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Robust Protective Effects of a Novel Multimodal Neuroprotectant Oxopropanoyloxy Benzoic Acid (a Salicylic Acid/Pyruvate Ester) in the Postischemic Brain

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ABSTRACT

Cerebral ischemia leads to brain injury via a complex series of pathophysiological events. Therefore, multidrug treatments or multitargeting drug treatments are attractive options in efficiently limiting brain damage. Here, we report a novel multifunctional compound oxopropanoyloxy benzoic acid (OBA-09), a simple ester of pyruvate and salicylic acid. This protective effect was manifested by recoveries from neurological and behavioral deficits. OBA-09 exhibited antioxidative effects in the postischemic brain, which was evidenced by remarkable reduction of lipid peroxidation and 4-hydroxy-2-nonenal staining in OBA-09-administered animals. Reactive oxygen species generation was markedly suppressed in primary cortical cultures under oxygen-glucose deprivation. More interestingly, OBA-09 was capable of scavenging hydroxyl radical in cell-free assays. High-performance liquid chromatography results demonstrated that OBA-09 was hydrolyzed to salicylic acid and pyruvate with $t_{1/2} = 43$ min in serum and 4.2 h in brain parenchyma, indicating that antioxidative function of OBA-09 is executed by itself and also by salicylic acid after the hydrolysis. In addition to antioxidative function, OBA-09 exerts anti-excitotoxic and anti-Zn^{2+}-toxic functions, which might be attributed to attenuation of ATP and nicotinamide adenine dinucleotide depletion and to the suppression of nuclear factor-κB activity induction. Together these results indicate that OBA-09 has a potent therapeutic potential as a multimodal neuroprotectant in the postischemic brain and these effects were conferred by OBA-09 itself and subsequently its hydrolyzed products.

Introduction

Ischemic stroke produces both immediate and long-term effects on neuronal death. Although it is initiated by the absence of a blood supply and the lack of oxygen delivery to affected brain regions, brain injury in the postischemic brain progresses through a complex series of pathophysiological events, involving glutamate excitotoxicity, oxidative stress, inflammation, and apoptosis, which cumulatively lead to neuronal death (Dirnagl et al., 1999). Excitotoxicity and Zn^{2+} toxicity are responsible for acute and massive neuronal death in the ischemic core (Lipton, 1999), whereas delayed neuronal injury that occurs in the surrounding regions, called "the penumbra," is caused by postischemic inflammation and apoptosis (Graham and Chen, 2001). The delayed neuronal death may occur over a few hours to days after the primary ischemic event and insidiously extends brain damage (Kirino, 2000).

We showed recently that combination treatment of ethyl pyruvate and aspirin (acetylsalicylic acid) provides synergistic neuroprotection in the postischemic brain (Kim et al., 2010). Ethyl pyruvate has been reported to potently suppress infarct formation in the postischemic brain (Kim et al., 2005;

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ABBREVIATIONS: OBA-09, oxopropanoyloxy benzoic acid; HPLC, high-performance liquid chromatography; ROS, reactive oxygen species; HNE, 4-hydroxy-2-nonenal; MCAO, middle cerebral artery occlusion; mNSS, modified neurological severity score; TTC, 2,3,5-triphenyl tetrazolium chloride; MEM, minimum essential medium; DIV, days in vitro; OGD, oxygen-glucose deprivation; EBSS, Earle's balanced salt solution; DCF, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-[4,5-dimethylthiazol-2-yo]-2,5-diphenyltetrazolium bromide; MDA, malondialdehyde; NF-κB, nuclear factor-κB; ESI, electrospray ionization; MS, mass spectrometry; LC, liquid chromatography; NMDA, *N*-methyl-D-aspartate.

Yu et al., 2005). In cells, ethyl pyruvate is converted to pyruvate, a well known H_2O_2 scavenger (Desagher et al., 1997) and ameliorator of zinc toxicity (Sheline et al., 2000). Aspirin has been used for primary and secondary stroke prevention for decades. It is beneficial for stroke patients in part due to its antiplatelet effect (Antithrombotic Trialists' Collaboration, 2002). In addition, aspirin also has anti-inflammatory (Pillinger et al., 1998; Feldman et al., 2001) and antiexcitotoxic effects (De Cristóbal et al., 2001; Castillo et al., 2003), which seem to complement the neuroprotective effects of ethyl pyruvate (Kim et al., 2010).

In view of the multifactorial nature of cerebral ischemia, a multimechanism-based approach to drug development might be needed to maximize the efficacy of the drug treatment. To find the best way to embody the synergistic neuroprotective effect afforded by the ethyl pyruvate/aspirin combination, we produced oxopropanoyloxy benzoic acid (OBA-09), a simple ester of pyruvate and salicylic acid, main metabolites of pyruvate and aspirin, respectively. OBA-09 was designed to incorporate pyruvate and salicylic acid and to release them slowly in vivo via ester hydrolysis. In the present study, we showed that OBA-09 exerted robust neuroprotective effect in the postischemic brain, resulting in marked reduction of infarct volume and substantial recovery of neurological and behavioral deficits. We also provided evidences that support that neuroprotective effects of OBA-09 were afforded via multiple mechanisms, which were executed by OBA-09 itself and also by pyruvate and salicylic acid after the hydrolysis.

Materials and Methods

Surgical Procedures for Middle Cerebral Artery Occlusion. Middle cerebral artery occlusion (MCAO) was carried out as described previously (Kim et al., 2006). In brief, male Sprague-Dawley rats (250–300 g) were anesthetized with 5% isoflurane and anesthesia was maintained using 0.5% isoflurane during operation. MCA occlusion was performed for 1 h using a nylon suture, and this was followed by reperfusion. The left femoral artery was cannulated for blood sampling to analyze pH, PaO_2, $PaCO_2$, and blood glucose concentration (I-STAT; Sensor Devises, Waukesha, WI). Regional cerebral blood flow was monitored using a laser Doppler flowmeter (Periflux System 5000; Perimed, Jarfalla, Sweden). A thermoregulated heating pad was used to maintain a rectal temperature of 37 ± 0.5°C. Sham group was operated in an identical manner but the MCA was not occluded. All experiments were carried out in accordance with The Guidelines for Animal Research issued by Inha University School of Medicine (Inchon, Republic of Korea).

Treatment with OBA-09, Sodium Pyruvate, or Salicylic Acid. Sodium pyruvate (5 mg/kg) and salicylic acid (5 mg/kg) were administered intravenously in a 0.3-ml injected volume 3 or 6 h after MCAO. OBA-09 (1, 2.5, 5, or 10 mg/kg) was administered intravenously in 0.3-ml injected volume 30 min before or 6 or 12 h after MCAO. OBA-09, sodium pyruvate, or salicylic acid was dissolved in distilled water.

Evaluation of a Modified Neurological Severity Scores. Neurological deficits were evaluated using modified neurological severity scores (mNSS) at indicated days as described previously (Chen et al., 2001). The mNSS system consists of motor, sensory, balance, and reflex tests, all of which are graded using a scale of 0 to 18 (normal, 0; maximal deficit, 18).

Rota-Rod Test. Twenty-four hours before MCAO, rats were conditioned on a rota-rod unit (Daejon Instrument, Seoul, Korea) at a constant 3 rpm until they were able to remain on the rotating spindle for 180 s. One day after MCAO, each rat was subjected to test trial on the rota-rod at 5 rpm. Thereafter, the residence times on the rota-rod at 10 or 15 rpm were measured with a 1-h intertrial interval.

Assessment of Infarct Volume. Rats were decapitated 2 days after MCAO, and whole brains were dissected coronally into 2-mm brain slices using a metallic brain matrix (RBM-40000; Arlington Scientific Inc., Springville, UT). Slices were immediately stained by immersion in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 15 min and then treated with 4% paraformaldehyde. The areas of infracted tissue were measured using Scion Image program (Scion Corporation, Frederick, MD). To count for cerebral edema and differential shrinkage resulting from tissue processing, areas of ischemic lesions were calculated as the ratio of the contralateral to ipsilateral hemisphere multiplied by the area of infarct. The infarct volumes were calculated (in cubic millimeters) by multiplying the summed section infarct areas by section thickness.

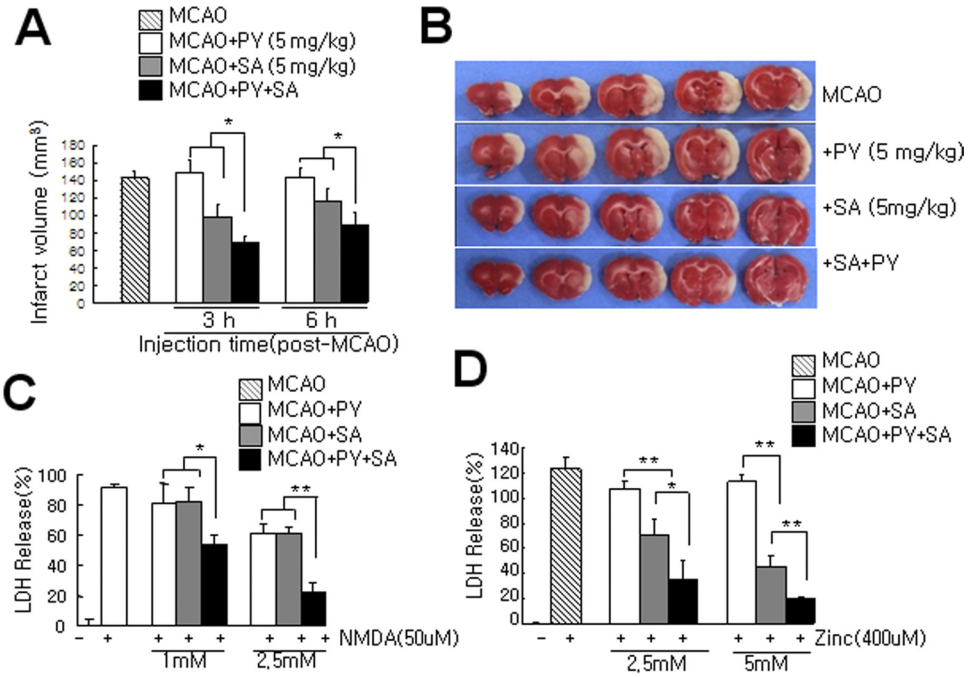

Fig. 1. Enhanced protective effect of combination treatment of pyruvate and salicylic acid in the postischemic brain and in primary cortical cultures. A, pyruvate (5 mg/kg) and salicylic acid (5 mg/kg) were administered intravenously individually or in combination at 3 or 6 h after 1 h of MCAO. Mean infarction volumes were assessed 2 days after MCAO by TTC staining and are presented as means ± S.E.M. (n = 4–6). B, representative images of infarctions on coronal brain section are presented. C and D, LDH levels in primary cortical cultures were measured 24 h after NMDA (50 μM, 10 min) (C) or Zn^{2+} (400 μM, 15 min) (D) treatment in the presence or absence of pyruvate and/or salicylic acid (1, 2.5, or 5 mM). LDH levels are presented as means ± S.E.M. (n = 4). MCAO, saline-treated MCAO group; +PY, pyruvate (5 mg/kg)-administered MCAO group; +SA, salicylic acid (5 mg/kg)-administered MCAO group; +PY+SA, pyruvate (5 mg/kg) and salicylic acid (5 mg/kg) coadministered MCAO group. *, $p < 0.05$; **, $p < 0.01$.

Preparation of Primary Cortical Cultures. Mixed cortical cultures, including neurons and astrocytes, were prepared from embryonic day 15.5 mouse cortices and cultured as described previously by Kim et al. (2006). Dissociated cortical cells were plated at a density of approximately 4×10^5 cells/well [five hemispheres per 24-well poly(D-lysine)- and laminin-coated plate]. Cultures were maintained without antibiotics in MEM containing 5% fetal bovine serum, 5% horse serum, 2 mM glutamine, and 21 mM glucose. At day 7 in vitro (DIV 7), when astrocytes had reached confluence underneath neurons, cytosine arabinofuranoside was added to a final concentration of 10 μM, and cultures were maintained for 2 days to halt microglial growth. Fetal bovine serum and glutamine were not supplemented from day 7, and media were changed every other day after day 7. Cultures were used at DIV 12 to 14.

NMDA and $ZnSO_4$ Treatment. Primary cortical cells were treated with serum-free MEM or HEPES-controlled salt solution containing 50 μM NMDA for 10 min or 400 μM $ZnSO_4$ (both from Sigma-Aldrich, St. Louis, MO) for 15 min. The medium was then removed and replaced with fresh MEM medium, and cells were cultured for 24 h.

Oxygen-Glucose Deprivation. Cultures of mixed cortical cells were prepared and used at DIV 12. The original media were removed, and the cell were washed with a glucose-free Earle's balanced salt solution (EBSS) at pH 7.4 and placed in fresh glucose-free EBSS. Cultures were then introduced into an incubator containing a mixture of 5% CO_2 and 95% N_2 at 37°C for 90 or 120 min. Control cultures were maintained in normal EBSS and in the incubator of normal conditions.

Cell Viability Assays. After treating cells with each chemical or oxygen-glucose deprivation (OGD) for the indicated times, 20 μl of Cell Counting Kit-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added. Cells were then incubated for 1 h, and optical densities were measured using a 96-well plate reader at 450 nm.

LDH Assays. After treating cells with each chemical or OGD for the indicated times, 50-μl aliquots of media and 50 μl of LDH assay reagent (Roche Diagnostics, Mannheim, Germany) were mixed in a 96-well plate and incubated for 1 h. Optical densities were measured using a 96-well plate reader at 490 nm.

Reactive Oxygen Species Quantification. Primary cortical cells were incubated for 30 min in MEM containing 5 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCF; Invitrogen). Cells were washed twice with phosphate-buffered saline, and fluorescence and differential interference contrast images were visualized under a Zeiss (Oberkochen, Germany) microscope. Quantitative analysis of the immunofluorescence data were carried out using ImageJ (http://rsbweb.nih.gov/ij/).

Sample Preparation for LC/ESI-MS. OBA-09 (15 mg/kg) was injected intravenously into naive animals. Plasma and brain tissue (cerebral cortex) samples were collected at 1, 2, 3, 4, 5, 10, 15, 20, and 25 h after the injection. All frozen samples were allowed to thaw on ice and were homogenized by vortexing. A 300-μl aliquot of plasma was transferred to a 1.5-ml centrifuge tube together with 20 μl of internal standard (5.0 μg/ml). For plasma, samples were centrifuged for 15 min at 5000 rpm. For brain homogenate, samples were centrifuged for 5 min at 1000 rpm, and the upper organic phase was transferred into clean tubes and evaporated in a vacuum oven at 40°C. The residues were then dissolved in 150 μl of acetonitrile. For both samples, the supernatant was transferred to an autosampler vial, and an aliquot of 10 μl was injected into the HPLC-MS/electrospray ionization (ESI) system for analysis. Protein precipitation by acetonitrile addition allows 2-(2-oxopropanoyloxy)benzoic acid (OBA) to be dissolved. For in vitro radical scavenging experiment, a mixture of 3.0 mM $FeCl_3$, 3.0 mM Na_2EDTA, and 30.0 mM H_2O_2 in 2 ml of Tris buffer, pH 7.4, was incubated with 2.5, 5.0, 10.0, or 20.0 mM OBA in the dark for 30 min at 37°C.

LC/ESI-MS. An LC/MS 2010EV liquid chromatography-mass spectrometer (Chiyoda-Ku, Tokyo, Japan) equipped with an ESI probe and QoQ system (Q-array-octapole-quadrupole mass analyzer) was used. The chromatographic system consisted of an LC-20AD pump, a DGU-20A3 degasser, a Shimadzu SIL-20A autosampler, a CTO-20A column oven, and an SPD-M20A UV/vis photodiode array detector. The column ZORBAX Eclipse-C18 (150 mm × 4.6 mm internal diameter packed with C18 silica, particle size of 5.0 μm; Agilent Technologies, Inc., Santa Clara, CA) was used. For detection of OBA-09 and salicylic acid, chromatography was carried out in isocratic mode with a 50:50 mixture of 0.1% formic acid acetonitrile and 0.1% formic acid water, and the flow rate was 0.3 ml/min. For detection of hydroxylated OBA-09, chromatography was carried out in isocratic mode with a 20:80 mixture of 0.1% formic acid acetonitrile and 0.1% formic acid water, and the flow rate was 0.4 ml/min.

Measurement of NAD Level. NAD^+ concentrations were determined by a cyclic enzymatic assay. Twenty-four hours after MCAO, both hemispheres were separated and weighed. Tissues were treated with 0.5 M perchloric acid (Sigma-Aldrich) for 15 min at 4°C and homogenized on ice. Homogenates were neutralized with 0.5 M KOH for 1 h on ice and centrifuged at 3000 rpm for 15 min at 4°C. Supernatants react with 100 μl of premix containing 0.2 mM phenazine ethosulfate, 0.5 mM 3-[4,5-dimethylthiazol-2-yo]-2,5-diphenyltetrazolium bromide (MTT), 600 mM ethanol (Merck, Darmstadt, Germany), 0.5 mM EDTA, and 4 U of alcohol dehydrogenase (Sigma-Aldrich) in 120 mM sodium/bicine buffer, pH 7.8, in the dark at 37°C for 1 h. The absorbance at 570 nm was measured.

ATP Assay. The brain tissue was homogenized on ice in 500 μl of 0.3% trichloroacetic acid and 1 mM EDTA, and distilled water was used to precipitate the proteins. This was followed by centrifugation at 10,000*g* for 3 min at 4°C, and the supernatant was mixed with buffer containing 0.1 M Tris-acetate, pH 7.75. The determination of ATP contained in the solution was carried out using a bioluminescence assay kit (Sigma-Aldrich) according to the manufacturer's instructions.

Quantification of Lipid Peroxidation. Lipid peroxidation levels were measured by malondialdehyde (MDA) assay using Bioxythech MDA-586 kit (OxisResearch, Portland, OR). Brain tissue was homoge-

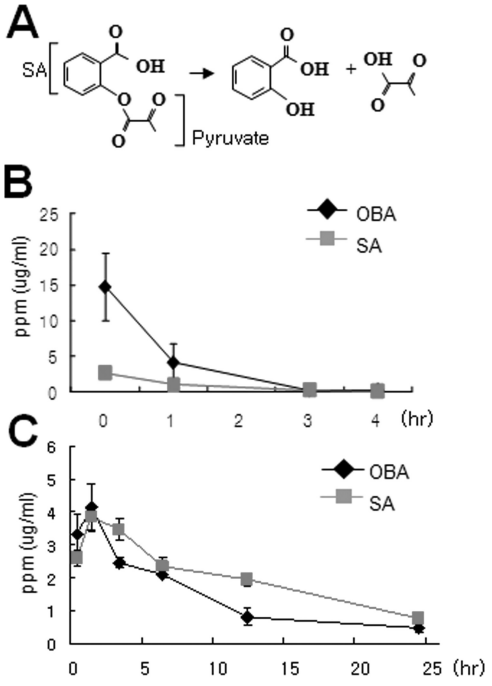

Fig. 2. Kinetics of the hydrolysis of OBA-09 and release of salicylic acid in vivo. A, structures of OBA-09 and its dissociated products, pyruvate and salicylic acid. B, OBA-09 (15 mg/kg) was injected intravenously into naive animals and concentration profiles of OBA-09 and salicylic acid in blood (B) and in brain tissue (cerebral cortex) (C) were monitored by LC/ESI-MS. Data are presented as means ± S.E.M. (n = 3).

nized in 4 volumes of ice-cold 20 mM phosphate-buffered saline containing 5 mM butylated hydroxytoluene. Homogenates were centrifuged at 3000*g* for 10 min at 4°C, and the supernatant was used for each assay. Equal amounts of proteins in each sample were reacted with a chromogenic reagent at 45°C for 60 min and centrifuged at 10,000*g* for 10 min. Supernatants were collected, and the absorbance at 586 nm was measured.

Anti-4-Hydroxy-2-nonenal Staining. Brains were fixed with 4% paraformaldehyde by transcardiac perfusion and stored in the same solution overnight at 4°C. Brain sections were prepared by cutting at 40 μm using a vibratome. Anti-4-hydroxy-2-nonenal (HNE) antibody (Alpha Diagnostic International, San Antonio, TX) was used at 1:100 of dilution. The number of HNE-positive cells in 0.1 mm^2 (0.32 $\times$ 0.32 mm^2) was quantified.

Nuclear Extract Preparation and NF-κB Activity Assay. Nuclear extracts were prepared using Nuclear Extraction kits (Imgenex, San Diego, CA) according to the manufacturer's instructions. NF-κB activity assays were carried out using NF-κB p65 Transcription Factor Assay Colorimetric kits (Millipore Bioscience Research Reagents, Temecula, CA) by following the manufacturer's instructions.

Western Blotting. Brain homogenates were immunoblotted as described previously (Kim et al., 2006). Primary antibodies were diluted at 1:1000 for anti-IκB-α, anti-p65 (both from Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α-tubulin (Calbiochem, San Diego, CA) antibodies and detected by using a chemiluminescence kit (Roche Diagnostics, Basel, Switzerland) using anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology).

Statistical Analysis. Statistical analysis was performed by analysis of variance followed by the Newman-Keuls test. All data are presented as means $\pm$ S.E.M., and a statistical difference was accepted at the 5% level.

Results

Synergistic Neuroprotective Effects of Pyruvate and Salicylic Acid in Primary Cortical Cultures and in the Postischemic Brain. In our previous report, we showed that combination treatment with ethyl pyruvate and aspirin enhances neuroprotection in the postischemic brain (Kim et al., 2010). Here, we confirmed the complementary effects of pyruvate and salicylic acid, the metabolic products of ethyl pyruvate and aspirin, respectively. Intravenous administrations of 5 mg/kg pyruvate 3 h after MCAO had no suppressive effect on infarct formation. However, when administered in combination with salicylic acid (5 mg/kg i.v., 3 h after

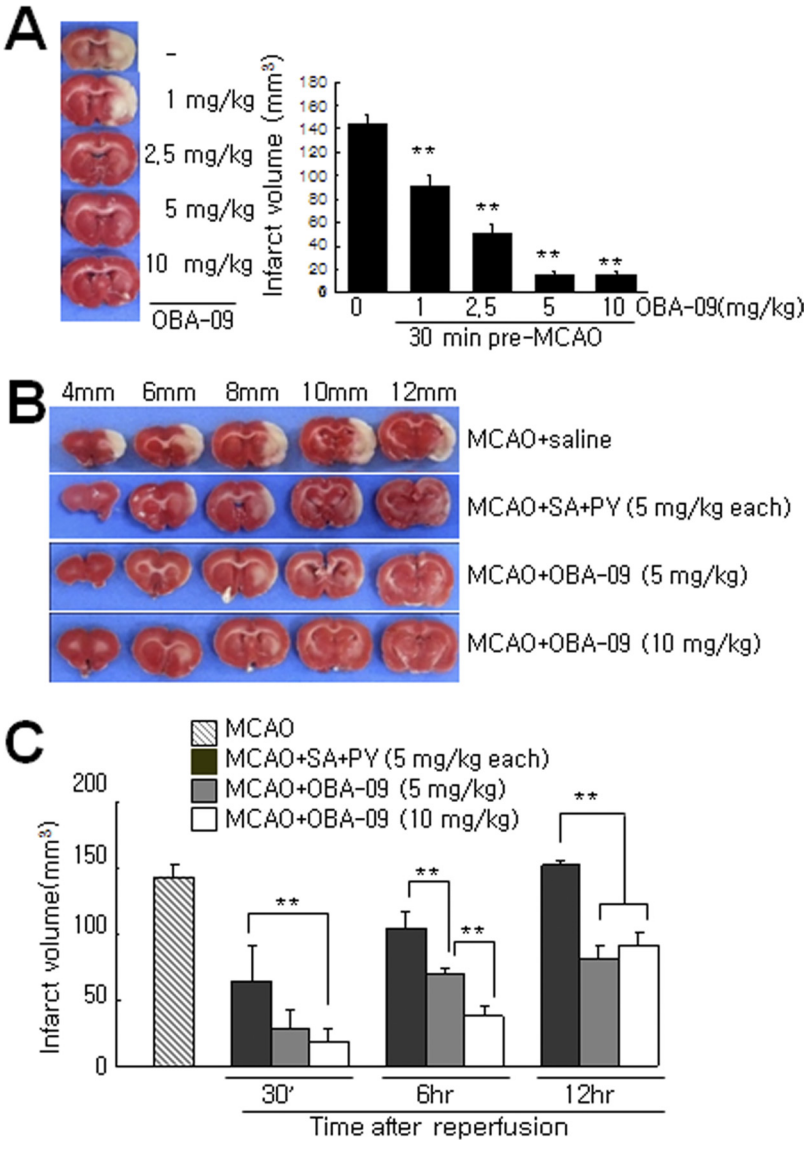

Fig. 3. Neuroprotective effects of OBA-09 in the postischemic brain. A, OBA-09 (1, 2.5, 5, and 10 mg/kg) was intravenously administered 30 min before MCAO. Mean infarction volumes were assessed at 2 days after MCAO by TTC staining. Data are presented as means $\pm$ S.E.M. (n = 5–6). Representative images of infarctions in coronal brain section are shown at the left. B and C, OBA-09 (5 or 10 mg/kg) or 5 mg/kg salicylic acid plus 5 mg/kg pyruvate were administered at 30 min, 6 or 12 h after MCAO (n = 6–8). Representative images of infarctions in coronal brain sections (10 mg/kg OBA-09, 30 min after administration) are presented (B). Mean infarction volumes assessed at 2 days after MCAO are presented as means $\pm$ S.E.M. (C; n = 6–8). MCAO, saline-treated MCAO group; MCAO+SA+PY, salicylic acid (5 mg/kg) and pyruvate (5 mg/kg) coadministered MCAO group; MCAO+OBA-09, OBA-09-administered MCAO group. **, $p < 0.01$.

MCAO), infarct volumes were reduced to 48.5 ± 4.3% ($n = 6$, $p < 0.01$) of that of untreated controls, which was significantly lower than that of salicylic acid-treated animals (68.5 ± 10.8%, $n = 6$) (Figs. 1A). Synergistic effects were also evident 6 h after treatment (Fig. 1, A and B). Likewise, a synergistic neuroprotective effect was observed in primary cortical cultures treated with NMDA (50 μM, 10 min) or Zn^{2+} (400 μM, 15 min). The protective effect of pyruvate plus salicylic acid was notably greater than the additive effects of pyruvate or salicylic acid (Fig. 1, C and D). Together these results indicate that treatment with pyruvate plus salicylic acid affords synergistic neuroprotection in vivo and in vitro.

The Extended Hydrolysis Kinetics of OBA-09 In Vivo. To find the best way to embody the synergistic neuroprotective effects of pyruvate plus salicylic acid, we generated OBA-09, an ester of pyruvate and salicylic acid (Fig. 2A). OBA-09 was designed to incorporate pyruvate and salicylic acid so as to release them slowly in vivo via ester hydrolysis (Fig. 2A). OBA-09 was subjected to LC/ESI-MS for monitoring the kinetics of the release of pyruvate and salicylic acid via ester hydrolysis in vivo. LC/ESI-MS analysis revealed that OBA-09

TABLE 1
Physiological parameters
Values are means ± S.D. ($n = 3$). One-way analysis of variance revealed no significant intergroup difference for any variance.

	Vehicle-Treated ($n = 3$)		OBA-09-Treated ($n = 3$)	
	Base	During Ischemia	Base	During Ischemia
Rectal temperature, °C	37.4 ± 0.15	37.5 ± 0.25	37.2 ± 0.15	37.5 ± 0.1
pH	7.5 ± 0.06	7.42 ± 0.03	7.47 ± 0.02	7.44 ± 0.03
pO_2, mm Hg	167 ± 11.5	172.7 ± 13.5	172.0 ± 7.2	174.3 ± 7.6
pCO_2, mm Hg	38.3 ± 5.2	39.9 ± 3.1	37.6 ± 2	43.9 ± 1.5
Glucose, mg/dl	123.6 ± 5.5	118 ± 11.3	130.6 ± 6.6	122.6 ± 3.2

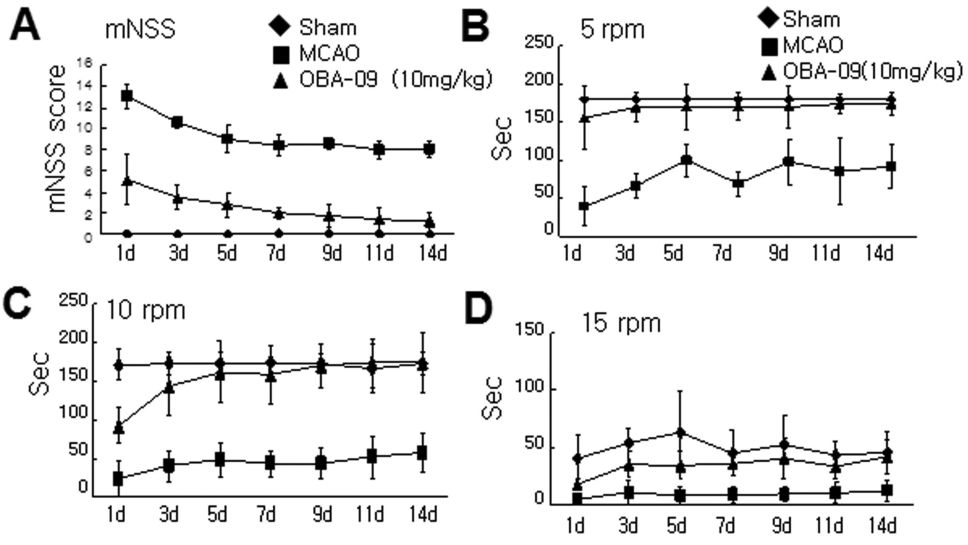

Fig. 4. Recovery of motor deficit by OBA-09. OBA-09 (10 mg/kg) was administered 1 h after MCAO. A, neurological deficits were evaluated using modified neurological severity scores at 1, 3, 5, 7, 9, 11, and 14 days after MCAO. B to D, the rota-rod test was performed at 5 (B), 10 (C), and 15 (D) rpm at 1, 3, 5, 7, 9, 11, and 14 days after MCAO. Sham, sham-operated group; MCAO, saline-treated MCAO group; MCAO+OBA-09, OBA-09-administered MCAO group. Data are presented as means ± S.E.M. ($n = 7$–12) *, $p < 0.05$; **, $p < 0.01$.

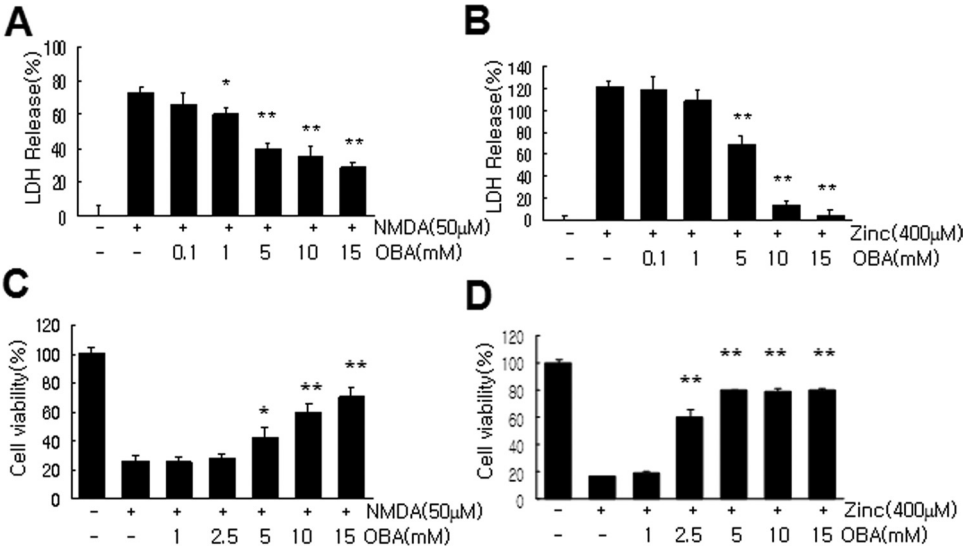

Fig. 5. Neuroprotective effects of OBA-09 in primary cortical cultures treated with NMDA or Zn^{2+}. LDH (A and B) and MTT assays (C and D) were carried out at 24 h after NMDA (50 μM, 10 min) (A and C) and Zn^{2+} (400 μM, 15 min) (B and D) treatment in the presence or absence of OBA-09 (0.1, 1, 5, 10, and 15 mM). The data are presented as means ± S.E.M. ($n = 7$–12). *, $p < 0.05$; **, $p < 0.01$.

decayed slowly with a $t_{1/2}$ of 42 min in serum and 4.2 h in brain parenchyma, and salicylic acid was released for up to 25 h after injection (Fig. 2B).

Robust Neuroprotective Effects of OBA-09 in the Postischemic Brain. When OBA-09 was administered intravenously at 1, 2, 5 or 10 mg/kg 30 min before MCAO, mean infarct volumes were reduced to 61.8 ± 6.6, 34.6 ± 5.1, 10.7 ± 1.9, and 10.1 ± 5.4%, respectively, of the untreated control (Fig. 3A). The administration of 10 mg/kg OBA-09 at 6 and 12 h after MCAO reduced mean infarct volumes to 27 ± 7.0 and 65 ± 10.2%, respectively (Fig. 3, B and C). Furthermore, the administration of 5 or 10 mg/kg OBA-09 even at 12 h after MCAO suppressed infarct volumes to 57.2 ± 9.9 and 65 ± 10.2% of the untreated controls (Fig. 3, B and C). Importantly, the efficacy of infarct suppression by OBA-09 (at 5 mg/kg) was far greater than combined treatment with salicylic acid (5 mg/kg) and pyruvate (5 mg/kg) at all time points examined (Fig. 3, B and C), implying additional beneficial functions of OBA-09 on top of those endowed by pyruvate and salicylic acid. Physiological parameters, namely, pH, PaO_2, $PaCO_2$, and blood glucose, were similar to those in OBA-09-treated and untreated animals (Table 1).

OBA-09 Improved Motor Impairment and Neurological Deficits of Animals with MCAO. Mean mNSS at 1 day after MCAO was 5.2 ± 2.3 when OBA-09 (10 mg/kg) was administered at 1 h after MCAO, and this value was significantly lower than that for the untreated MCAO group (13.1 ± 1.1) (Fig. 4A). mNSS for both untreated and OBA-09-treated animals gradually recovered, but the recovery of OBA-09 group was more dramatic and reached to the near normal level at 14 days after MCAO (Fig. 4A). Motor activities were assessed using the rota-rod test at a speed of 5 rpm and subsequently at 10 and 15 rpm (with a 1-h interval between tests) also showed notably better motor skills for OBA-09-treated animals (Fig. 4, C and D). This behavioral improvement shown by OBA-09-administered animals lasted for 14 days (Fig. 4, A–D).

Antiexcitotoxic and anti-Zn^{2+}-Toxic Effects of OBA-09 in Primary Cortical Cultures. Because pyruvate and salicylic acid are known to exert protective effects against excitotoxicity and Zn^{2+} toxicity (Maus et al., 1999; Moro et al., 2000; Sheline et al., 2000), we examined whether OBA-09 has similar functions. In NMDA-treated primary cortical cultures, OBA-09 reduced neuronal cell death in a dose-dependent manner, wherein 15 mM OBA-09 blocked cell death almost to 37.3% of the untreated control (Fig. 5A). Likewise, OBA-09 blocked Zn^{2+}-induced neuronal cell death in a dose-dependent manner, wherein 10 mM OBA-09 blocked cell death almost to the basal level (Fig. 5B). Protective effects of OBA-09 were confirmed by cell survival assay (Fig. 5, C and D). These results indicated that OBA-09 was endowed with antiexcitotoxic and anti-Zn^{2+}-toxic effects.

The Neuroprotective Effects of OBA-09 and Its Reactive Oxygen Species Scavenging Function in Primary Cortical Cultures. Next, we investigated whether OBA-09 confers antioxidative function. Primary cortical cultures were subjected to 90 or 120 min of OGD, and levels of cell death and reactive oxygen species (ROS) generation were examined. OBA-09 blocked OGD-induced cell death in a dose-dependent manner, wherein at 10 or 15 mM, OBA-09 blocked cell death almost to the basal level (Fig. 6A). Protective effects of OBA-09 were further confirmed by cell survival assay (Fig. 6B). The antioxidative potency of OBA-09 was examined by DCF staining. An increase in DCF fluorescence was detected 30 min after OGD (120 min) (Fig. 6, C and D). Treatment with 10 mM OBA-09 decreased DCF fluorescence to 51.7%, and treatment with 15 mM OBA-09 further decreased it to 27.9% (Fig. 6, C and D). These results demonstrated that OBA-09 has an antioxidative effect.

Suppression of Metabolic ROS Generation by OBA-09 in the Postischemic Brain. Antioxidative function of OBA-09 was further confirmed in the postischemic brains. Levels of lipid peroxidation (MDA assay) and HNE staining, two hallmarks of oxidative damage, were examined. MDA assay revealed that lipid peroxidation in penumbra in the superior prefrontal cortex (Fig. 7A) at 12 h after MCAO increased to 2.9-fold of the control (Fig. 7B). Intravenous administration of OBA-09 (5 mg/kg) significantly reduced lipid peroxidation levels to 1.3-fold of the

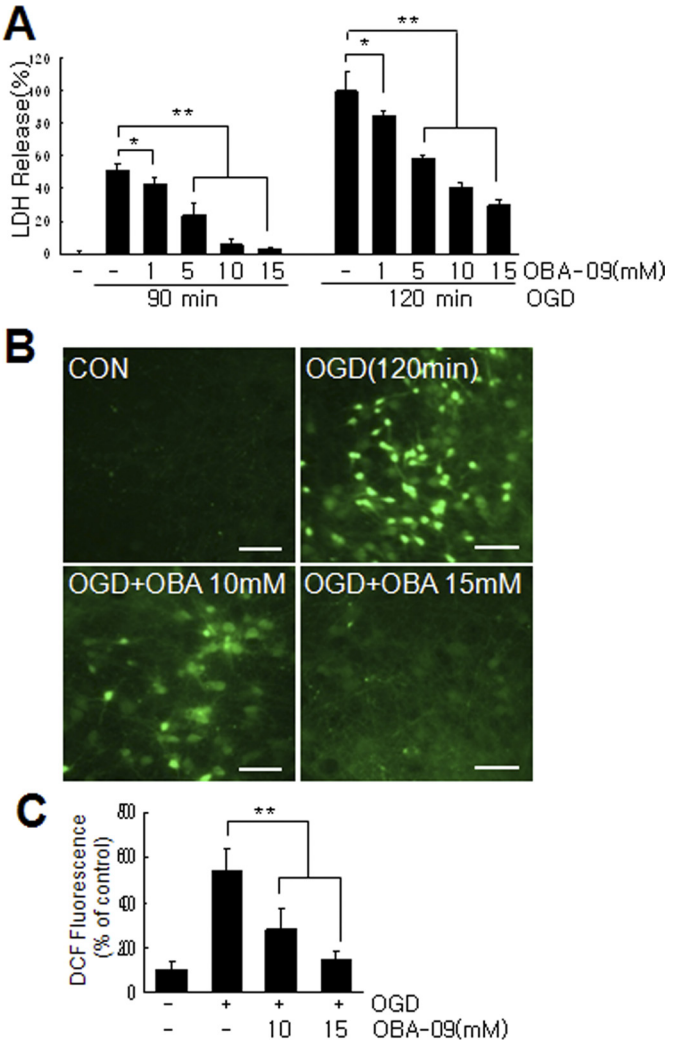

Fig. 6. OBA-09 inhibited OGD-induced ROS production in primary cortical cultures. A and B, LDH (A) and MTT assays (B) were carried out at 24 h after 90 or 120 min of OGD in the presence or absence of OBA-09 (1. 5, 10, and 15 mM). The data are presented as means ± S.E.M. ($n = 6$); *, $p < 0.05$; **, $p < 0.01$. C and D, intracellular ROS levels were assayed using 10 μM DCF 30 min after 120 min of OGD. Fluorescence (DCF) images were taken using a confocal laser microscope (C). Representative images from at least three independent experiments are presented. Quantitative analysis of the immunofluorescence data were carried out using ImageJ and the data are presented as means ± S.E.M. ($n = 6$) (D). Scale bars, 20 μm. *, $p < 0.05$; *, $p < 0.01$.

control (Fig. 7B). In addition, immunohistochemistry with anti-HNE antibody showed that the number of HNE-positive cells in penumbra in the superior prefrontal cortex was also reduced after treatment of 5 mg/kg OBA-09, and it was further reduced by 10 mg/kg OBA-09 (Fig. 7, C and D). Together, these results show that OBA-09 functions as a potent antioxidant in vivo and in vitro.

Hydroxyl Radical Scavenging by OBA-09 before the Hydrolysis. It has been reported that salicylic acid reacts with hydroxyl radical to form 2,3- and 2,5-dihydroxybenzoic acid (Sagone and Husney, 1987; Zhang and Piantadosi, 1994; Globus et al., 1995). The extended hydrolysis kinetics of OBA-09 in vivo (Fig. 2) prompted us to examine the possibility that OBA-09 uses antioxidative function before it is hydrolyzed. Results obtained from radical generation reaction in cell-free condition followed by HPLC analysis demonstrated that OBA-09 scavenged hydroxyl radical, generating 4-hydroxylated OBA-09 and 2-hydroxylated OBA-09 (Fig. 8, A and B). The amounts of hydroxylated OBA-09 were proportional to the amount of OBA-09, and 20 mM OBA-09 gave rise to 74 μg/ml 4-hydroxylated OBA-09, which is a main form of hydroxylated OBA-09, at 30 min after the incubation (Fig. 8C). Because this assay was carried out in a cell-free system, the result indicated that OBA-09 is able to scavenge hydroxyl radicals directly.

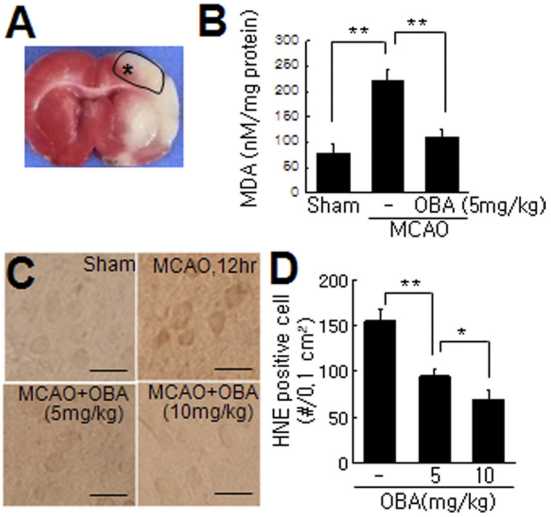

Fig. 7. ROS scavenging effects of OBA-09 in the postischemic brain. OBA-09 (5 or 10 mg/kg) was pretreated at 30 min before MCAO, and MDA assay and HNE staining were carried out 12 h after MCAO. B, MDA levels were examined in penumbras (indicated area in A) in postischemic brains treated or not with OBA-09 (5 mg/kg) ($n = 4$). C, representative images for 4-HNE staining in penumbras (asterisk in A) are presented. D, quantitative assessment of 4-HNE-positive cells was carried out in the indicated region (0.32×0.32 cm^2) ($n = 6$ or 7). Scale bars in C, 50 μm. *, $p < 0.05$; **, $p < 0.01$.

OBA-09 Prevented NAD and ATP Depletions in the Postischemic Brain. It has been reported that pyruvate attenuates zinc-induced neuronal death by inhibiting the depletion of NAD and ATP levels (Paschen et al., 1983; Eliasson et al., 1997; Sheline et al., 2000). Therefore, we investigated whether replenishment of ATP and NAD levels serve as a molecular mechanism whereby OBA-09 exerts neuroprotection in Zn^{2+}-treated primary cortical cultures and in the postischemic brain. ATP level in the cortex penumbra in ischemic hemispheres (Fig. 9A) was decreased to 59.4% of the normal brain 24 h after MCAO (Fig. 9B). It was recovered by OBA-09 (5 mg/kg) almost to the normal level, and the efficacy was greater than the combination treatment of salicylic acid plus pyruvate (5 mg/kg each) (Fig. 9B). Likewise, NAD level in ischemic hemispheres, which was decreased to 49.1% of that of contralateral hemisphere 24 h after MCAO, was also restored by OBA-09 (5 mg/kg i.v.) to 88.6% of the control (Fig. 9C). The efficacy was greater than that (74.1%) achieved by combination treatment of salicylic acid plus pyruvate (5 mg/kg each) (Fig. 9C).

OBA-09 Reduced NF-κB Activity in the Postischemic Brain. Salicylic acid suppresses NMDA-induced neuronal death by inhibiting IκB kinase-α (Ko et al., 1998; Yin et al., 1998). Four hours after MCAO, the amount of IκB-α in cytoplasm was significantly lower in cortex penumbra of ischemic hemispheres (Fig. 10, A and B). However, these decreases were suppressed by OBA-09 (5 mg/kg) and the suppression was similar to those obtained after administrating salicylic acid plus pyruvate (5 mg/kg each) (Fig. 10B). In contrast, levels of α-tubulin in cytoplasm were unchanged in all cases (Fig. 10B). Consistent with IκB-α regulation, the enhanced NF-κB activity in the ischemic cortex (8.3-fold of the control 12 h after MCAO) was suppressed to 2.7-fold of the control by OBA-09 treatment (5 mg/kg) (Fig. 10C). These results indicate that OBA-09 suppresses NF-κB activity by suppressing IκB-α degradation like salicylic acid. Together, these results indicate that OBA-09 uses molecular mechanisms similar to those of pyruvate and salicylic acid.

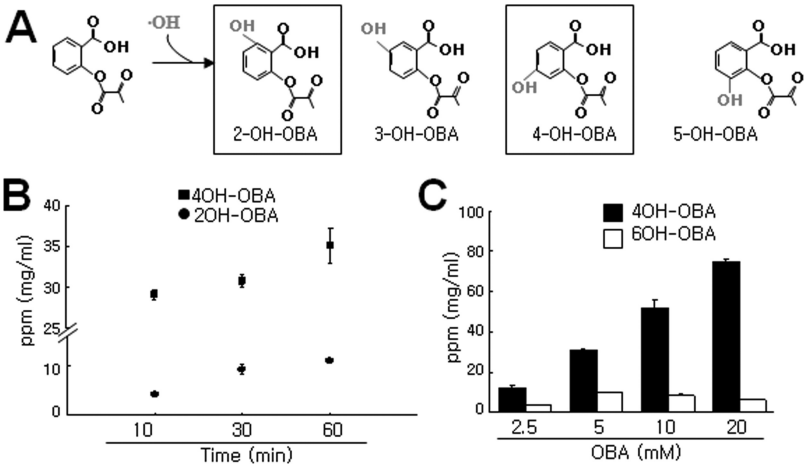

Fig. 8. Hydroxy radical scavenging by OBA-09. A, four hydroxylated OBA-09 products. B and C, hydroxylated OBA-09 was measured in a cell-free condition by HPLC. B, production of 4OH-OBA and 2OH-OBA at 10, 30, and 60 min after incubation ($n = 5$ or 6). C, 4OH-OBA and 2OH-OBA generations were examined after incubation of increasing concentrations of OBA-09 with hydroxyl radical for 30 min ($n = 3$ or 4).

Discussion

The present study demonstrates that OBA-09 affords robust neuroprotection in the postischemic brain with a wide therapeutic window. The results also indicate that those effects are achieved via several mechanisms, which were performed by OBA-09 itself and its hydrolyzed products, pyruvate and salicylic acid. Once being administered into the brain, pyruvate and salicylic acid are released from OBA-09 via ester hydrolysis in an extended time window ($t_{1/2}$ of 43 min in serum) (Fig. 2). The continuous supply of pyruvate at low levels is especially beneficial, because pyruvate has poor stability in solution and is spontaneously converted to parapyruvate (an inhibitor of a key step in the tricarboxylic acid cycle), which limits the usefulness of pyruvate as a therapeutic agent (Montgomery and Webb, 1956; Vonkorff, 1964). In this regard, it is worth noting that the dissociation constant of OBA-09 in brain tissue ($t_{1/2}$ of 4.2 h) was much higher than that in blood (Fig. 2). Delayed but sustained provision of salicylic acid and pyruvate explains the neuroprotective potency of OBA-09 after a single bolus administration. In addition, such a prolonged supplementation of pyruvate and salicylic acid might serve as the basis of the higher potency of OBA-09 compared with salicylic acid/sodium pyruvate cotreatment at equivalent concentrations. In addition, because OBA-09 is an ester, it can probably penetrate cells more rapidly, although this aspect requires further investigation.

Given the fact that excitotoxicity-induced acute neuronal death is followed by slowly occurring delayed neuronal death in the postischemic brain (Kirino, 2000), the observed wide therapeutic window of OBA-09 is of considerable importance in the postischemic brain. The delayed neuronal death in the penumbra persists for hours to days after the primary ischemic event and results in an expansion of the infarct and an inevitable worsening of neurological outcome (Dirnagl et al., 1999; Kirino, 2000). Multiple mechanisms, including postischemic inflammation, apoptosis (Graham and Chen, 2001), and oxidative stress (Chan, 2001), might be involved in the delayed neuronal injury. Moreover, it has been reported that energy deficiency occurring during ischemic insult impairs Na^+ and K^+-ATPase activity, which exacerbates cellular responses to oxidative stress and apoptotic insult (Chinopoulos et al., 2000; Wang et al., 2003). Therefore, remarkable ATP-replenishing effect of OBA-09 (Fig. 9), which is probably supplied by pyruvate derived from OBA-09, might antagonize the Na^+, K^+-pump failure and alleviate the delayed neuronal damage. Sustained supply of pyruvate, which functions also as a metabolic substrate, contributes to the delayed neuroprotective effect of OBA-09.

One of the observed beneficial effects of OBA-09 was its ability to reduce ROS, which we confirmed in vivo and in vitro (Figs. 6 and 7). ROS are produced during cerebral ischemia in various ways, especially after reperfusion, and perturb the prooxidant-antioxidant balance and damage cellular macromolecules, such as lipids, proteins, and nucleic acids (Love, 1999; Chan, 2001). In addition, oxidative stress also indirectly causes cellular damage, such as apoptosis and inflammation (Chan,

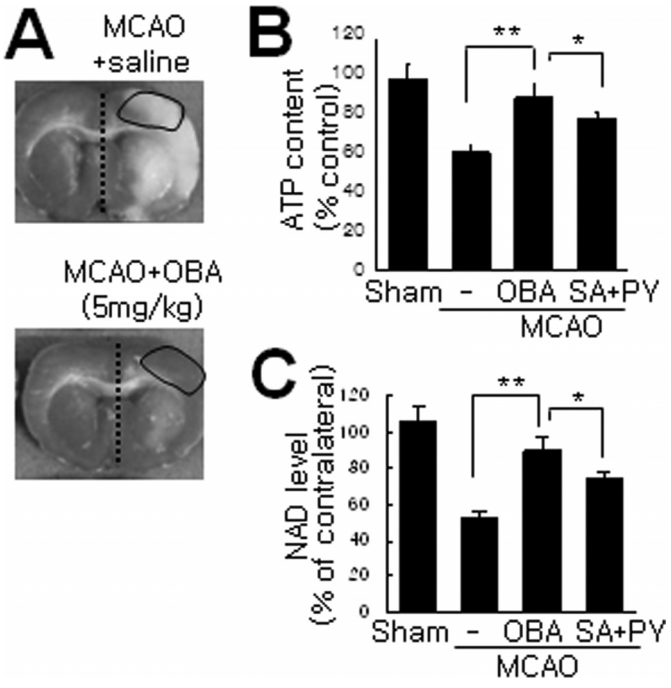

Fig. 9. Recovery from ATP and NAD depletion by OBA-09 in the postischemic brain. A, representative images of infarctions in animals with MCAO with or without OBA-09. OBA-09 (5 mg/kg) was administered 5 min after MCAO. B and C, ATP (B) and NAD (C) levels were examined 24 h after MCAO. OBA-09 (5 mg/kg) or salicylic acid plus pyruvate (5 mg/kg each) was administered 5 min after MCAO. Levels of ATP and NAD were examined in the indicated area and ischemic hemisphere as marked in A. Data are expressed as means ± S.E.M. ($n = 5$). *, $p < 0.05$; **, $p < 0.01$. Sham, sham-operated group; MCAO, saline-treated MCAO group; OBA, OBA-09-administered MCAO group; SA+PY, salicylic acid and pyruvate-coadministered MCAO group.

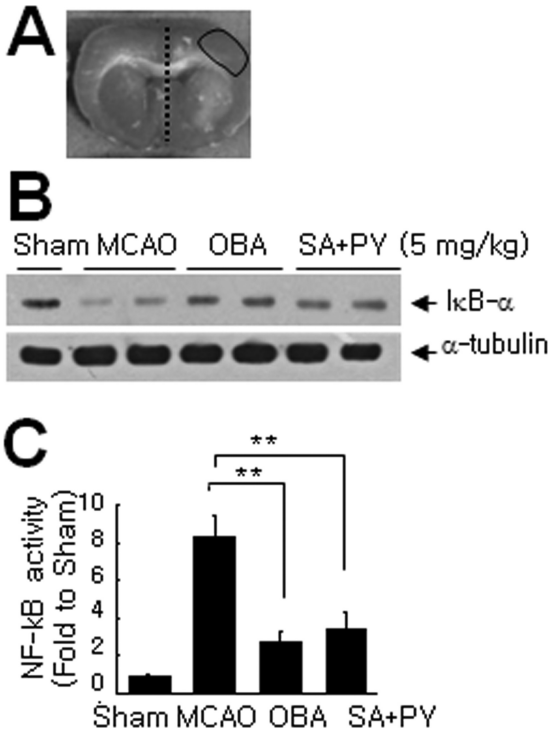

Fig. 10. Inhibition of NF-κB activation by OBA-09 in the postischemic brain. A, representative images of infarctions in animals with MCAO with OBA-09. OBA-09 (5 mg/kg) was administered 5 min after MCAO. B, Western blots showing cytosolic levels of IκB-α 4 h after MCAO. C, NF-κB activities were examined 12 h after MCAO. OBA-09 (5 mg/kg) or pyruvate plus salicylic acid (5 mg/kg each) was administered 5 min after MCAO. The brain region examined was from the indicated area in A. Data are expressed as means ± S.E.M. ($n = 5$). *, $p < 0.05$; **, $p < 0.01$. Sham, sham-operated group; MCAO, saline-treated MCAO group; OBA, OBA-09-administered MCAO group; SA+PY, salicylic acid and pyruvate-coadministered MCAO group.

2001; Chamorro, 2004). Among various sources of free radicals, OBA-09 effectively reduced the levels of hydroxyl radicals via hydroxyl radical scavenging function, which is a well known function of salicylic acid (Sagone and Husney, 1987). It is interesting to note here that OBA-09 is capable of exerting the hydroxyl radical scavenging function as it is (i.e., without being dissociated into pyruvate and salicylic acid) (Fig. 8). Thus, OBA-09 seems to exert antioxidative function initially probably through the salicylic acid moiety of the hybrid molecule and later through dissociated salicylic acid.

Excitotoxicity and Zn^{2+} toxicity are responsible for acute and massive neuronal death in the ischemic core of the postischemic brain (Lipton, 1999). In the present study, we showed that OBA-09 suppressed NMDA- and Zn^{2+}-induced neuronal cell death dose-dependently (Fig. 5). Regarding the molecular mechanism underlying these neuroprotective effects, it has been reported that salicylic acid and pyruvate use different mechanisms, namely, via the suppression of IκB-degradation in the cytoplasm by IκB kinase-β inhibition and via the suppression of NAD and ATP depletion, respectively (Yin et al., 1998; Maus et al., 1999; Moro et al., 2000). Here, we showed that OBA-09 recovered both NAD and ATP levels to almost the basal level and effectively suppressed IκB-α degradation, which suppresses NF-κB activity in the postischemic brain (Figs. 9 and 10). The suppression of NAD depletion also plays a critical role in protecting cells from Zn^{2+} toxicity (Sheline et al., 2000). Thus, the remarkable protective effects of OBA-09 in the postischemic brain seem to be derived in part by antiexcitotoxic and anti-Zn^{2+}-toxic effects initiated by pyruvate and salicylic acid.

In addition to antiexcitotoxic and antioxidative effects, we found that OBA-09 markedly suppressed LPS-induced microglia activation (S.-W. Kim and J.-K. Lee, unpublished data), which might be attributed in part by antioxidative function or NF-κB-inhibiting functions of OBA-09. Considering that oxidative stress triggers infiltration and migration of neutrophils and other leukocytes (Crack and Taylor, 2005), antioxidative effects of OBA-09 might be responsible for anti-inflammatory effects. Therefore, we speculate that the neuroprotective mechanism of OBA-09 in vivo might be produced through multiple mechanisms initiated by pyruvate and salicylic acid; among them, antioxidative and antiexcitotoxic functions were demonstrated in the current study. Although more targets of OBA-09 that produces neuroprotection remain to be elucidated, our results suggest a value of OBA-09 as a multimechanism-based therapeutic means to suppress cerebral ischemic injury with a wide therapeutic window.

Authorship Contributions

Participated in research design: S.-W. Kim, H. J. Kim, Han, Yoon, and J.-K. Lee.

Conducted experiments: S.-W. Kim, H. J. Kim, I.-D. Kim, and J.-E. Lee.

Contributed new reagents or analytic tools: H. J. Kim, J.-E. Lee, Yoon, and J.-K. Lee.

Performed data analysis: S.-W. Kim, H. J. Kim, J.-H. Kim, I.-D. Kim, Han, Yoon, and J.-K. Lee.

Wrote or contributed to the writing of the manuscript: Han and J.-K. Lee.

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