischemia. The results suggested that acetaminophen might reduce extracellular oxidative stress through its antioxidant property and attenuate the activation of ASM.

ASM has been reported to be involved in some neuronal disorders, including depression, Alzheimer's disease (AD), and ischemia[12]. In a recent prospective study, ASM activity has been found to be higher than normal in a population of patients experiencing a major depressive episode and who were free of antidepressant drugs[17]. Regarding AD, several reports have found higher levels of ceramide in the brains of AD patients, even in the earliest stages of the disease[18]. Recently, it has been found that ASM activities are increased in the brains of AD patients compared to those of healthy, age-matched brains, providing a possible mechanism for the increased ceramide[12]. In addition, using cultured rat neurons, it has been found that amyloid beta peptide, which is the main constituent of AD plaques, induced increase in the levels of expression of ASM[12]. ASM has also been reported to play a role in cerebral ischemia. In wild-type mice and not ASM-knockout mice, an experimental model of transient focal cerebral ischemia resulted in increased levels of ASM and ceramide, and the production of inflammatory cytokines. In addition, wild-type mice displayed larger infarct sizes and worse behavioral outcomes than ASM knockout mice[19]. These reports suggest that ASM plays a complex role in the pathogenesis of several common neurological diseases. Our results were comparable to the data shown in a report on an experimental model of transient focal cerebral ischemia, which suggested the involvement of ASM in the pathogenesis of neonatal hypoxic-ischemic brain injury.

Recently, many functional inhibitors of ASM have been reported to be potential clinical medications, because ASM has been shown to play a role in the pathogenesis of common diseases, including cardiovascular diseases, diabetes, pulmonary diseases, and neuronal diseases[20,21]. Our results suggested an indication of these ASM inhibitors for the treatment of neonatal hypoxic-ischemic brain injury.

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