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# SERINE RACEMASE INHIBITION INDUCES NITRIC OXIDE-MEDIATED NEUROVASCULAR PROTECTION DURING CEREBRAL ISCHEMIA

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**Abstract—There are no effective neuroprotectant drugs for acute cerebral ischemia. Serine racemase (SR) synthesizes D-serine, which is involved in N-methyl-D-aspartate (NMDA) receptor-induced neurotoxicity. Recently, SR deletion was reported to protect against focal cerebral ischemia. However, regulatory mechanisms controlling SR-activity in the neurovascular unit (NVU) during cerebral ischemia remain to be clarified. We investigated the effects of SR inhibition on neurovascular protection after ischemia. The SR inhibitor phenazine methosulfate (PMS) alleviated neuronal damage in an *ex vivo* ischemic model (oxygen glucose deprivation [OGD]) using primary neuronal cultures, and in an *in vivo* mouse model of ischemia (middle cerebral artery occlusion [MCAO]). Ischemic preconditioning (IP) and PMS-treatment inhibited SR phosphorylation after ischemia *ex vivo*. In addition, SR phosphorylation after MCAO was also decreased in PMS-treated mice. Reductions in regional cerebral blood flow (CBF) after MCAO were improved by administration of PMS. Treatment with PMS increased phosphorylation of endothelial nitric oxide synthase (eNOS) in the ischemic core and penumbra region. In neuron-endothelial cell co-cultures, PMS promoted nitric oxide production after OGD. These findings indicate that SR inhibition acts as a neuroprotectant in the NVU and ameliorant of CBF abnormalities post-stroke. Thus, pharmacologic SR inhibition has potential clinical applications.** 

**Key words: brain ischemia, endothelium, ischemic preconditioning and induced tolerance, nitric oxide, serine racemase.**

## INTRODUCTION

In order to improve treatment of acute ischemic stroke, it is very important to develop both better methods for restoring cerebral blood flow (CBF) by vascular recanalization and pharmacological agents for neuronal protection. Whereas tissue plasminogen activator (t-PA) and intra-arterial thrombectomy with novel devices have greatly advanced the efficacy of recanalization therapies, (Berkhemer et al., 2015; Saver et al., 2015) potential neuroprotectants for treating stroke have failed to demonstrate clinical efficacy (Ikonomidou and Turski, 2002; Lo et al., 2003). In stroke, excessive release of glutamate during cerebral ischemia induces overstimulation of N-methyl-D-aspartate (NMDA) type glutamate receptors and leads to neuronal toxicity and death; thus, NMDA receptors play an essential role in ischemic neuronal injury (Bonventre et al., 1997). However, NMDA receptor antagonists have failed to prove protective in clinical trials despite promising preclinical data (Ikonomidou and Turski, 2002). Most of the compounds that have so far undergone clinical trials are antagonists of the glutamate binding site of the NMDA receptor (Ikonomidou and Turski, 2002). Therefore, it is important to investigate potential neuroprotectants that inhibit excitotoxicity by mechanisms other than preventing glutamate binding to NMDA receptors.

Recently, D-serine has emerged as an important mediator of NMDA receptor activity. D-serine is the optical isomer of L-serine and had been considered an unnatural amino acid, absent in higher animals (Van Horn et al., 2013). However, the development of more sensitive analytical techniques and instruments revealed that D-serine was abundant in the cerebral cortex. Further investigations have shown that D-serine binds to the glycine binding site of NMDA receptors and potentiates neurotransmission to a greater degree than glycine (Mothet et al., 2000). Moreover, D-serine has been found to be necessary for normal NMDA receptor-mediated neuronal transmission, which generates long-term potentiation and is essential for memory (Basu et al., 2009). D-serine is also involved in NMDA receptor-induced neurotoxicity. (Katsuki et al., 2004, 2007). Thus, it is notable that serine racemase (SR), which converts L-serine to D-serine, is involved in various neurological disorders (Shleper et al., 2005; Inoue et al., 2008), and cerebral ischemic infarct volume following middle cerebral artery occlusion (MCAO) is reduced in SR knockout mice (Mustafa et al., 2010).

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*Abbreviations:* CBF, cerebral blood flow; eNOS, endothelial nitric oxide synthase; IP, ischemic preconditioning; MCAO, middle cerebral artery occlusion; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NVU, neurovascular unit; OGD, oxygen glucose deprivation; PMS, phenazine methosulfate; SR, serine racemase; t-PA, tissue plasminogen activator.

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Accumulating evidence indicates that dynamic interactions between neuronal, glial, and vascular networks (together called the neurovascular unit [NVU]) play a fundamental role in stroke pathology and in maintaining normal brain function. The release of neurotransmitter from neurons has been shown to contribute to vasomotor responses through neurovascular coupling (Drake and Iadecola, 2007). Nitric oxide (NO) signaling in particular plays a central role in NVU and stroke. Excessive NMDA receptor stimulation and neuronal nitric oxide synthase (nNOS) activation after stroke exacerbate neuronal damage (Huang et al., 1994; Zhou et al., 2010). Endothelial nitric oxide synthase (eNOS)-derived NO maintains vascular homeostasis. Activation of eNOS improves CBF and reduces infarct volume (Huang et al., 1996; Atochin et al., 2007).

In light of these findings, neurovascular targets, including SR, may hold promise for the treatment of cerebral ischemia. However, little is known about the regulatory mechanisms of SR activity in the NVU and how the alteration of the SR-serine axis might contribute to the regulation of CBF under ischemic conditions. In this study, we found that SR activity in the NVU is involved in NO regulation after ischemia. Our results further demonstrated that the pharmacological inhibition of SR by phenazine methosulfate (PMS) (Kim et al., 2005; Sasabe et al., 2007; Esposito et al., 2012; Hagiwara et al., 2013) reduced neurovascular damage and improved functional outcome, thereby indicating that SR may be a novel therapeutic target for stroke.

## EXPERIMENTAL PROCEDURES

### Primary cortical neuron cultures

Primary cultures of rat cortical neurons were obtained from embryonic day 16 Wistar rats (Charles River) as described previously.(Mabuchi et al., 2001) Briefly, cortices were dissociated with a papain dissociation system (Worthington Biochemical Corporation). Cells were then seeded at a density of 7 × 105 cells/ml in 6-well plates and 60-mm dishes coated with polyethylenimine in high-glucose DMEM (Sigma) containing 5% fetal bovine serum (Invitrogen) and 1% Antibiotic–Antimycotic (Invitrogen). At 24 h after seeding, the medium was changed to Neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen) and 1% Antibiotic–Antimycotic. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Assays of cultured cells were conducted after 10–12 days.

### Endothelial cell cultures

Mouse immortalized cerebral endothelial cells (b.End3) (ATCC No) were incubated (4 × 105 cells/ml) in 6-well plates and 60-mm dishes in high glucose DMEM (Sigma) containing 10% fetal bovine serum(Invitrogen), 1%MEM Non-Essential Amino Acids (Life Technologies) and 1% Antibiotic–Antimycotic (Invitrogen). Cells were routinely subcultured when confluent. Cultures were grown for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO2 before use in experiments.

### Oxygen-glucose deprivation

For oxygen-glucose deprivation(OGD)/reoxygenation, cells were washed with phosphate-buffered saline and incubated with glucose-free Earle's balanced salts solution (Biological Industries Israel Beit Haemek Ltd) in an anaerobic chamber containing 5% CO2 and 95% N2 (< 1% O2) as previously reported (Sasaki et al., 2011). After OGD, the medium was changed to the original medium and the cells were placed in a normoxic chamber.

In cultures of primary neurons, sublethal ischemia was applied by OGD for 45 min, and lethal ischemia was applied for 180 min. To investigate the involvement of SR on ischemic tolerance, cultures were incubated with the following chemicals: L-serine (10 mmol/L) and/or, a potent SR inhibitor PMS (1 μmol/L) (Kim et al., 2005; Sasabe et al., 2007; Esposito et al., 2012; Hagiwara et al., 2013).

Cultures of b.End3 cells were subjected to 180 min OGD. To examine the association between L-serine and NO production, these cultures were incubated with L-serine (3 mmol/L) after OGD.

### Cell viability assays

Neuronal cell death after ischemia was assessed by measuring lactate dehydrogenase (LDH) activity using a cytotoxicity detection kit (Roche Applied Science). Collected culture medium was centrifuged at 300 *g* for 5 min before conducting assays according to the manufacturer's instruction. In parallel cultures, 100% cell death was induced by applying 2 mmol/L NMDA and relative assessments of neuronal injury were normalized by comparison with 100% cell death.

### NO fluorescent assays

NO production by cultured endothelial cells was monitored with diaminofluorescein-FM (DAF-FM; SEKISUI MEDICAL), which emits fluorescence in response to NO (Kojima et al., 1999). After 180 min OGD, b.End 3 cells were incubated in medium containing 1.2 μM DAF-FM. The supernatant of the medium was analyzed for fluorescence activity of DAF-FM by a microplate reader 3, 12, and 24 h after OGD. To evaluate intracellular NO levels by fluorescence microscopy, b.End 3 cells were incubated at 37 °C in a humidified atmosphere of 95% and 5% $CO_2$ in medium containing 2.0 μM Diaminofluorescein-FM diacetate (DAF-FM DA; SEKISUI MEDICAL) for 24 h following 180 min OGD (Kojima et al., 1999).

### Western blot analysis

Cells were lysed with lysis buffer composed of 20 mmol/L Tris–HCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Nonidet P-40, protease inhibitor cocktail (Complete Mini; Roche Applied Science) and phosphatase inhibitor cocktail (PhosSTOP; Roche Applied Science). DNA in the lysate was sheared using a syringe with an 18-guage needle. Samples were also isolated from mouse cortex. The 10 μg of proteins was subjected to electrophoresis in a 7.5% Tris–HCl polyacrylamide gel and transferred to a