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Robust protective effects of a novel multi-modal neuroprotectant OBA-09 (a salicylic acid/pyruvate ester) in the postischemic brain

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Running title; Neuroprotection by OBA-09 in the postischemic brain

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ABSTRACT

Cerebral ischemia leads to brain injury via a complex series of pathophysiological events. Therefore, multi-drug treatments or multi-targeting drug treatments are attractive options in efficiently limiting brain damage. Here, we report a novel multi-functional 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 anti-oxidative effects in the postischemic brain, which was evidenced by remarkable reduction of lipid peroxidation and 4-hydroxy-2-nonenal **(**HNE) staining in OBA-09-administered animals. ROS 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 (HPLC) 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 anti-oxidative function of OBA-09 is executed by itself and also by salicylic acid after the hydrolysis. In addition to anti-oxidative function, OBA-09 exerts anti-excitotoxic and anti-Zn^{2+}-toxic functions, which might be attributed to attenuation of ATP and nicotinamide adenine dinucleotide (NAD) depletion and to suppression of NF-κB activity induction. Together these results indicate that OBA-09 has a potent therapeutic potential as a multi-modal neuroprotectant in the postischemic brain and these effects were conferred by OBA-09 itself and subsequently its hydrolyzed products.

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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 recently showed 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 (Yu et al., 2005; Kim 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 (Feldman et al., 2001; Pillinger et al., 1998) and anti-excitotoxic effects (De Cristobal et al., 2001; Castillo et al., 2003), which appear to complement the neuroprotective effects of ethyl pyruvate (Kim et al., 2010).

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In view of the multifactorial nature of cerebral ischemia, a multi-mechanism-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 which 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 MCA 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 (rCBF) 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.

Treatment with OBA-09, sodium pyruvate, or salicylic acid.

Sodium pyruvate (5 mg/kg) and salicylic acid (5 mg/kg) was administered intravenously in 0.3 ml of injected volume at 3 or 6 hrs post-MCAO. OBA-09 (1, 2.5, 5, or 10 mg/kg) was administered intravenously in 0.3 ml of injected volume at 30 min before or 6 or 12 hrs post-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

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(normal: 0, maximal deficit: 18).

Rota-rod test.

Twenty four hours before MCAO, rats were conditioned on a rota-rod unit 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. Subsequently, the residence times on the rota-rod at 10 or 15 rpm were measured with a 1 h inter-trial interval.

Assessment of infarct volume.

Rats were decapitated at 2 days after MCAO, and whole brains were dissected coronally into 2-mm brain slices using a metallic brain matrix (RBM-40000, ASI, 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 the Scion Image program (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 contra-lateral to ipsilateral hemisphere multiplied by the area of infarct. The infarct volumes were calculated (in mm^3) by multiplying summed section infarct areas by section thickness.

Preparation of primary cortical cultures.

Mixed cortical cultures, including neurons and astrocytes, were prepared from embryonic day 15.5 (E15.5) mouse cortices and cultured as described previously by Kim *et al*. (2006). Dissociated cortical cells were plated at a density of approximately $4x10^5$ cells per well (five hemispheres per 24-well poly-D-lysine and laminin-coated plate). Cultures were maintained

without antibiotics in MEM containing 5% FBS, 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 (ara-C) was added to a final concentration of 10 μM, and cultures were maintained for 2 days to halt microglial growth. Fetal bovine serum (FBS) and glutamine were not supplemented from day 7, and media were changed every other day after day 7. Cultures were used at DIV 12-14.

NMDA and $ZnSO_4$ treatment.

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

Oxygen-glucose deprivation (OGD)

Cultures of mixed cortical cells were prepared, used at 12 days in vitro (DIV). The original media were removed, 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℃ 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 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.

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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, 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.

ROS Quantification

Primary cortical cells were incubated for 30 min in MEM containing 5 μM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Eugene, OR). Cells were washed twice with PBS, and fluorescence and differential interference contrast (DIC) images were visualized under a Zeiss (Oberkochen, Germany) microscope. Quantitative analysis of the immunofluorescence data was carried out using image J (Window version; National institutes of Health)

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 hrs after the injection. All frozen samples were allowed to thaw on ice and homogenized by vortexing. A 300 μl aliquot 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 vacuum oven at 40 °C. The residues were then dissolved in 150 μl acetonitrile. For both samples, the supernatant was transferred

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to an autosampler vial and an aliquot of 10 μl was injected into the HPLC-MS/ESI system for analysis. Protein precipitation by acetonitrile addition allows 2-(2-oxopropanoyloxy)benzoic acid (OBA) 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, 20.0 mM OBA in the dark for 30 min at 37 °C.

LC/ESI-MS

A LC/MS 2010EV liquid chromatography-mass spectrometer (Chiyoda-Ku, Tokyo, Japan) equipped with an electrospray ionization (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 a SPD-M20A UV/vis photodiode array detector. The column ZORBAX Eclipse-C18 (150 mm × 4.6 mm i.d.; packed with C18 silica; particle size 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, St. Louis MO) 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

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ethosulfate, 0.5 mM MTT (3-[4,5-dimethylthiazol-2-yo]- 2,5-diphenyltetrazolium bromide), 600 mM ethanol (Merck, Darmstadt, Germany), 0.5 mM EDTA, and 4 U alcohol dehydrogenase (Sigma, St. Louis MO) 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% tricloroacetic acid and 1 mM EDTA, and distilled water 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, St. Louis, MO) according to the manufacturer's instruction.

Quantification of lipid peroxidation

Lipid peroxidation levels were measured by malondialdehyde (MDA) assay using Bioxythech MDA-586 kit (OxisResearch, Portland, OR). Brain tissue was homogenized in 4 volumes of ice-cold 20 mM PBS containing 5 mM butylated hydroxytoluene. Homogenates were centrifuged at 3,000 g for 10 min at 4℃ 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 minutes 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 (HNE) staining.

Brains were fixed with 4% paraformaldehyde by transcardiac perfusion and post-fixed in the

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same solution overnight at 4°C. Brain sections were prepared by cutting at 40 μm using a vibratome. Anti-HNE antibody (Alpha diagnostic, Woodlake, SA) was used at 1:100 of dilution. The number of HNE positive cells in 0.1 mm^2 (0.32 x 0.32 mm^2) were 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 (Chemicon, Temecula, CA) by following the manufacturer's instruction.

Western blotting.

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

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by the Newman-Keuls test. All data were presented as means±SEM and a statistical difference was accepted at the 5% level.

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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 of pyruvate 3 hrs post-MCAO had no suppressive effect on infarct formation. However, when administered in combination with salicylic acid (5 mg/kg. i.v., 3 hrs-post 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 at 6 hrs-post treatment (Figs. 1A and B). Similarly, a synergistic neuroprotective effect was observed in primary cortical cultures treated with NMDA (50 uM, 10 min) or Zn^{2+} (400 uM, 15 min). The protective effect of pyruvate plus salicylic acid was notably greater than the additive effects of pyruvate or salicylic acid (Figs. 1C 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

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kinetics of the release of pyruvate and salicylic acid via ester hydrolysis *in vivo*. LC/ESI-MS analysis revealed that OBA-09 decayed slowly with a $t_{1/2}$ of 42 min in serum and 4.2 hr in brain parenchyma, and salicylic acid was released for up to 25 hrs of post-injection (Fig. 2B).

Robust neuroprotective effects of OBA-09 in the postischemic brain

When OBA-09 was administered intravenously (i.v.) at 1, 2, 5 or 10 mg/kg at 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 of OBA-09 at 6 and 12 hrs post-MCAO reduced mean infarct volumes to 27±7.0% and 65±10.2%, respectively (Figs. 3B and C). Furthermore, the administration of 5 or 10 mg/kg of OBA-09 even at 12 hrs post-MCAO suppressed infarct volumes to 57.2±9.9% and 65±10.2% of the untreated controls (Figs. 3B 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 (Figs. 3B 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 modified neurological severity score (mNSS) at 1 day post-MCAO was 5.2±2.3 when OBA-09 (10 mg/kg) was administered at 1 hr post-MCAO, and this value was significantly lower than untreated MCAO group (13.1±1.1) (Fig. 4A). mNSSs 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 post-MCAO (Fig. 4A). Motor activities were assessed using the rota-rod test at a speed of 5 rpm, and subsequently at 10

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rpm and 15 rpm (with a 1 hr interval between tests) also showed notably better motor skills for OBA-09 treated animals (Figs. 4C and D). This behavioral improvement shown by OBA-09-administered animals lasted for 14 days (Fig. 4A-D).

Anti-excitotoxic and anti-Zn^{+2}-toxic effects of OBA-09 in primary cortical cultures

Since pyruvate and salicylic acid are known to exert protective effects against excitotoxicity and Zn^{+2}-toxicity (Moro et al., 2000; Maus et al., 1999; 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 un-treated control (Fig. 5A). Similarly, 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. 5C and D). These results indicated that OBA-09 was endowed with anti-excitotoxic and anti-Zn^{+2}-toxic effects

The neuroprotective effects of OBA-09 and its ROS scavenging function in primary cortical cultures

Next, we investigated whether OBA-09 confers anti-oxidative function. Primary cortical cultures were subjected to 90 min or 120 min of oxygen-glucose deprivation (OGD) and levels of cell death and 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 anti-oxidative potency of OBA-09 was examined by DCF staining. An increase in DCF fluorescence was detected 30 min after OGD (120 min) (Figs.

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6C 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% (Figs. 6C and D). These results demonstrated that OBA-09 has antioxidative effect.

Suppression of metabolic ROS generation by OBA-09 in the postischemic brain

Anti-oxidative 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 hrs post-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 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 of OBA-09, and it was further reduced by 10 mg/kg of OBA-09 (Figs. 7C 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 (DHBA) (Sagone and Husney, 1987; Globus et al., 1995; Zhang and Piantadosi, 1994). The extended hydrolysis kinetics of OBA-09 *in vivo* (Fig. 2) prompted us to examine the possibility that OBA-09 employs anti-oxidative 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. 8A and B). The amounts of hydroxylated OBA-09 were proportional to the amount of OBA-09 and 20 mM of OBA-09

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gave rise to 74 ug/ml of 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.

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 neuroptrotection 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 at 24 hrs post-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). Similarly, NAD level in ischemic hemispheres, which was decreased to 49.1% of that of contralateral hemisphere at 24 hrs post-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 (Yin et al., 1998; Ko et al., 1998). At 4 hrs post-MCAO, the amount of IκB-α in cytoplasm was significantly lower in cortex penumbra of ischemic hemispheres (Figs. 10A 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

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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 at 12 hrs post-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 the results from previous and this sections indicate that OBA-09 employs similar molecular mechanisms that pyruvate and salicylic acid have.

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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, since pyruvate has poor stability in solution and is spontaneously converted to parapyruvate (an inhibitor of a key step in the TCA cycle), which limits the usefulness of pyruvate as a therapeutic agent (Montgomery and Webb, 1956; von Korff, 1964). In this regard, it is worth to note here 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 as compared with salicylic acid/sodium pyruvate co-treatment at equivalent concentrations. In addition, since 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 occurred during ischemic insult impairs Na^+, 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 reactive oxygen species (ROS), which we confirmed *in vivo* and *in vitro* (Figs. 6 and 7). ROS are produced during cerebral ischemia in various ways, especially following reperfusion, and perturb the pro-oxidant-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, 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 appears to exert anti-oxidative function initially probably through the salicylic acid moiety of the hybrid molecule and later through dissociated salicylic acid.

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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 employ 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 (Moro et al., 2000; Yin et al., 1998; Maus et al., 1999). 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 appear to be derived in part by anti-excitotoxic and anti-Zn^{2+}-toxic effects initiated by pyruvate and salicylic acid.

In addition to anti-excitotoxic and anti-oxidative effects, we found that OBA-09 markedly suppressed LPS-induced microglia activation (unpublished), which might be attributed in part by anti-oxidative function or NF-kB inhibiting functions of OBA-09. Considering oxidative stress triggers infiltration and migration of neutrophils and other leukocytes (Crack and Taylor, 2005), anti-oxidative 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 anti-oxidative and anti-excitotoxic 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 multi-mechanism-based therapeutic means to suppress cerebral ischemic injury with a wide therapeutic window.

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Authorship contributions

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

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

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

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

Wrote or contributed to the writing of the manuscript: P-L Han, J-K Lee

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Footnotes

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Disclosure/Conflict of Interest

The authors declare no conflict of interest

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FIGURE LEGENDS

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 (i.v.) individually or in combination at 3 h or 6 h after 1 h of MCAO. Mean infarction volumes were assessed at 2 days post-MCAO by TTC staining and are presented as means±SEM (n=4-6). (B) Representative images of infarctions on coronal brain section are presented. (C,D) LDH levels in primary cortical cultures were measured at 24 h after NMDA (50 uM, 10 min) (C) or Zn^{2+} (400 uM, 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±SEM. (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) co-administered MCAO group.* $p < 0.05$, ** $p < 0.01$.

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±SEM (n=3).

Fig. 3. Neuroprotective effects of OBA-09 in the postischemic brain.

(A) OBA-09 (1, 2.5, 5, 10 mg/kg) was intravenously administered at 30 min before MCAO. Mean infarction volumes were assessed at 2 days post-MCAO by TTC staining. Data are presented as means±SEM (n=5-6). Representative images of infarctions in coronal brain

section are shown in the left panel. (B,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 hrs post-MCAO (n=6-8). Representative images of infarctions in coronal brain sections (10 mg/kg OBA-09, 30 min post-administration) are presented (B). Mean infarction volumes assessed at 2 days post-MCAO are presented as means±SEM (C; n=6-8). MCAO, saline-treated MCAO group; MCAO+SA+PY, salicylic acid (5 mg/kg) and pyruvate (5 mg/kg) co-administered MCAO group; MCAO+OBA-09, OBA-09-administered MCAO group. ** $p < 0.01$.

Fig. 4. Recovery of motor deficit by OBA-09.

OBA-09 (10 mg/kg) was administered at 1 hr post-MCAO. (A) Neurological deficits were evaluated using modified neurological severity scores at 1, 3, 5, 7, 9, 11, and 14 days post-MCAO. (B-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 post-MCAO. Sham, sham-operated group; MCAO, saline-treated MCAO group; MCAO+OBA-09, OBA-09-administered MCAO group. Data are presented as means±SEM (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, B) and MTT assays (C, D) were carried out at 24 hrs after NMDA (50 uM, 10 min) (A, C) and Zn^{2+} (400 uM, 15 min) (B, D) treatment in the presence or absence of OBA-09 (0.1, 1, 5, 10, 15 mM). The data are presented as means±SEM (n=7-12) * $p < 0.05$, ** $p < 0.01$.

Fig. 6. OBA-09 inhibited OGD-induced ROS production in primary cortical cultures

(A, B) LDH (A) and MTT assays (B) were carried out at 24 hrs after 90 min or 120 min oxygen-glucose deprivation (OGD) in the presence or absence of OBA-09 (1. 5, 10, 15 mM). The data are presented as means±SEM (n=6) * $p < 0.05$, ** $p < 0.01$. (C,D) Intracellular ROS levels were assayed using 10 μM DCF 12 hrs after 120 min OGD. Fluorescence (DCF) images were taken using a confocal laser microscope (C). Representative images from at least three independent experiments were presented. Quantitative analysis of the immunofluorescence data was carried out using image J and the data are presented as means±SEM (n=6) (D). Scale bars, 20 μm. * $p < 0.05$, *p<0.01.

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 at 1 day post-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) were presented. (D) Quantitative assessment of 4-HNE positive cells was carried out in the indicated region (0.32x0.32 cm^2) (n=6 or 7). Scale bars in C represent 50 μm. * $p < 0.05$, ** P<0.01.

Fig. 8. Hydroxy radical scavenging by OBA-09.

(A) Four hydroxylated OBA-09 products. (B, 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).

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 at 5 min post-MCAO. (B-C) ATP (B) and NAD (C) levels were examined at 24 hrs post-MCAO. OBA-09 (5 mg/kg) or salicylic acid plus pyruvate (5 mg/kg each) were administered at 5 min post-MCAO. Levels of ATP and NAD were examined in indicated area and ischemic hemisphere as marked in A. Data are expressed as means±SEM. (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 co-administered 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 at 5 min post-MCAO. (B) Western blots showing cytosolic levels of IκB-α at 4 hrs post-MCAO. (C) NF-κB activities were examined by at 12 hrs post-MCAO. OBA-09 (5 mg/kg) or pyruvate plus salicylic acid (5 mg/kg each) were administered at 5 min post-MCAO. Brain region examined was from the indicated area in (A). Data are expressed as means±SEM. (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 co-administered MCAO group.

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Table 1. Pysiological parameters

	Vehicle-treated group (n=3)		OBA-09-treated group (n=3)	
	Base	During ischemia	Base	During ischemia
Rectal Temperature(℃)	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 mmHg	167±11.5	172.7±13.5	172.0±7.2	174.3±7.6
pCO_2 mmHg	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

Values are Means±SD (N=3). One way analysis of variance revealed no significant intergroup difference for any variance.

Figure 1

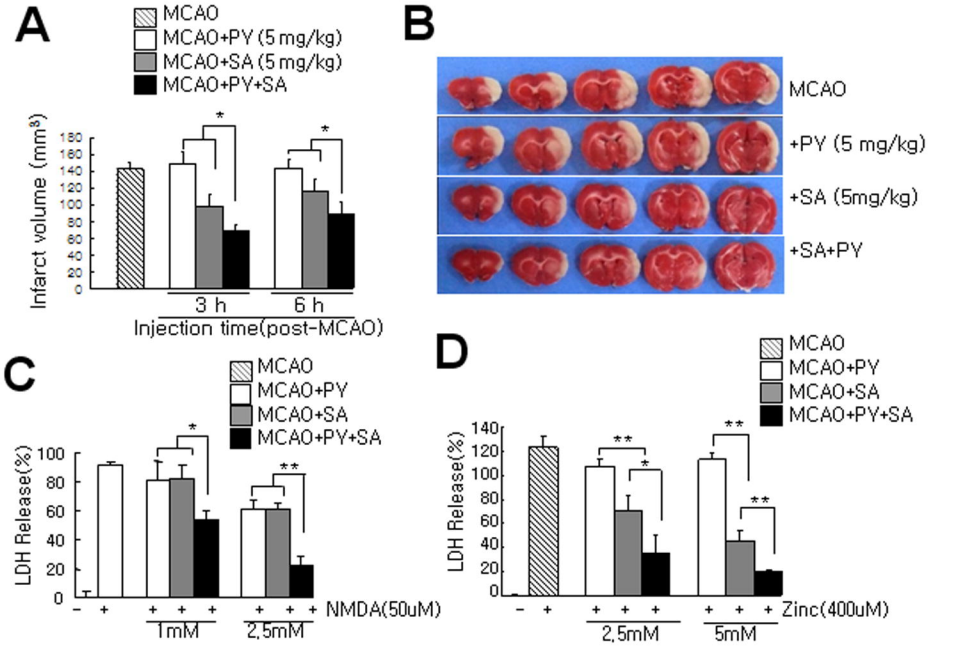

A
MCAO
MCAO+PY (5 mg/kg)
MCAO+SA (5 mg/kg)
MCAO+PY+SA
Infarct volume (mm³)
3 h
6 h
Injection time(post-MCAO)
B
MCAO
+PY (5 mg/kg)
+SA (5mg/kg)
+SA+PY
C
MCAO
MCAO+PY
MCAO+SA
MCAO+PY+SA
LDH Release(%)
NMDA(50uM)
1mM
2,5mM
D
MCAO
MCAO+PY
MCAO+SA
MCAO+PY+SA
LDH Release(%)
Zinc(400uM)
2,5mM
5mM

Figure 2

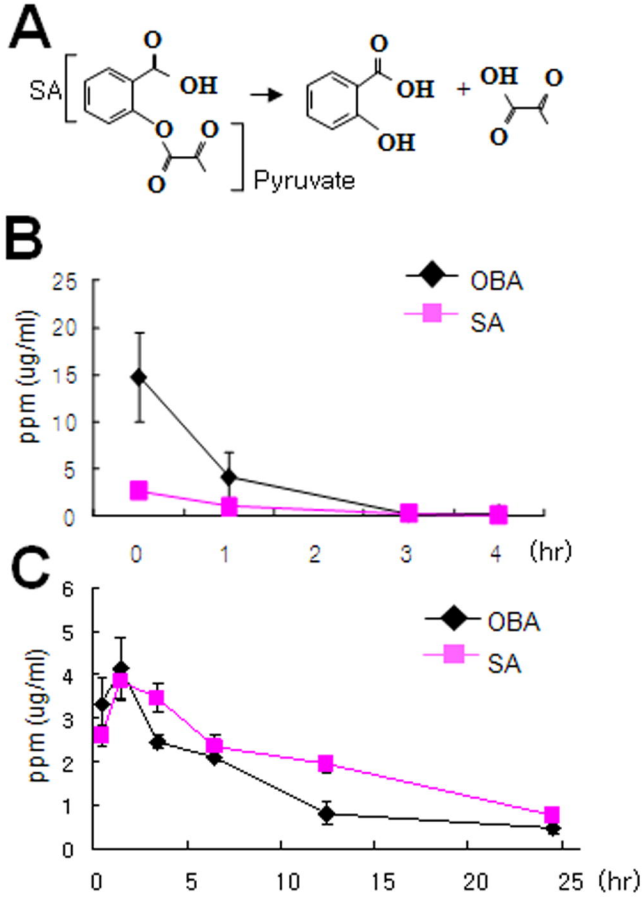
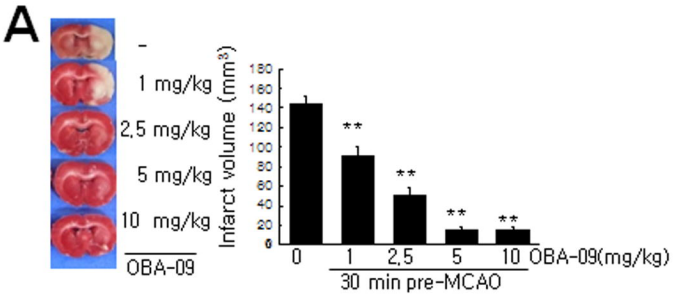
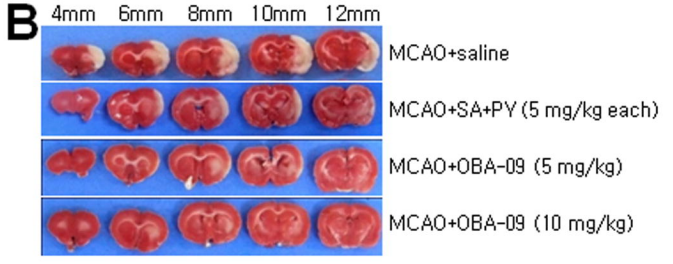

A
SA
OH
O
O
O
O
Pyruvate
OH
OH
OH
O
O
+
B
ppm (ug/ml)
25
20
15
10
5
0
OBA
SA
0
1
2
3
4
(hr)
C
ppm (ug/ml)
6
5
4
3
2
1
0
OBA
SA
0
5
10
15
20
25
(hr)

Figure 3


A
-
1 mg/kg
2.5 mg/kg
5 mg/kg
10 mg/kg
OBA-09
Infarct volume (mm³)
180
160
140
120
100
80
60
40
20
0
**
**
**
**
0
1
2,5
5
10
OBA-09(mg/kg)
30 min pre-MCAO


B
4mm
6mm
8mm
10mm
12mm
MCAO+saline
MCAO+SA+PY (5 mg/kg each)
MCAO+OBA-09 (5 mg/kg)
MCAO+OBA-09 (10 mg/kg)

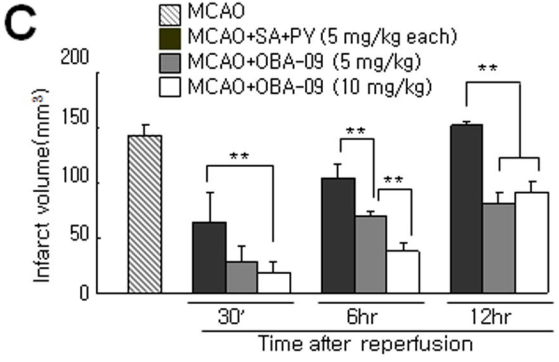

C
MCAO
MCAO+SA+PY (5 mg/kg each)
MCAO+OBA-09 (5 mg/kg)
MCAO+OBA-09 (10 mg/kg)
Infarct volume(mm³)
200
150
100
50
0
**
**
**
**
30'
6hr
12hr
Time after reperfusion

Figure 4

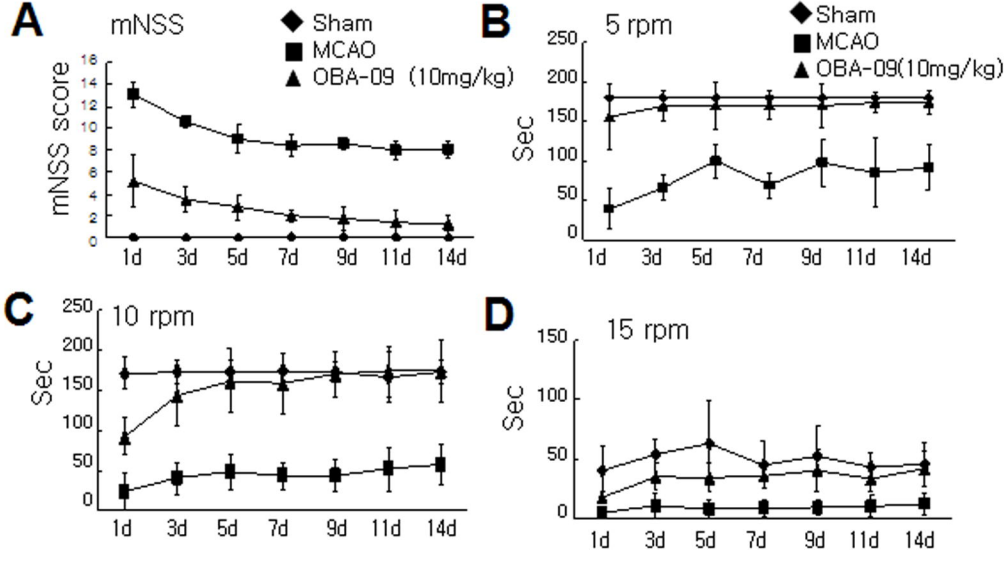

A
mNSS
Sham
MCAO
OBA-09 (10mg/kg)
mNSS score
16
14
12
10
8
6
4
2
0
1d
3d
5d
7d
9d
11d
14d
B
5 rpm
Sham
MCAO
OBA-09(10mg/kg)
Sec
250
200
150
100
50
0
1d
3d
5d
7d
9d
11d
14d
C
10 rpm
Sec
250
200
150
100
50
0
1d
3d
5d
7d
9d
11d
14d
D
15 rpm
Sec
150
100
50
0
1d
3d
5d
7d
9d
11d
14d

Figure 5

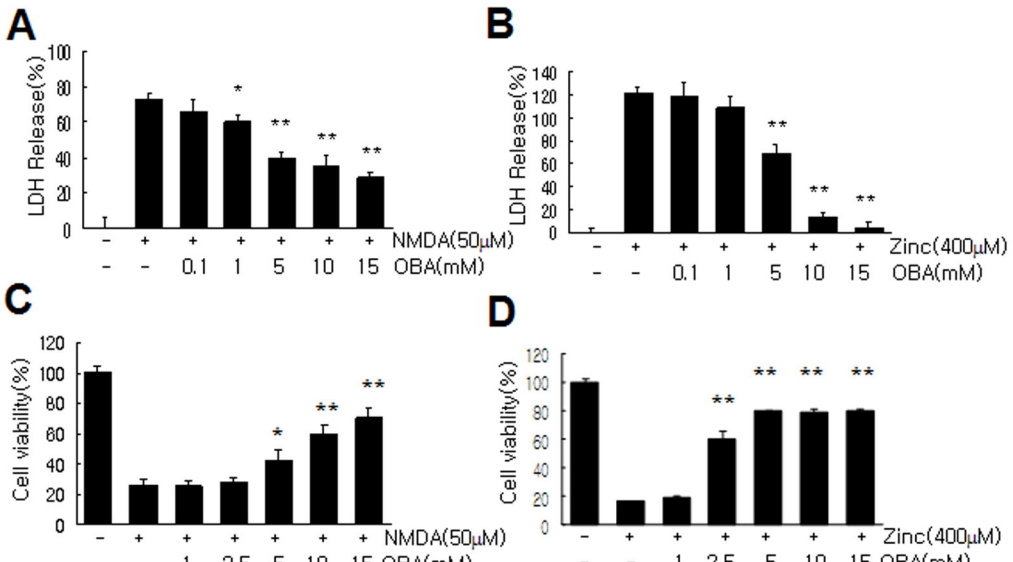
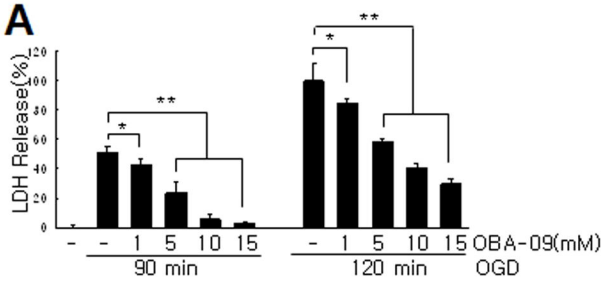
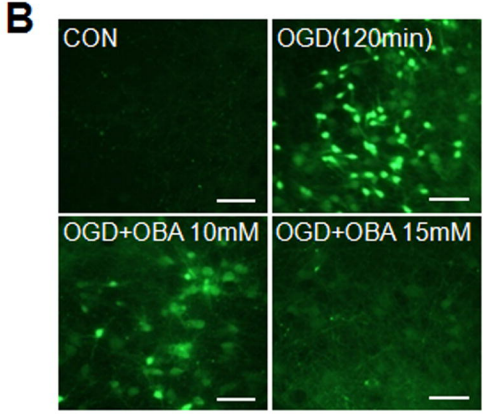

A
LDH Release(%)
100
80
60
40
20
0
*
**
**
**
- + + + + + + NMDA(50μM)
- - 0.1 1 5 10 15 OBA(mM)
B
LDH Release(%)
140
120
100
80
60
40
20
0
**
**
**
- + + + + + + Zinc(400μM)
- - 0.1 1 5 10 15 OBA(mM)
C
Cell viability(%)
120
100
80
60
40
20
0
*
**
**
- + + + + + + NMDA(50μM)
- - 1 2.5 5 10 15 OBA(mM)
D
Cell viability(%)
120
100
80
60
40
20
0
**
**
**
**
- + + + + + + Zinc(400μM)
- - 1 2.5 5 10 15 OBA(mM)

Figure 6


A
LDH Release(%)
120
100
80
60
40
20
0
**
*
**
*
- - 1 5 10 15 OBA-09(mM)
90 min
120 min
OGD


B
CON
OGD(120min)
OGD+OBA 10mM
OGD+OBA 15mM

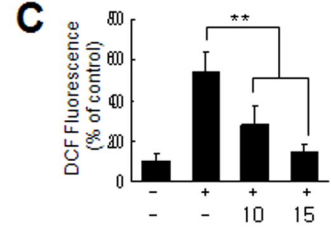

C
DCF Fluorescence
(% of control)
800
600
400
200
0
**
- + + + OGD
- - 10 15 OBA-09(mM)

Figure 7

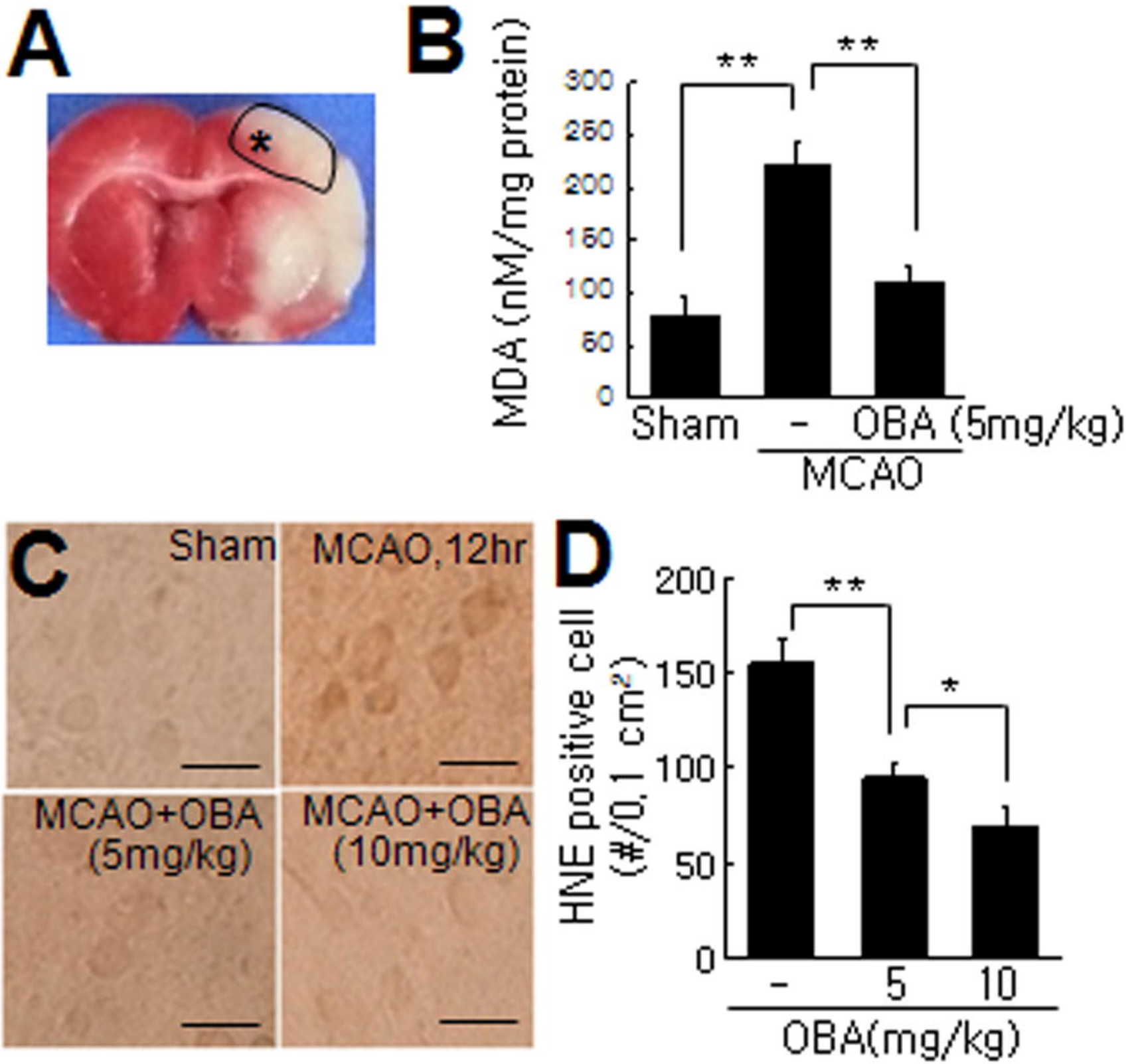
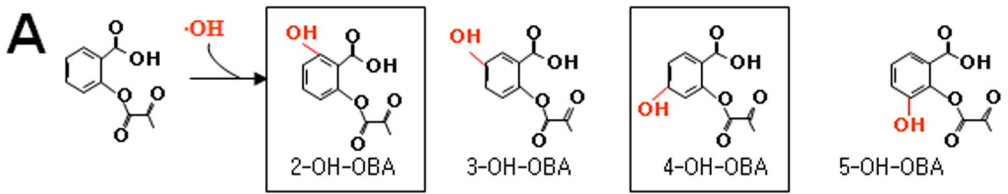
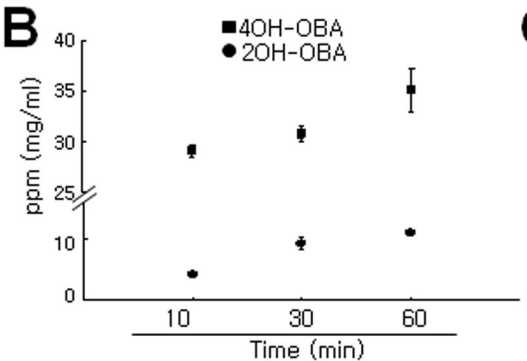

A
*
B
MDA (nM/mg protein)
300
250
200
150
100
50
0
**
**
Sham
-
OBA (5mg/kg)
MCAO
C
Sham
MCAO,12hr
MCAO+OBA
(5mg/kg)
MCAO+OBA
(10mg/kg)
D
HNE positive cell
(#/0.1 cm²)
200
150
100
50
0
**
*
-
5
10
OBA(mg/kg)

Figure 8


A
·OH
2-OH-OBA
3-OH-OBA
4-OH-OBA
5-OH-OBA


B
4OH-OBA
2OH-OBA
ppm (mg/ml)
40
35
30
25
10
0
10
30
60
Time (min)

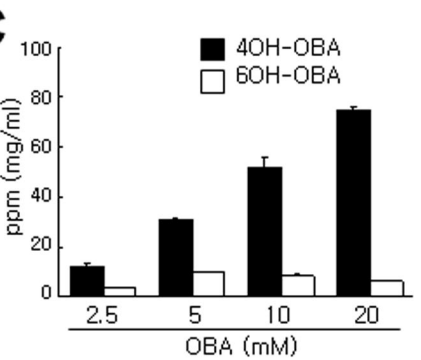

C
4OH-OBA
6OH-OBA
ppm (mg/ml)
100
80
60
40
20
0
2.5
5
10
20
OBA (mM)

Figure 9

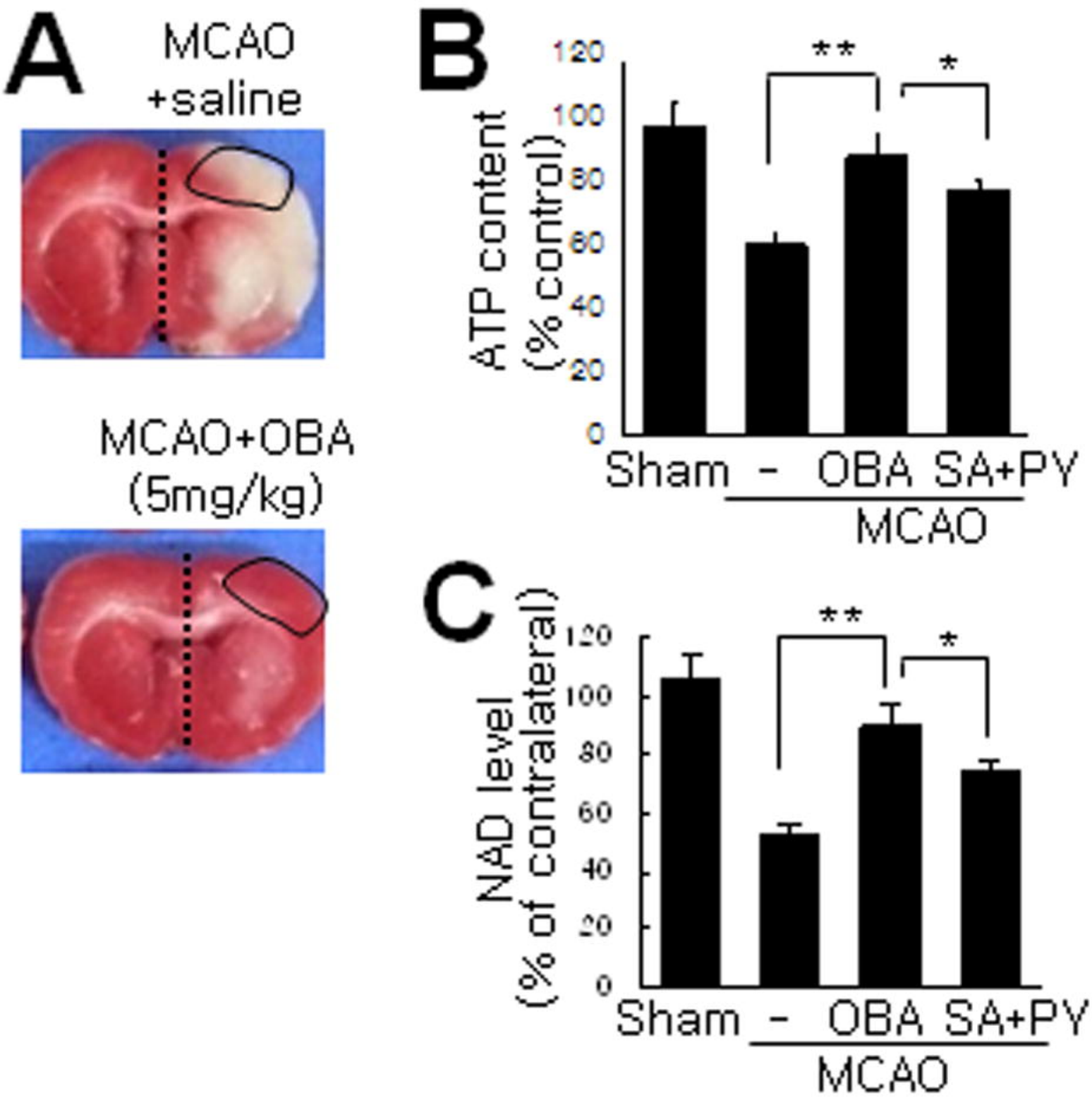
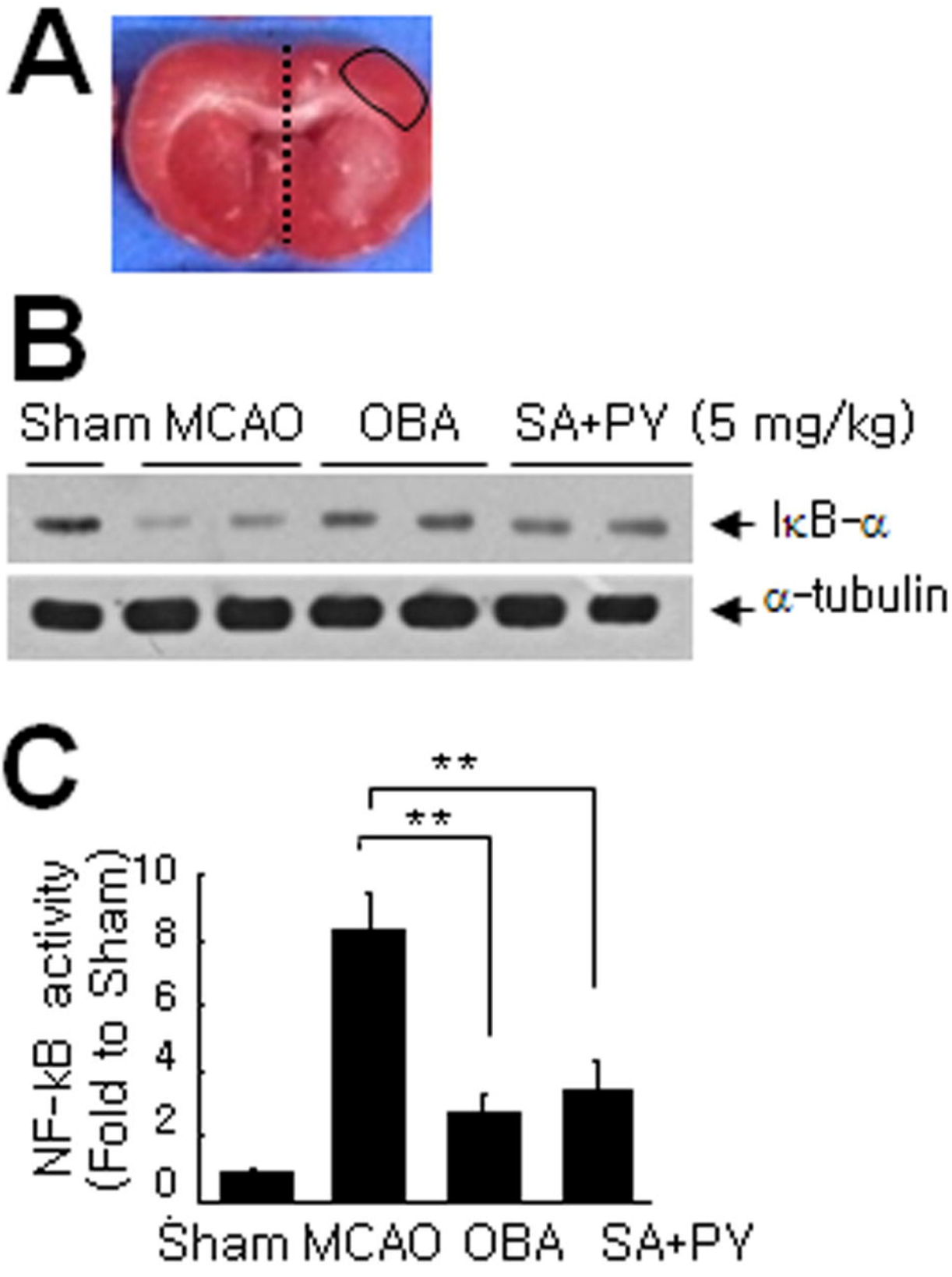

A
MCAO
+saline
MCAO+OBA
(5mg/kg)
B
ATP content
(% control)
120
100
80
60
40
20
0
**
*
Sham - OBA SA+PY
MCAO
C
NAD level
(% of contralateral)
120
100
80
60
40
20
0
**
*
Sham - OBA SA+PY
MCAO

Figure 10


A
B
Sham
MCAO
OBA
SA+PY (5 mg/kg)
IκB-α
α-tubulin
C
**
**
NF-kB activity
(Fold to Sham)
10
8
6
4
2
0
Sham
MCAO
OBA
SA+PY