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Therapeutic time window and dose dependence of neuroprotective effects of sodium orthovanadate following transient middle cerebral artery occlusion in rats

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Abbreviations

CREB, cyclic AMP-responsive element-binding protein; ERK, extracellular-signal regulated kinase; JNK, c-Jun N-terminal kinase; MCAO, middle cerebral artery occlusion; MAPK, mitogen-activated protein kinase; PI3-K, phosphoinositide 3-kinase; PTPs, protein tyrosine phosphatases; rCBF, regional cerebral blood flow; TTC, 2,3,5-triphenyltetrazolium chloride

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Abstract

Vanadium is widely distributed in the environment and exhibits various biological and physiological effects in the human body. We previously documented the neuroprotective effect of sodium orthovanadate (SOV) against in rodents intravenously injected with 2ml/kg 50 mM SOV just after the induction of middle cerebral artery occlusion (0 min post-MCAO). To evaluate its potential clinical use, we here determined therapeutic time window (0, 45, and 90 min post-MCAO) and the neuroprotective dose (2ml/kg of 12,5, 25, 37.5, and 50 mM) of SOV in rats. A single injection of 50 mM SOV at 0 or 45 min post-MCAO produced similar neuroprotective effects and even 50 mM delivered 90 min post-MCAO exerted significant neuroprotection. Although the maximal neuroprotective effect was obtained at 50 mM SOV, 25 mM injected once, and 12.5 mM delivered at 0 and 45 min post-MCAO significantly reduced the infarct volume. We also documented that SOV treatment ameliorates ischemic neuronal cell injury via the activation of both Akt and ERK, inhibits serum glucose , and elicits the gradual recovery of rCBF after transient MCAO in rats. To elucidate the important factor(s) involved in the neuronal protection afforded by SOV we measured Akt and ERK activity, physiological parameters, blood glucose levels, and rCBF following various SOV treatments. In conclusion, Akt activation was the most important factor in SOV-induced neuroprotection; ERK activation, the gradual recovery of rCBF, and decreased blood glucose were weak contributors.

Introduction

Cerebral ischemia is a major cause of death in humans (Hankey and Warlow, 1999). After arterial occlusion, necrotic cell death is predominant in the ischemic core (Garcia et al., 1995). In contrast, apoptotic cell death accounts for delayed neuronal death in the penumbra (Charriaut-Marlangue et al., 1996) where anti-apoptotic agents rescue cells from neuronal death. Although restoration of the cerebral blood flow immediately after ischemia reduces the infarct size in the ischemic penumbra, reperfusion-induced injuries have been observed (Marchal et al., 1999). Despite efforts to develop novel drugs to rescue neurons from delayed neuronal death in the penumbral region (Zivin, 1997), few currently available drugs are effective in stroke patients.

The transition metal vanadium (atomic weight 50.9415) is widely distributed in the environment; it exhibits various biological and physiological effects in the human body (Elberg et al., 1994; Brichard and Henquin, 1995). Vanadium compounds mimic many of the physiological actions of insulin; they lead to an increase in hexose uptake, glycogen synthesis, glycolysis, and fatty acid synthesis in insulin-responsive tissues (Shechter, 1990). Vanadate inhibits protein tyrosine phosphatases (PTPs) by acting as a transition state of a phosphate analogue and by forming a reversible bond through cysteine residues (Morinville et al., 1998). Moreover, it indirectly activates non-receptor protein tyrosine kinase in cell-free systems (Elberg et al., 1994). Thus, vanadate increases tyrosine phosphorylation via the inhibition of non-selective PTPs and activation of tyrosine kinases.

Akt (protein kinase B) and Extracellular signal-regulated kinase (ERK) are kinases known to inhibit apoptosis. A number of studies have indicated that activated Akt and ERK promote neuroprotection during cerebral ischemia (Li et al., 2003; Kilic et al., 2005). We documented that sodium orthovanadate (Na_3VO_4 : SOV) activates Akt through PI3-K and ERK, thereby eliciting neuroprotection against hippocampal delayed neuronal death in gerbils: There was a correlation between the activity of Akt and ERK and the neuroprotective efficacy of SOV and the administration of both SOV and the kinase inhibitors decreased the protective effects (Kawano et al., 2001). We also demonstrated that SOV treatment ameliorates ischemic neuronal injury following transient middle cerebral artery occlusion (MCAO) in rats via both Akt and ERK activation, that it results in a lower blood glucose level, and that it elicits gradual regional cerebral blood flow (rCBF) recovery (Hasegawa et al., 2003).

In addition to evoking therapeutic efficacy, vanadium compounds have also been shown to

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produce a variety of side-effects, including hematologic and biochemical alterations, renal toxicity, immunotoxicity, and mutagenicity (Jandhyala and Hom, 1983; Zaporowska and Wasilewski, 1992; Sakurai, 1994; Domingo et al., 1995). Thus, safety and therapeutic time window should be determined in experimental animals.

In this study we evaluated the potential clinical usefulness of SOV. We determined the effective dose and therapeutic time window to elicit the neuroprotective effect of SOV in rats. We also measured physiological parameters, blood glucose levels, and rCBF.

Materials and Methods

Experimental animals

All experiments were approved by the Animal Care and Use Committee of Kumamoto University. Male Wistar rats weighing 330-400 g were kept under constant environmental conditions (temperature 22°C ± 2°C, humidity 55% ± 5%, 12:12 hour light:dark cycle) in the Animal Research Center of Kumamoto University. They had free access to food and water before and after all procedures. Anesthesia was induced with 4%- and maintained with 2-2.5% halothane, 30% oxygen, and 70% nitrous oxide via a facemask. The left femoral artery was cannulated to measure arterial blood gases. Samples were analyzed in a blood gas analyzer (SL Blood Analysis System Series 2000, Diametrics Medical, MN, USA). During surgery, their rectal temperature was monitored and kept at 38.0°C ± 0.5°C with a feedback-regulated heating system (Small Animals Heat Controller, Unique Medical, Tokyo, Japan). Their blood pressure was monitored intermittently via a tail artery (TK-340 Rat-Mouse Manometer-Tachometer, Unicom, Chiba, Japan); blood glucose levels were measured via a tail vein (Precision Q.I.D. System, Abbott Laboratories, IL, USA). We chose the following time points : 0 min post-MCAO ; just after the induction of MCAO, 45 min post-MCAO ; 45 min after MCAO, 90 min post-MCAO ; just prior to release of the occlusion.

Regional cerebral blood flow (rCBF) measurements

Changes in rCBF were recorded on the dura of the left parietal cortex using a laser Doppler flowmeter (ALF21, Advance, Tokyo, Japan) attached to a hollow plastic tube. The rats were placed in a stereotaxic frame and craniectomy (2 mm lateral and 1 mm caudal to the bregma) was performed carefully using a drill. After the tube was fixed to the bone with resin, the flowmeter probe was inserted. Changes in rCBF were expressed as a percentage of the pre-treatment basal level.

Induction of focal ischemia

The rats were removed from the stereotaxic apparatus and reversible focal ischemia was induced by occluding the MCA using the intraluminal technique (Hasegawa et al., 2003). Briefly, under an operating microscope, the left common carotid artery was exposed through a midline incision in the neck. About 20 mm of 3-0 nylon suture coated with poly-L-lysine were introduced

into the left internal- through the common carotid artery. The animals were allowed to recover from anesthesia as soon as occlusion of MCA. In this study, most of animals recovered from anesthesia within 15 min. Ninety-minute later, they were again briefly anesthetized for removal of the nylon suture to allow reperfusion. Neurologic examinations were performed 10 min before reperfusion using a modification of the neurologic score of Bederson et al. (1986). Accordingly, grade 0 was recorded in the absence of observable deficits, grade 1 in the presence of forelimb flexion, grades 2 and 3 when there was decreased resistance to lateral pushing in the absence or presence of circling, respectively, and grade 4 was assigned to comatose animals. Rats with grades 0 and 4 were excluded from further experiments.

Measurement of the volume of ischemic brain injury

The animals were decapitated 24 hr post-MCAO. Their brains were quickly removed, placed in cold saline solution for 10 min, and then cut into 2 mm-thick coronal slices using a rodent brain matrix. Six selected sections (± 5 mm, ± 3 mm, and ± 1 mm from the bregma) were stained for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C. The area of ischemic brain injury was measured using the NIH image program. Infarct areas were corrected to compensate for edema formation by subtracting the area of the intact ipsilateral hemisphere from the area of the intact contralateral hemisphere. Then the total infarct areas on each slice were added together and multiplied by slice thickness to obtain the infarct volume.

Determination of the therapeutic time window

At 0-, 45-, or 90-min post-MCAO, groups of 8 rats each were intraperitoneally (i.p.) injected with 2 ml/kg saline or 50 mM SOV. The infarct volume was determined 24 hr post-MCAO by TTC staining.

Dose-dependence and effect of repeat-administrations of low-dose SOV

Groups of 8 rats with transient MCAO received 2 ml/kg saline or 12.5- (4.6 mg/kg), 25- (9.2 mg/kg) or 37.5 mM (13.8 mg/kg) SOV i.p. at 0 min post-MCAO. The data of infarct volume of 50 mM (18.4 mg/kg) SOV administrated group obtained from the study of therapeutic time window were used as the data of 50 mM SOV. Another group was injected with 2 ml/kg 12.5 mM SOV at 0- and again at 45 min post-MCAO. The infarct volume was determined 24 hr post-MCAO by TTC staining.

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Effect of blood glucose level during MCAO on infarct size

SOV leads to a decrease in blood glucose. Therefore, to examine the effect of decreased blood glucose on the results obtained in ischemic SOV-treated rats, we performed a set of experiments shown in Fig. 3. We injected 3 groups of 8 rats each with saline (2 ml/kg, controls) or 2 ml/kg 12.5 mM SOV without (group B) or with (group C) the additional administration of 10% glucose and measured their blood glucose levels every 15 min during a 90-min period. Identical experiments were performed on 3 groups of rats subjected to 90-min MCAO and the infarct volume in these animals was determined at 24 hr post-MCAO.

Gel electrophoresis and immunoblotting

Samples were obtained from the peri-infarct cortex on the ipsilateral side (Hasegawa et al., 2003). At 45 and 90 min post-MCAO or at pre-MCAO time points, 4 rats in each group were decapitated, their brains removed, and the indicated regions dissected out in cold saline under a microscope. Each sample was kept at –80°C until use. Frozen tissues were homogenized and sonicated with a Biorupture instrument (UCD-200TM, Cosmo Bio, Tokyo, Japan) at 0°C in 0.2 ml of homogenization buffer containing 50 mmol/L Tris-HCl (pH 7.5), 0.5% Triton X-100, 4 mmol/L EGTA, 10 mmol/L EDTA, 0.5 mol/L NaCl, 1 mmol/L Na_3VO_4, 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor, and 1 mmol/L dithiothreitol. Insoluble materials were removed by 15-min centrifugation at 15000 *g*. The protein content in each supernatant fraction was determined using Bradford's solution. Individual samples were applied to a 10% acrylamide denaturing gel, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then the proteins were transferred for 1 hr at 70V to an Immobilon PVDF membrane (Millipore Corp., Bedford, MA, USA). Blotting membranes were incubated for 1 hr with 5% nonfat milk in PBS containing 0.1% Tween-20 (PBST) at room temperature and then incubated overnight at 4°C with a 1:200 dilution of anti-phospho-Akt (Ser473) antibody (Cell Signaling Technology, Beverly, MA, USA) or a 1:500 dilution of anti-MAP kinase activated (diphosphorylated) ERK-1 & 2 antibody (Sigma-Aldrich, St. Louis MO, USA) in nonfat milk in PBST. After several washes with PBST, the membranes were incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit antibody (Vector, CA, USA) diluted 1:5000 and processed with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The images were scanned and analyzed semiquantitatively using the NIH image software. Changes in the phosphorylation of

Akt-Ser-473 and ERK were expressed as a percentage of the pre-ischemia level.

Statistical Analysis

All values are expressed as the mean ± SD. Blood gases, blood glucose levels, and rCBF were analyzed by one-way ANOVA, infarct volume in measurement of therapeutic time window and dose dependency and phosphorylation of Akt (Ser473) and ERK were analyzed by non-repeated one-way ANOVA; for statistical comparison of groups at each time point we used the unpaired t-test. Differences of $p<0.05$ were considered statistically significant.

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Results

Therapeutic time window

The infarct volume in the control (saline) group was 121 ± 27 mm^3 (Fig. 1B). As previously reported (Hasegawa et al., 2003), at 50 mM, SOV delivered at 0 min post-MCAO resulted in a remarkable decrease in the infarct volume (42 ± 34 mm^3, 34 ± 28%). SOV was neuroprotective even when delivered as late as 90 min post-MCAO (83 ± 25 mm^3, 69 ± 21%). Delivery at 0 min produced protective effects similar to those observed in rats injected at 45 min post-MCAO (47 ± 34 mm^3, 35 ± 29%).

Dose dependence of neuroprotection afforded by SOV

The neuroprotective effect of SOV delivered at 0 min post-MCAO was dose-dependent (Fig. 2). The infarct volume was 77 ± 34 mm^3, 53 ± 26 mm^3, and 42 ± 34 mm^3 at 25-, 37.5- and 50 mM SOV, respectively, and significantly different from the controls (121 ± 27 mm^3). Although a single injection of 12.5 mM SOV (93 ± 41 mm^3) was not neuroprotective, 2 injections delivered at 0 and 45 min post-MCAO produced a significant reduction in the infarct volume (67 ± 45 mm^3).

Physiological parameters

Blood gas analysis (Table 1) showed that the controls manifested mild alkalosis at 90 min post-MCAO. In rats treated with 50 mM SOV we observed mild CO_2 elevation leading to mild acidosis at 90 min post-MCAO. The injection of SOV at 45 or 90 min post-MCAO produced a dose-dependent increase in PO_2. There were no statistically significant changes in hematocrit (data not shown).

In the controls, the blood glucose level pre- and at 45- and 90 min post-MCAO was not significantly different (Table 2). However, in all SOV-treated rats it was significantly lower at 45 min post-MCAO and the decrease was SOV dose-dependent. At 90 min post-MCAO, it recovered to the pre-MCAO level in rats injected with 12.5 or 25 mM SOV. Blood glucose in rats treated with 37.5 mM or 50 mM SOV and in those injected twice with 12.5 mM SOV was significantly lower than in the controls.

As shown in Table 2, at 45 min post-MCAO, the groups did not differ significantly with respect to rCBF. At 90 min, only rats treated with 50 mM SOV had significantly lower rCBF than the controls. The saline- and SOV-treated rats did not differ significantly in their neurological

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scores and blood pressure readings (data not shown).

Effect of maintained blood glucose level on infarct volume

As the SOV-induced decrease in blood glucose may affect its neuroprotection (Hasegawa et al., 2003), we compared the infarct volume in SOV-treated rats that did, or did not, receive an injection of glucose. In addition, we measured blood glucose in non-MCAO rats that did, or did not receive SOV (Figs. 3A, 3B). Glucose-untreated rats injected at 0- and 45 min post-MCAO with 12.5 mM SOV (group B in Fig. 3A) showed a gradual decrease in the blood glucose level. On the other hand, there was no significant difference in blood glucose between the controls (group A) and rats treated with both SOV (12.5 mM, 0 and 45 min post-MCAO) and glucose (10%, 30 and 75 min post-MCAO) (group C).

The administration of glucose to SOV-treated ischemic rats did not significantly affect the infarct volume. However, the administration of SOV with or without additional glucose did result in a significant reduction in the infarct volume compared to the controls (Fig. 3C).

Western blot analysis of Akt and ERK-2 phosphorylation after transient MCAO

We already reported that SOV was neuroprotective only in the penumbra area of the ipsilateral cortex and that the activation of Akt and ERK was correlated with its neuroprotective effect (Hasegawa et al., 2003). Here we studied the time course of Akt and ERK activities (phosphorylation) in rats treated with SOV immediately after MCAO. Fig. 4A shows the changes in Akt-Ser-473 phosphorylation in the penumbra area. In rats treated with saline only (control) and those injected with 12.5 mM SOV at 0 min post-MCAO, Akt-Ser-473 phosphorylation was significantly decreased at 45- (40 ± 18% vs. 55 ± 19%) and 90 min (54 ± 22% vs. 58 ± 24%) post-MCAO. On the other hand, in rats treated with 50 mM SOV at 0 min post-MCAO it was not significantly different from the pre-MCAO level at 45- and 90 min post-MCAO (81 ± 11% and 130 ± 23%, respectively). Interestingly, rats treated with repeated injection of 12.5 mM SOV at 0- and again at 45 min post-MCAO maintained Akt phosphorylation at 90 min that was similar to the pre-MCAO level (108 ± 14%).

In gerbils, the phosphorylation of ERK-1 (44 kDa) and ERK-2 (42 kDa) was decreased following ischemia induction (Kawano et al., 2001). Therefore, we also assessed ERK-2 phosphorylation in our rats. As shown in Fig. 4B, in the controls and in rats treated with a single injection of 12.5 mM SOV at 0 min post-MCAO, there was a significant decrease in ERK-2

phosphorylation at 45- (35 ± 23% vs. 46 ± 32%) and 90 min (43 ± 10% vs. 56 ± 18%) post-MCAO. On the other hand, in rats treated with 50 mM SOV, ERK-2 phosphorylation was maintained to the pre-MCAO level by 45 and 90 min post-MCAO (101 ± 46% and 118 ± 34%, respectively). The changes in ERK-2 and Akt phosphorylation were similar. However, a second injection of 12.5 mM SOV at 45 min post-MCAO failed to restore ERK-2 phosphorylation by 90 min post-MCAO (77 ± 9%).

Discussion

We confirmed that SOV had a dose-dependent neuroprotective effect in ischemic rats. Although maximal neuroprotection was obtained with 50 mM SOV, 25 mM delivered once, and 12.5 mM injected at 0 and 45 min post-MCAO significantly reduced the infarct volume. Regarding the therapeutic time window, the injection of 50 mM SOV at 0- and 45 min post-MCAO afforded similar neuroprotective effects and SOV was significantly neuroprotective even in rats treated at 90 min post-MCAO. These findings indicate that SOV has a wide therapeutic time window and suggest that it may be clinically useful in patients with cerebral ischemia. A major problem with vanadium compounds is their potential toxicity (Domingo et al., 1995). Their mitogenic effects may be due to their inhibition of tyrosine phosphatases and their potentiation of the autophosphorylation of tyrosine kinases of growth hormone receptors on the cell membrane (Tracey and Gresser, 1986). Orthovanadate reportedly induced cell death and exhibited anti-neoplastic properties (Cruz et al., 1995). Vanadium compounds may exert inhibiting effects on ion transport-coupled ATPase (Cantley et al., 1978) or induce p53 activation mainly through H_2O_2 generation (Huang et al., 2000) which result in cell death. The morphological changes and cytotoxic effects seen in cells may depend on the SOV concentration and the length of exposure (Cruz et al., 1995; Figiel and Kaczmarek, 1997). As the period of administration is short, the mitogenic effect(s) of these agents do not need to be considered in stroke patients. Our previous histological study disclosed no evidence of neurotoxicity in the contralateral hemisphere of ischemic rats treated with 50 mM orthovanadate 28 days earlier (Hasegawa et al., 2003). Furthermore, our present results indicate that the SOV dose could be reduced without compromising its neuroprotective effects.

We previously reported that the anti-apoptotic effects of Akt and ERK resulted in neuroprotection against hippocampal delayed neuronal death in gerbils (Kawano et al., 2001). We also documented that SOV ameliorated ischemic neuronal cell injury, and that its effect was associated with the combined activation of Akt and ERK, a decrease in blood glucose levels, and the gentle recovery of rCBF (Hasegawa et al., 2003). In the current study we focused on determining which factor(s) was the most important for SOV-induced neuroprotection.

We found that rats treated at 0 min post-MCAO with 50 mM SOV manifested significantly lower rCBF at 90 min post-MCAO than the controls. The gentle recovery of rCBF may contribute to neuroprotection by avoiding the generation of free radicals or inflammatory

cytokines. However, the rCBF in rats treated with 37.5 mM SOV, which exerted the same level of neuroprotection as 50 mM, was not statistically different from the controls. Therefore, we postulate that the gradual rCBF recovery contributes only weakly to the neuroprotection induced by SOV.

Hypoglycemic ischemia leads to major metabolic derangement and an increase in brain tissue damage (Vannucci et al., 1980; Wass and Lanier, 1996). In contrast, hyperglycemia adversely affects the energy metabolism in patients with cerebral ischemia due to severe lactic acidosis and results in poor clinical outcomes (Gardiner et al., 1982; Nedergaard, 1987). As normoglycemic cats with MCAO experienced less brain damage and lower mortality (de Courten-Myers et al., 1994), the blood glucose revel was thought to be a major factor in their prognosis.

Based on our previous observation that in rats, 50 mM SOV significantly reduced the blood glucose level although it remained within the normal range (Hasegawa et al., 2003). We hypothesized that the lowering of blood glucose was one important effect of SOV in the prevention of ischemic neuronal injury. Therefore, in the current study, we attempted to maintain the blood glucose level by injecting SOV-treated rats with glucose. However, we observed no significant difference in the infarct volume of rats treated at 0- and 45-min post-MCAO with 12.5 mM SOV, irrespective of whether they were or were not additionally injected with 10% glucose. Based on this observation we postulated that the SOV-induced decrease in blood glucose did not contribute strongly to its neuroprotective effect.

In growth factor-mediated signaling cascades, Akt is involved in anti-apoptotic signaling downstream from PI3-K. The direct or indirect phosphorylation by active Akt of BAD, caspase-9, cyclic AMP-responsive element binding protein (CREB), nuclear factor-κB (NF-□B), and forkhead transcription factors results in anti-apoptotic effects (Datta et al., 1997; Cardone et al., 1998; Du and Montminy, 1998; Brunet et al., 1999; Romashkova and Makarov, 1999). We speculated that SOV stimulated the Akt-related anti-apoptotic transcription factor pathway and rescued penumbra regions affected by apoptotic mechanisms (Kawano et al., 2001) and that Akt activation underlies the effect of SOV observed in our study (Hasegawa et al., 2003). ERK, also known as mitogen-activated protein kinase, is normally activated in response to growth and differentiation factors; its increased phosphorylation has been reported in rodents with transient MCAO (Alessandrini et al., 1999). We have shown that SOV activates ERK, resulting in neuroprotection against forebrain ischemia in gerbils (Kawano et al., 2001). The current study

revealed that in rats treated with a single injection of 12.5 mM SOV, Akt and ERK phosphorylation was not maintained at 45 min post-MCAO. Although a second injection of 12.5 mM SOV had no effect of ERK activation at 90 min post-MCAO, it did activate Akt. Therefore, we suggest that Akt activation was mainly involved in the observed SOV-induced neuroprotection. We further postulate that the recovery of Akt activity in the early stage of ischemia may further enhance the neuroprotective effects of SOV.

In conclusion, SOV belongs to a novel class of compounds available for the treatment of stroke patients. It also manifests a therapeutic time window of at least 90 min . Less than 50 mM SOV, especially twice administration of 12.5 mM or single injection of 25 mM SOV, may help to reduce the SOV dose. Vanadium compounds have been used to treat patients with diabetes mellitus without lethal side effects (Goldfine et al., 1995; Boden et al., 1996). As the required treatment period in patients with cerebral ischemia is short and temporary (1-2 weeks), SOV may be of use in the clinical setting. Studies are ongoing in our laboratory to determine whether stroke patients can be treated safely and effectively with SOV.

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Footnotes

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Figure Legends

Figure 1

(A)

Representative ischemic lesions at +1mm from the bregma in coronal section from SOV (injection at 0, 45, and 90 min post-MCAO) and saline treated rats at 1 day (TTC) after ischemia.

(B)

Quantitative analysis of total infarct volume to determine the therapeutic time window in groups of 8 rats treated with saline (controls) or SOV at 24-hr post-MCAO. Values are the mean ± SD; asterisks denote significant differences between the controls and the SOV-treated groups (*, $p<0.05$; **, $p<0.01$).

Figure 2

Quantitative analysis of total infarct volume to determine the SOV dose-dependence in groups of 8 rats treated with saline (controls) or SOV at 24-hr post-MCAO. Values are the mean ± SD; asterisks denote significant differences between the controls and the SOV-treated groups (*, $p<0.05$; **, $p<0.01$).

Figure 3

Effect of 10% glucose administration during MCAO on total infarct volume

(A)

Group A (n=8): controls (saline only)

Group B (n=8): 12.5 mM SOV at 0 and 45 min post-MCAO

Group C (n=8): 12.5 mM SOV at 0 and 45 min post-MCAO plus 10% glucose at 30- and 75 min post-MCAO

(B)

Time course of glucose level measured at 15-min intervals in groups of rats treated as in Fig. 2A but not subjected to MCAO

(C)

Quantitative analysis of total infarct volume in groups of rats treated as in Fig. 2A. Values are the mean ± SD for 8 rats/group

†, $p<0.05$; ††, $p<0.01$ vs. pre-MCAO

*, $p<0.05$; **, $p<0.01$ vs. controls

Figure 4

Changes in Akt-Ser-473 (A) and ERK-2 (B) phosphorylation in the penumbra after cerebral ischemia.

Top: Representative Western blots of Akt-Ser-473 (A) and ERK-2 (B) phosphorylation.

Bottom: Quantitative analysis of relative Akt-Ser-473 and ERK-2 phosphorylation in groups of 4 rats treated with saline, SOV, or at pre-MCAO

Values are the mean ± SD

†, $p<0.05$; ††, $p<0.01$ vs. pre-MCAO

*, $p<0.05$; **, $p<0.01$ saline vs. SOV groups

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Table 1

Blood gas analysis before and after MCAO ; pH, PO_2, and PCO_2 were measured before and 45- and 90 min post MCAO

Group	Saline	SOV			
		12.5 mM	25 mM	37.5 mM	50 mM
pre-MCAO					
pH	7.42 ± 0.04	7.43 ± 0.04	7.41 ± 0.07	7.43 ± 0.05	7.41 ± 0.06
PCO_2 (mmHg)	44 ± 6	42 ± 6	44 ± 6	44 ± 6	46 ± 6
PO_2 (mmHg)	150 ± 36	148 ± 37	145 ± 26	150 ± 31	145 ± 34
45 min post-MCAO					
pH	7.44 ± 0.02	7.44 ± 0.01	7.42 ± 0.03	7.39 ± 0.04	7.32 ± 0.02**
PCO_2 (mmHg)	43 ± 1	41 ± 5	46 ± 2	49 ± 6	47 ± 4
PO_2 (mmHg)	155 ± 9	156 ± 38	162 ± 9	163 ± 14	175 ± 10*
90 min post-MCAO					
pH	7.47 ± 0.01†	7.44 ± 0.07	7.44 ± 0.05	7.41 ± 0.05	7.33 ± 0.04**
PCO_2 (mmHg)	39 ± 2	39 ± 10	42 ± 6	45 ± 5	49 ± 4**
PO_2 (mmHg)	147 ± 5	160 ± 10**	168 ± 5**	174 ± 5**	182 ± 7**†

Values are the mean ± SD for 5 rats/group.

†, $p<0.05$; ††, $p<0.01$ vs. pre-MCAO, *, $p<0.05$; **, $p<0.01$ (controls vs. SOV group)

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Table 2

Blood glucose levels and regional CBF before and 45- and 90 min post-MCAO

Group	Saline	SOV				
		12.5 mM (x 1)	12.5 mM (x 2)	25 mM	37.5 mM	50 mM
Blood glucose (mg/dl)						
pre-MCAO	125 ± 18	125 ± 9	122 ± 9	127 ± 12	122 ± 12	122 ± 18
45 min post-MCAO	128 ± 10	113 ± 6*†	110 ± 7*†	99 ± 6**††	76 ± 5**††	71 ± 8**††
90 min post-MCAO	136 ± 7	128 ± 6	95 ± 9**††	122 ± 15	101 ± 10**†	76 ± 15**†
Cerebral blood flow (%)						
pre-MCAO	100	100	100	100	100	100
0 min post-MCAO	18 ± 19	18 ± 9	14 ± 12	19 ± 8	11 ± 10	14 ± 12
45 min post-MCAO	50 ± 13	42 ± 20	43 ± 13	40 ± 9	39 ± 10	34 ± 14
90 min post-MCAO	52 ± 11	42 ± 11	44 ± 14	38 ± 20	42 ± 8	33 ± 12*

The values are the mean ± SD for 8 and 5 rats/group, respectively.
†, $p<0.05$; ††, $p<0.01$ vs. pre-MCAO, *, $p<0.05$; **, $p<0.01$ (controls vs. SOV group)

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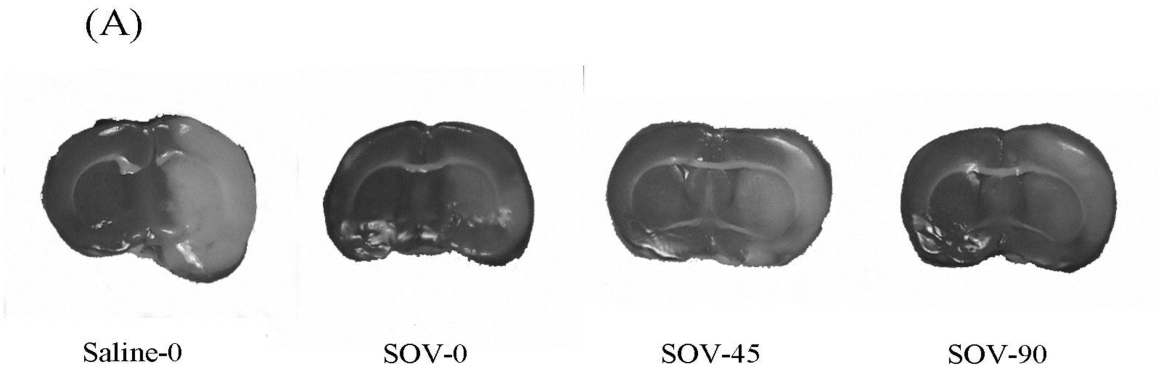

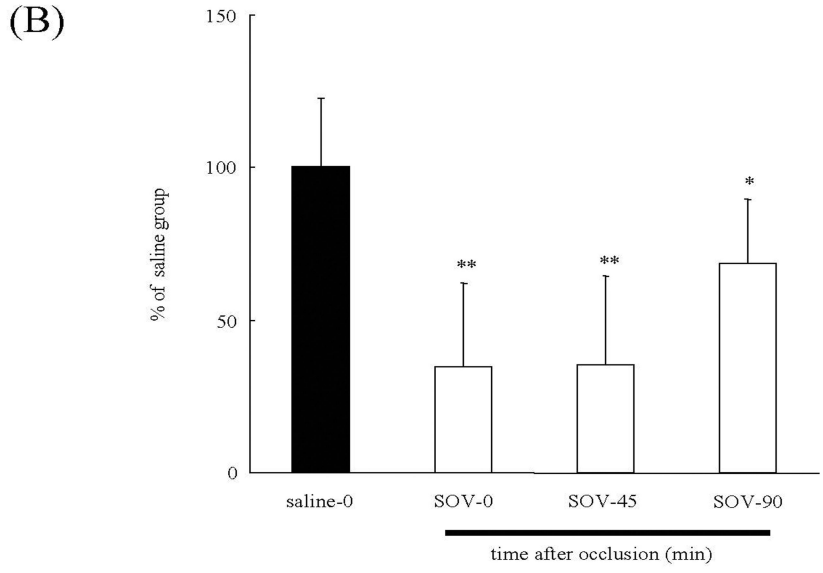

Figure 1

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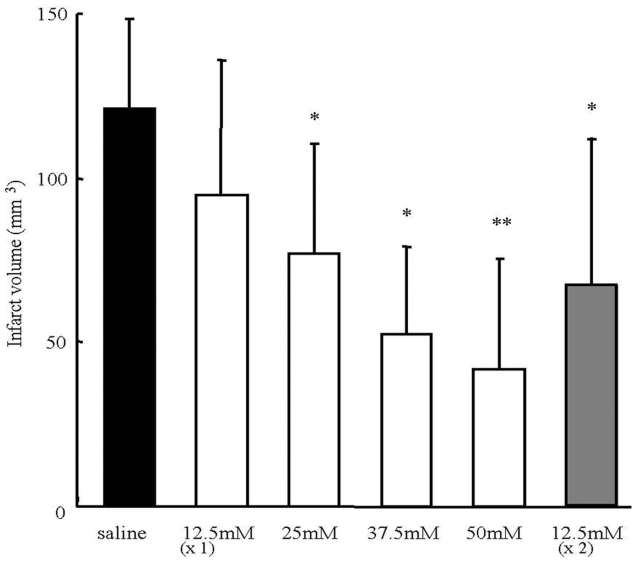

Figure 2

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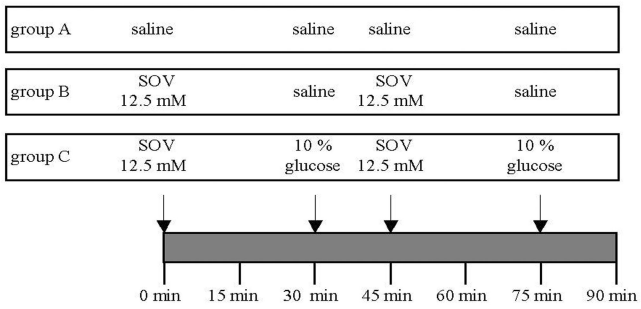

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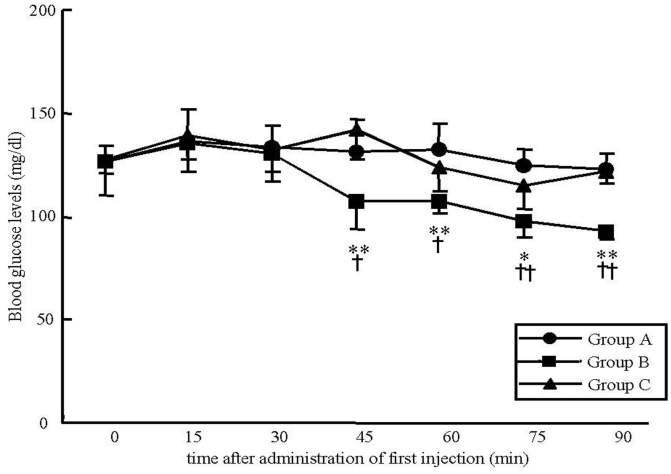

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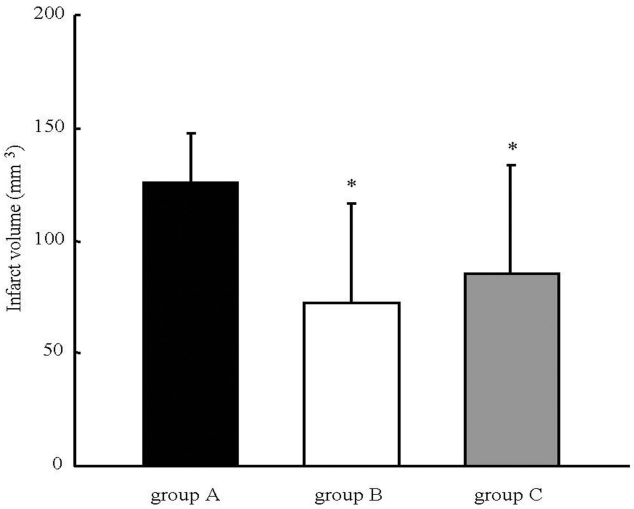

Figure 3

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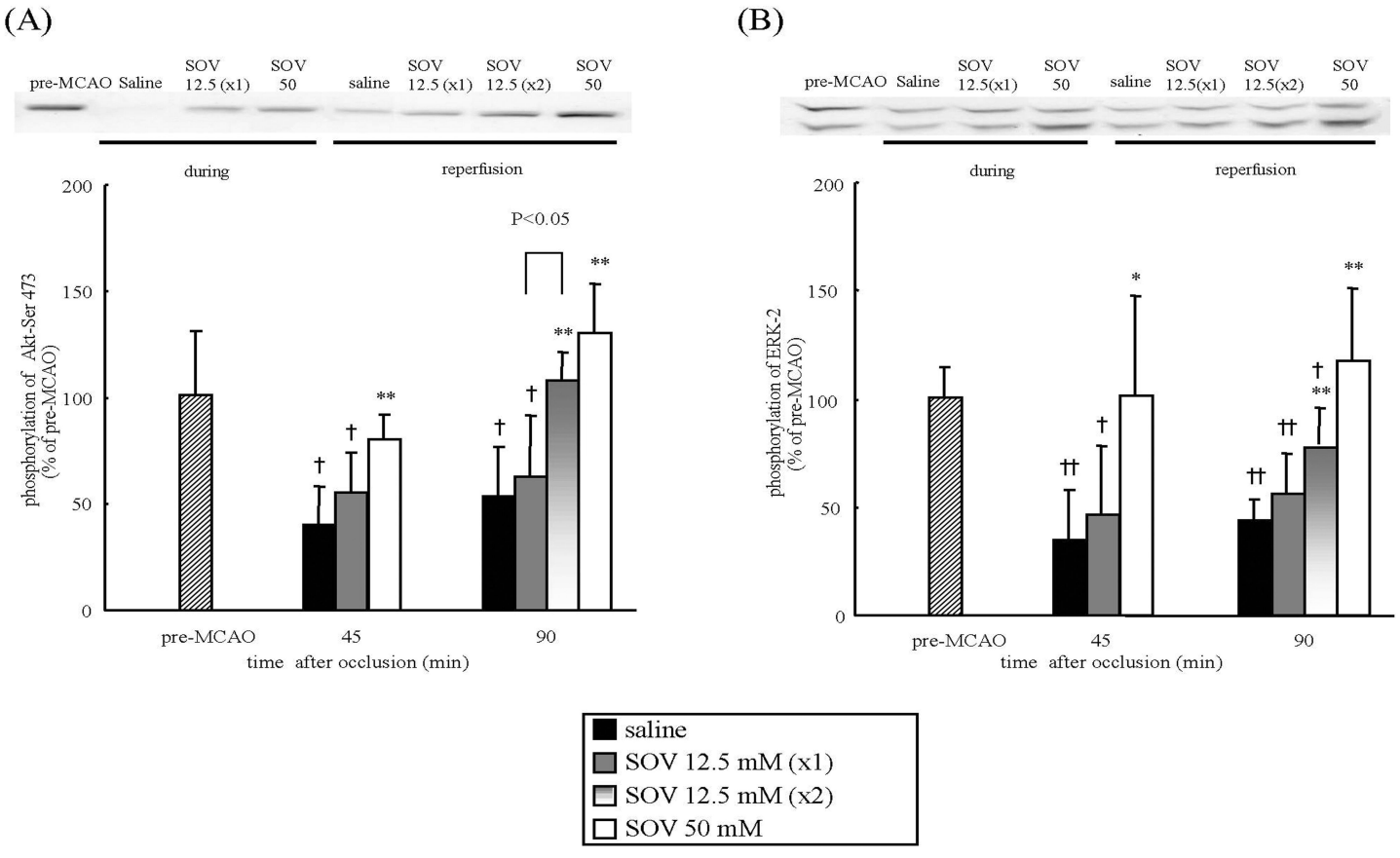

Figure 4