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An *S*-Nitrosylated Hemoglobin Derivative Protects the Rat Hippocampus From Ischemia-Induced Long-Term Potentiation Impairment With a Time Window

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Abstract. Evidence suggests that *S*-nitrosylation is a biological process involved in cerebral ischemia. The aim of the present study was to elucidate the effects of *S*-nitrosylated (SNO) polyethylene glycol-conjugated (PEG) hemoglobin (Hb) developed as an artificial oxygen carrier, which can absorb free NO and translocate NO to a sulfhydryl (SH) moiety, on ischemic cerebral dysfunction. Long-term potentiation (LTP) in the perforant path-dentate gyrus synapses of the rat hippocampus was evaluated as functional outcome 4 days after transient incomplete cerebral ischemia (2-vessel occlusion: 2VO, 10 min). SNO-PEG-Hb (250 mg/kg, i.v.) administered on Day 0, 1, 2, or 4 (immediately, 24 h, 48 h, or 96 h after reperfusion, respectively) alleviated 2VO-induced LTP impairment with a therapeutic time window. The effect was significant when SNO-PEG-Hb was administered on Day 1 or 2. SNO-PEG-Hb altered NOS features observed in the vehicle-treated 2VO rat, upregulation of eNOS, nNOS, and iNOS expressions at mRNA and protein levels; SNO-PEG-Hb further upregulated eNOS and nNOS and downregulated iNOS expressions. These findings suggest that SNO-PEG-Hb might have protective effects on the rat hippocampus from ischemia/reperfusion-induced functional damages, thereby increasing the therapeutic potential as an artificial oxygen carrier for use in the area of oxygen therapy.

Keywords: *S*-nitrosylated hemoglobin derivative, nitric oxide, long-term potentiation, 2-vessel occlusion

Introduction

Nitric oxide (NO) is a signaling molecule that regulates many biological processes in the brain, including neurotransmitter release, several types of synaptic plasticity such as long-term potentiation (LTP), and apoptosis (1 – 4). NO is known to modulate the biological activity of membrane-bound, cytosolic, and nuclear proteins, including NMDA receptor, hemoglobin (Hb), matrix metalloproteinases, and transcription factors such as NF-κB, by reacting with thiols to form an *S*-nitrosylated derivative (3, 5 – 7).

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Evidence accumulated that NO produced both during and after cerebral ischemia may be an important factor in the pathogenesis of neuronal ischemic injury (8 – 10). Indeed, cerebral ischemia alters the activity of the L-arginine-NO pathway, determined by the amount of the final NO metabolic products NO_2^- and NO_3^- (11, 12). Although the mechanisms underlying the ischemic brain damage are complex, it is conceivable that *S*-nitrosylation processes triggered by nitrosative ischemic insult could be involved in the pathogenesis of the cerebral ischemia (3, 5).

NO is synthesized endogenously by the conversion of L-arginine to citrulline via three different types of NO synthase (NOS), the constitutive calcium/calmodulin-dependent endothelial NOS (eNOS), neuronal NOS

(nNOS), and the inducible calcium-independent NOS (iNOS), encoded by a gene located on chromosome 7, 12, and 17, respectively (13, 14). Evidence suggests differential contributions of NOS isoforms or their temporal NO production to the pathogenesis of post-ischemic brain injury; during the early stages of cerebral ischemia, eNOS and nNOS are continuously activated and produce NO. In the late stages of ischemia, activated iNOS produced excessive amount of NO. Thereby, eNOS-derived NO is beneficial in promoting collateral circulation and microvascular flow, whereas nNOS- and iNOS-derived NO are detrimental in the postischemic brain (9, 10, 12, 15 – 17). However, temporal roles of NOS-derived NO in the pathology of the cerebral ischemia remain controversial since the effects of NOS inhibitors depend on the animal model used, NOS isoenzyme specificity, and the timing and/or dosage administered (18 – 22).

A cellular modified Hb developed as an oxygen carrier is known to cause vasoconstriction (23, 24) and platelet stimulation (25) due to its NO scavenging action. Recently, new insights on the NO/Hb interaction have been proposed that NO covalently binds to a cysteine residue on the β-chain of Hb (Cysβ93). This *S*-nitrosylated Hb (SNO-Hb) retains EDRF/NO-like bioactivity and is capable of transferring NO to molecules containing low molecular weight thiol (6, 26 – 30). Thus, SNO-Hb might act as an NO donor as well as an oxygen carrier in vivo. Based on these findings, we have recently developed an *S*-nitrosylated (SNO)-pegylated (PEG)-Hb derivative (SNO-PEG-Hb) as a new candidate for an artificial oxygen carrier (31, 32). This new type of Hb derivative with high molecular weight could deliver oxygen and translocate NO to an SH moiety. Our results indicated that this compound can prevent the undesirable adverse effects of free Hb such as vasoconstriction and renal toxicity, and it can carry out its functions without accumulating in the reticuloendothelial system (31, 32). Recent evidence that *S*-nitrosylation is a critical process involved in some physiological and pathophysiological states (3, 5 – 7) suggested to us that SNO-PEG-Hb might have additional therapeutic potential under cerebral ischemia, where NOS-derived NO could act as a neuronal modulator.

The aim of the present study was to elucidate the effects of SNO-PEG-Hb on hippocampal neuronal dysfunction after transient cerebral ischemia. LTP in the perforant path-dentate gyrus synapses of the rat hippocampus was evaluated as the functional outcome after incomplete cerebral ischemia (2-vessel occlusion: 2VO, 10 min), where NOS-derived NO exerted a pivotal role on LTP impairment (10, 12). The effects of SNO-PEG-Hb were further evaluated as a measure of mRNA and protein expressions for three NOS isoforms, eNOS, nNOS, and iNOS, and key angiogenic molecules, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), in the hippocampus of the 2VO rat.

Materials and Methods

Animals

All experiments were performed on male Wistar rats (weighing 300 to 350 g, Shizuoka) in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee at the Hokkaido University Graduate School of Medicine.

Induction of transient cerebral ischemia

All procedures followed our previous studies (10). Briefly, an incomplete cerebral ischemia was induced by clamping of bilateral common carotid arteries of rats under 1% halothane anesthesia. The bilateral common carotid arteries were exposed and clamped with a clip for 10 min. The clip was then removed and recirculation within the carotid artery was allowed. The same surgical procedure was conducted in the sham-operated rats without carotid artery occlusion. Rectal temperature was monitored throughout surgery by a rectal probe and maintained at 37°C to 38°C with an electric heating pad. They were then returned to their home cage and allowed free access to food and water.

Experimental protocol

The rats received SNO-PEG-Hb or PEG-Hb immediately after reperfusion (Day 0), at a dose of 250 mg /kg, via the tail vein. Sham-operated rats were treated with the same volume of saline (2.5 ml/kg, i.v.) as a control. To evaluate the time window for treatment, SNO-PEG-Hb was also injected at other time points of postischemia, 24 h (Day 1), 48 h (Day 2), or 96 h (Day 4) after reperfusion. LTP recording, immunoblot analysis, and quantitative real-time RT-PCR were conducted on Day 4.

Preparation of Hb solutions

Hb derivatives were prepared from the hemolysate of outdated human red cell products by an ultrafiltration method as previously reported (31, 33). Briefly, Hb (0.25 mM) was dissolved in 10 mM sodium phosphate, pH 8.5, containing 0.438 mM pyridoxal-5' phosphate. Pyridoxalation and PEG modifications of Hb was performed under fully anaerobic conditions, according to the method of Iwasaki and Iwashita (34) with modifications; deoxygenation was achieved by flushing with

argon gas for more than 10 h in the presence of caprylic alcohol as an antifoaming agent; thereafter, the reaction was started by the addition of sodium borohydrate (2.5 mM) as a 1 μM sodium hydroxide solution and left for 1 h with stirring. Pyridoxalated Hb was pegylated by addition of the activated ester of PEG-bis (succinimidyl succinate) (Sunbright DEAC-30HS, MW 3.0 kDa; NOF, Tokyo) to the Hb solution with stirring. After 4 h reaction, glycine (5 mM) was added to inactivate the residual esters. *S*-nitrosylation was performed according to the modified method of McMahon and Stamler (35); PEG-Hb was diluted to 0.05 mM with 0.1 M sodium phosphate buffer, 1 mM EDTA, and 0.5 mM diethylenetriaminepentaacetic acid with the desired pH. *S*-Nitrosoglutathione (Dojin, Kumamoto), which was dissolved in 0.01 nM hydrochloric acid, was then added (0.25 – 1.00 mM). After the reaction, the mixture was left for the desired time with stirring, and the solution was concentrated with an ultrafiltration membrane having a cutoff point of 30 kDa (YM-30; Nihon Millipore, Tokyo) to remove unreacted PEG and other low molecular weight compounds. These *S*-nitrosylation and dialysis/concentration processes were performed under the flow of pure oxygen gas. The final concentration of Hb and the pH of the solution were adjusted to 10% and 7.40, respectively. The solution was filtered through a sterilization membrane and saved at −80°C until use. The yield of *S*-nitrosylation was estimated using high-performance liquid chromatography (HPLC) coupled with flow reactors of metal and Griess reagent (36).

Electrophysiological recording

In order to assess the functional outcome, hippocampal LTP was recorded 4 days after transient cerebral ischemia (Day 4). Anesthesia was induced by 1% halothane in a mixture of 20% O_2 and 80% N_2. The rats were tracheotomized, and artificially respirated. Extracellular recordings of field EPSPs were made from the granule cell body layer of the dentate gyrus (DG) (coordinates: 3.5 mm posterior, 2.0 mm lateral to the bregma, 3.3 mm ventral from the cortical surface) after electrical stimulation of the perforant-path (coordinates: 8.1 mm posterior, 4.4 mm lateral to the bregma, 2.5 mm ventral from the cortical surface), according to the atlas of Paxinos and Watson (37), at a baseline frequency of 0.033 Hz and 250 μs in duration as described previously (38). The evoked responses were amplified and monitored with an oscilloscope (VC-10; Nihon Kohden, Tokyo) and were averaged with a data analysis system (MASSCOMP; Concurrent, Tokyo). The tetanus consisted of 10 trains at 1 Hz, each composed of 8 pulses at 400 Hz and at the baseline stimulation voltage. After tetanic stimulation, the population spike (PS) amplitude was measured for 1 h and the values were normalized relative to the mean values obtained during the 5-min recording before tetanic stimulation. The time course changes and the area under the curve (AUC) from 0 to 60 min after tetanic stimulation were determined to evaluate the ensemble effect of Hb-derivatives.

Western blot analysis

To clarify the molecular basis of the effects of SNO-PEG-Hb, we also performed immunoblot analysis for angiogenic growth factors, VEGF and bFGF, and for NOS, eNOS, iNOS, and nNOS, in the hippocampus of 2VO rats. The animals were sacrificed under ketamine anesthesia (100 mg/kg, i.p.) 4 days after reperfusion (Day 4, n = 8). Samples were also obtained from sham-operated animals (n = 8). The quickly removed hippocampus was rinsed in sterilized water on ice and then frozen in liquid nitrogen and kept at −80°C until used. Hippocampal tissues were minced with scissors, homogenized, and then centrifuged at 500 g for 15 min to pellet any insoluble material. The protein concentration of supernatant was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples were run on SDS-PAGE, using 7.5 – 15% polyacrylamide gel, and electrotransferred to polyvinylidine diflouride filter (PVDF) membranes. To reduce non-specific binding, the PVDF was blocked for 2 h at room temperature with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (TPBS). Thereafter, the PVDF was incubated overnight at 4°C with specific antibodies for VEGF (anti-human VEGF rabbit polyclonal antibody; Immunological Laboratories, Fujioka), bFGF (anti-human bFGF rabbit polyclonal antibody; Affinity BioReagents, Golden, CT, USA), eNOS (anti-human eNOS rabbit polyclonal antibody, Affinity BioReagents), nNOS (anti-human nNOS rabbit polyclonal antibody; ZYMED Laboratories, San Francisco, CA, USA), and iNOS (anti-rabbit iNOS mouse monoclonal antibody, Affinity BioReagents) each at 1:100 – 1,000 dilution in TPBS. After three times washing with TPBS, the PVDF was incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham; Little Chalfont, Buckghamshine, UK), anti-mouse (Amersham) or anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:2,000 – 10,000 in TPBS at room temperature for 60 min. Then, the PVDF was washed five times in TPBS. The blots were developed using the enhanced chemiluminescence detection system (Amersham). The chemiluminescence was visualized using a Lumino Image Analyzer (LAS1000; Fuji Photo Film, Tokyo) or

exposed to X-ray film (Fuji Photo Film). To check for protein loading/transfer variations, all blots were stained with Ponceau Red (washable, before incubation with antibodies) and with Coomassie brilliant blue (permanent, after the enhanced chemiluminescence detection system). Intensity of total protein bands per lane was analyzed using free software NIH image produced by Wayne Rasband (National Institute of Health, Bethesda, MD, USA). The results are expressed as a percentage of the band obtained with sham-operated controls (sham) in each experiment. Negligible loading/transfer variation was observed between samples.

Real-time quantitative PCR

Total RNA samples were prepared from tissues by the guanidinium thiocyanate-phenol-chloroform single-step extraction method with Isogen (Nippon Gene, Toyama). After being isolated, treated with DNase I, and quantified, RNA was reverse-transcribed to cDNA by the use of a ReverTra Ace (TOYOBO, Osaka). The single-stranded cDNA was then used in real-time quantitative PCR for evaluation of relative expression levels of the 5 genes of interest. Selected genes and primers are shown in Table 1. DNA amplification was performed in the Applied Biosystems (ABI 7900HT) real-time PCR instrument with the GeneAmp 7900HT Sequence detection system software (Perkin-Elmer Corp., Foster City, CA, USA), and the detection was made by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. The PCR reactions were set up in microtubes in a volume of 20 μl. The reaction components were 2 μl of cDNA synthesized as above, 10 μl of 2 × SYBR Green master mix (Perkin-Elmer Corp.), and 0.4 μM of each pair of oligonucleotide primers (Table 1). The program was as follows: an initial step at 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. Regression curves were drawn for each sample and its relative amount was calculated from the threshold cycles with the instrument's software (SDS 2.0) according to the manufacturer's instructions. The PCR products were analyzed by gel electrophoresis to confirm the specificity of generated products. Relative expression levels of the target genes were normalized to the geometric mean of the two internal control genes, β-actin and G3PDH.

Statistical analyses

All values are expressed as mean ± S.E.M. The statistical significance of differences between group means was determined by a one-way analysis of variance (ANOVA). To determine the group differences in the time courses of the PS amplitude, analysis was carried out using repeated measures ANOVA. The significance of differences among all groups was assessed with the Tukey's post-hoc multiple comparison test. *P*-values <0.05 were considered significant.

Results

Effects on 2VO-induced hippocampal LTP impairment

As shown in Fig. 1A, tetanic stimulation produced a long-lasting increase in the PS amplitude, LTP, in the perforant-path/DG synapses of the sham-operated rat hippocampus. Transient (10 min) 2VO significantly impaired LTP formation in this synapse, which was recorded 4 days after reperfusion (Day 4). SNO-PEG-Hb (250 mg/kg, i.v.) alleviated 2VO-induced LTP impairment with a therapeutic time window; the effects increased along with the treated time-point (Day 0, 1, and 2) after reperfusion. Significant alleviating effects of SNO-PEG-Hb were observed in the group treated on Day 1 or 2, when the time course changes were evaluated using repeated measure ANOVA. The maximum and significant effect in the AUC, reflecting the ensemble effect of time course changes in LTP, was obtained in the group treated on Day 2 (Fig. 1B). LTP formation in the 2VO rat treated with SNO-PEG-Hb on Day 4 was not significantly different from that in the vehicle-treated 2VO rat.

On the contrary, a non-*S*-nitrosylated Hb derivative PEG-Hb (250 mg/kg, i.v.) administered on Day 0 exacerbated 2VO-induced LTP impairment (Fig. 2). The AUC obtained during the 60 min after tetanic stimula-

Table 1. Primers and conditions for real-time quantitative PCR analyses

Gene	Primers (5'-3')		Product size
	Sense	Antisense	bp
VEGF	GTACCTCCACCATGCCAAGT	GCATTAGGGGCACACAGGAC	194
bFGF	CCCGCCCACTTCAAGGATCC	TAGCAGACATTGGAAGAAAC	300
eNOS	CTGCTGCCCCAGATATCTTC	CAGGTACTGCAGTCCCTCCT	230
nNOS	CCGGCTACACTTCTCCTCAC	CACGAAGCAGGGGACTACAT	210
iNOS	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTTGAAGGC	426

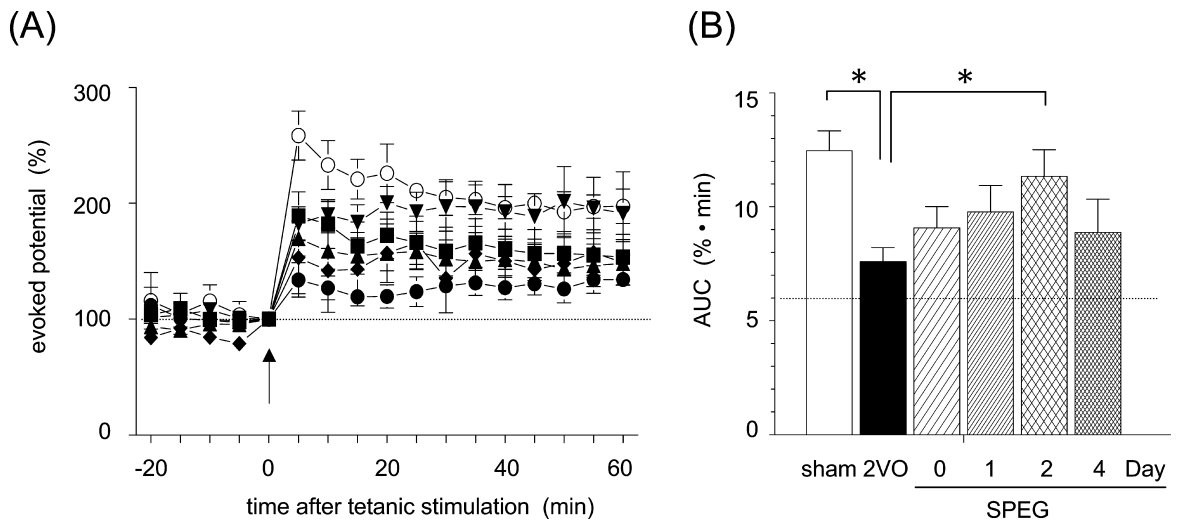

Fig. 1. Temporal effects of an *S*-nitrosylated-hemoglobin derivative on hippocampal long-term potentiation (LTP) in the perforant path-dentate gyrus synapses of the 2VO rat hippocampus. LTP was recorded 4 days after 2-vessel occlusion (2VO, 10 min). The effects were evaluated by time course changes (A) and the area under the curve (AUC) (B) of the population spike amplitude for the 60-min period after tetanic stimulation. *S*-Nitrosylated-polyethylene glycol-conjugated Hb (SPEG; 250 mg/kg, i.v.) was injected immediately (Day 0, filled triangles), 24 h (Day 1, filled squares), 48 h (Day 2, filled and inversed triangles), or 96 h (Day 4, 20 min before tetanic stimulation; filled diamonds) after reperfusion. Sham-operated rats (sham, open circles) and 2VO rats (2VO, filled circles) were treated with the same volume of vehicle (2.5 ml/kg, i.v.). The responses are expressed as % means ± S.E.M. (n = 6 – 12) of the basal population spike amplitude obtained before tetanic stimulation. **P*<0.05 *vs* vehicle-treated 2VO rats. In panel A, statistical significance, determined by repeated measures ANOVA followed by Tukey's post-hoc multiple comparison test, was observed between vehicle-treated 2VO rats and SPEG-treated 2VO rats on Day 1 (*P*<0.05) or 2 (*P*<0.01). The marks indicating statistical significance were not presented in the figure to avoid complexity.

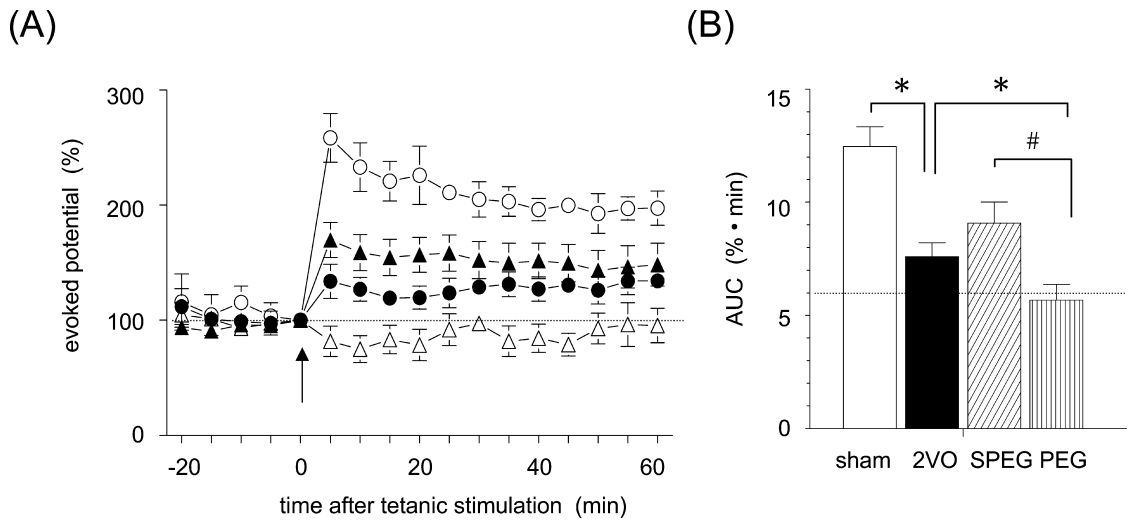

Fig. 2. Effects of Hb derivatives with and without *S*-nitrosylation on hippocampal long-term potentiation (LTP) in the perforant path-dentate gyrus synapses of the 2VO rat hippocampus. LTP was recorded 4 days after 2-vessel occlusion (2VO, 10 min). The effects were evaluated by time course changes (A) and the area under the curve (AUC) (B) of the population spike amplitude for the 60-min period after tetanic stimulation. Hb derivatives (250 mg/kg, i.v.) were injected immediately (Day 0) after reperfusion. Sham-operated rats (sham, open circles) and 2VO rats (2VO, filled circles) were treated with the same volume of vehicle (2.5 ml/kg, i.v.). The responses are expressed as % means ± S.E.M. (n = 6 – 9) of the basal population spike amplitude obtained before tetanic stimulation. *S*-Nitrosylated-polyethylene glycol-conjugated Hb: SPEG or filled triangles; polyethylene glycol-conjugated Hb: PEG or open triangles. **P*<0.05 *vs* 2VO and $^{\#}P$<0.05 *vs* SPEG. In panel A, statistical significance, determined by repeated measures ANOVA followed by Tukey's post-hoc multiple comparison test, was observed between PEG-treated 2VO rats and vehicle-treated (*P*<0.05) or SPEG-treated 2VO rats (*P*<0.05). The marks indicating statistical significance were not presented in the figure to avoid complexity.

tion decreased significantly, when compared to those in the vehicle-treated and SNO-PEG-Hb-treated 2VO group (Fig. 2B).

Effects on hippocampal expression of angiogenic molecules, VEGF and bFGF

Immunoblot analysis using the antisera raised against human VEGF and bFGF showed a single band with a molecular mass of approximately 39 and 18 kDa, which was referred to as VEGF and bFGF, respectively, in the rat hippocampus (Fig. 3: A and B). The bands obtained from the 2VO rat were evidently stronger than those from the sham-operated rat. Densitometric quantification of the signals revealed that VEGF and bFGF protein levels 4 days after 2VO were 237 ± 11% and 569 ± 23% (n = 5) of the control level, respectively The increased expression level of VEGF and bFGF significantly reduced to 199 ± 9% and 349 ± 39%, respectively, of the control level, when 2VO rats were treated with SNO-PEG-Hb (250 mg/kg, i.v.) on Day 0 (n = 5). The effects of SNO-PEG-Hb were more evident when treated on Day 1; VEGF expression decreased to

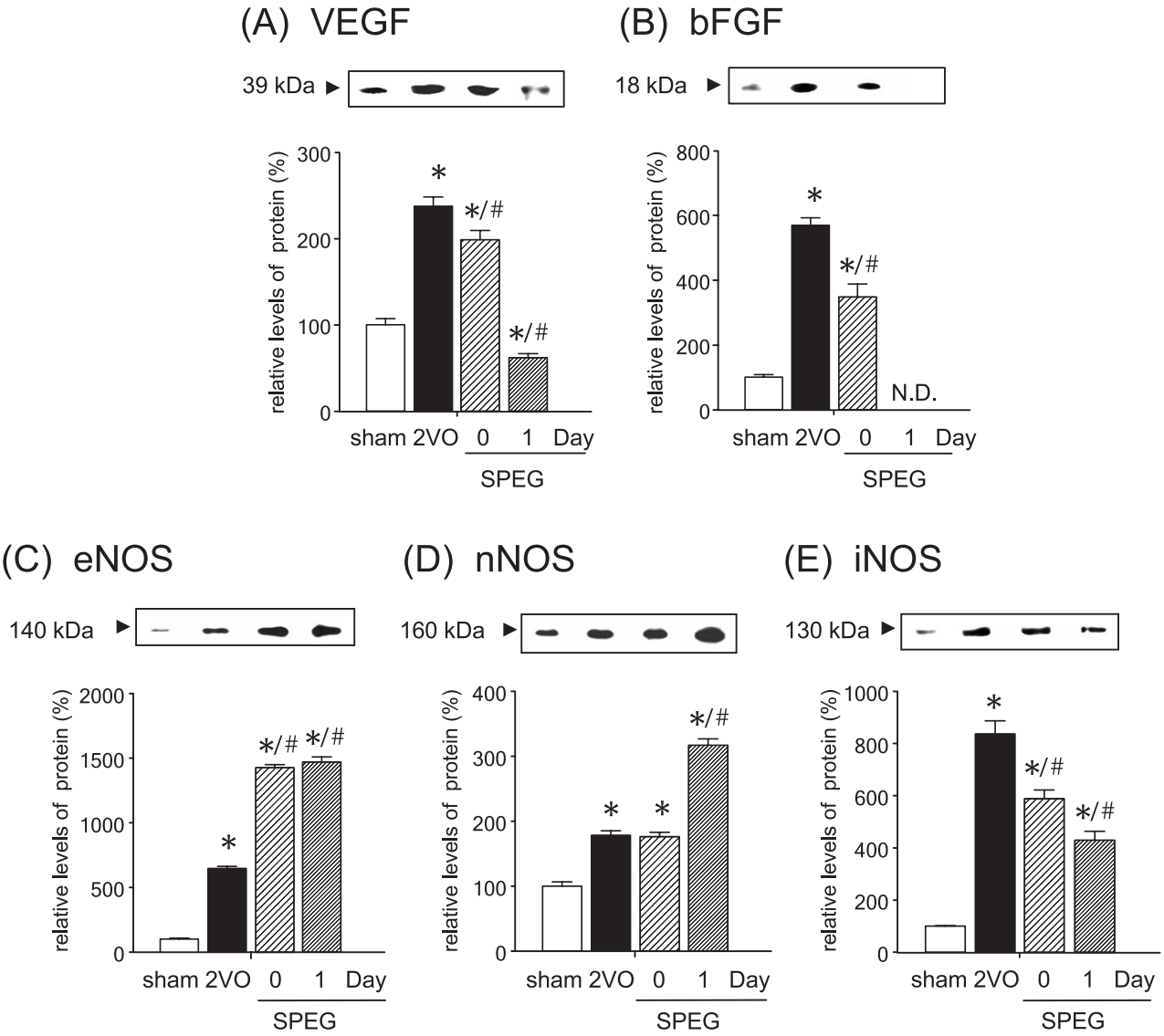

Fig. 3. Immunoblot analyses of angiogenic molecules and NO synthase isoforms in the hippocampus of the 2VO rat treated with an *S*-nitrosylated-hemoglobin derivative. A: VEGF, B: bFGF, C: eNOS, D: nNOS, and E: iNOS proteins. Upper panels: Representative Western blot; Lower panels: Bar graph comparing the immunostained band for each protein in the four groups of the hippocampus. Densitometric results are expressed as percent of the band obtained with sham-operated controls (sham) in each experiment. Bars are means ± S.E.M. of 5 separate experiments. **P*<0.05 and $^{\#}P$<0.05 *vs* the value obtained in sham-operated (sham) and vehicle-treated 2VO (2VO) rats, respectively.

62 ± 5% (n = 5), of the control level, and the bFGF band was not detectable.

Gene expressions of VEGF and bFGF in the hippocampus from sham-operated control rats and vehicle- and SPEG-treated 2VO rats were analyzed by the real-time quantitative PCR (Table 2). β-actin and G3PDH mRNA was used as an internal standard and for adjustment of sample-to-sample variations. The change in the transcript band for VEGF and bFGF was comparable with that obtained from Western blot analysis (Fig. 3: A and B). Namely, densitometric analysis showed that in vehicle-treated 2VO rats, VEGF and bFGF increased to 205% and 250% of sham-operated rats, respectively, at the mRNA level. SNO-PEG-Hb treatment on Day 0 or 1 decreased the mRNA level of both VEGF and bFGF. Especially, the effects of SNO-PEG-Hb were more evident in the rat treated with SNO-PEG-Hb on Day 1; VEGF and bFGF expressions decreased to 75% and 30% of sham-operated rats, respectively.

Effects on hippocampal expression of NOS isoforms

The NOS protein in the hippocampus was assessed using Western blot analysis. Bands for eNOS, nNOS, and iNOS were detected in the sham-operated rat hippocampus, with the predicted size of 140, 160, and 130 kDa, respectively (Fig. 3: C – E). In 2VO rats, the bands became stronger, indicating that hippocampal NOS expressions increased 4 days after cerebral blood flow restoration.

SNO-PEG-Hb (250 mg/kg, i.v.) treatment either on Day 0 or 1 altered these NOS features observed in 2VO rats; SNO-PEG-Hb further upregulated eNOS and nNOS and downregulated iNOS expression with time-dependency (Fig. 3: C – E). Namely, SNO-PEG-Hb treatment on Day 0 or 1 significantly increased the density of the eNOS band (1431 ± 27% and 1473 ± 44%, respectively, n = 5) when compared with that in the vehicle-treated 2VO rats (646 ± 17%, n = 5). Concerning nNOS, the band density in 2VO rats treated with SNO-PEG-Hb on Day 0 (177 ± 6%, n = 5) was comparable to that in vehicle-treated 2VO rats (178 ± 7%, n = 5), while in 2VO rats treated on Day 1, it was significantly increased (317 ± 11%, n = 5). On the contrary, SNO-PEG-Hb treatment on Day 0 or 1 in 2VO rats significantly decreased the density of the iNOS band (589 ± 36% and 431 ± 38%, respectively, n = 5), when compared with that in vehicle-treated 2VO rats (837 ± 50%, n = 5). Thus, the effects of SNO-PEG-Hb on nNOS and iNOS were more evident in 2VO rats treated on Day 1.

The changes in NOS mRNA levels of the hippocampus, which were quantitatively confirmed by analysis using the real-time PCR method, correlated well with NOS protein levels (Table 2). Namely, in vehicle-treated 2VO rats, the mRNA level of all NOS isoforms increased to different degrees as compared to the sham-operated control; 450% for eNOS, 160% for nNOS, and 600% for iNOS, respectively. SNO-PEG-Hb treatment on Day 0 or 1 further increased eNOS and nNOS and decreased iNOS transcripts in the hippocampus, compared with vehicle-treated 2VO rats. The effects were evident in 2VO rats treated with SNO-PEG-Hb on Day 1; eNOS and nNOS increased to 915% and 257%, respectively, and iNOS decreased to 265% of sham-operated rats.

Table 2. Relative amounts of mRNAs in the 2VO rat hippocampus

Gene	Sham	2VO		
		Vehicle	SPEG0	SPEG1
VEGF	100%	205%	160%	75%
bFGF	100%	250%	150%	30%
eNOS	100%	450%	850%	915%
nNOS	100%	160%	165%	257%
iNOS	100%	600%	375%	265%

Total RNA was extracted from the hippocampus and subjected to real-time PCR quantification, as described under the Materials and Methods. Values represent the amount of mRNA relative to that in sham-operated controls, which is considered as 100%. Sham-operated control rats (sham), 2VO rats [vehicle-treated (Vehicle), SNO-PEG-Hb (250 mg/kg)-treated on Day 0 (SPEG0), on Day 1 (SPEG1)].

Discussion

In the present study, we demonstrated that an *S*-nitrosylated Hb derivative, SNO-PEG-Hb (250 mg/kg, i.v.), alleviated LTP impairment in the dentate gyrus synapses of the rat hippocampus which was observed 4 days after transient cerebral ischemia (10 min). The effects were of time-dependent; impaired LTP was ameliorated along with the time-point of treatment (Day 0, 1, 2, and 4) after reperfusion. The significant effect was obtained by SNO-PEG-Hb treatment on Day 1 and 2. A non-*S*-nitrosylated Hb derivative, PEG-Hb (250 mg/kg, i.v.; Day 0), inversely deteriorated 2VO-induced LTP impairment.

The oxidation to nitrate by oxyHb or quenching to the α-subunit ferrous hemes has been considered to be the major pathway for NO elimination from the body. Recent studies have provided new insights on the NO and Hb interaction that NO binds to the cysteine thiols within the β-subunit of Hb (Cysβ93) in the tissue with high oxygen tension such as lung (6, 26 – 30). This SNO-Hb retains EDRF/NO-like bioactivity since it induces relaxation of pre-capillary vessels and inhibits platelet aggregation (6, 26, 39). In addition, SNO-Hb is

capable of transferring NO to the SH moiety. In the ischemic tissue with low oxygen tension, SNO-Hb is likely to release NO, dilate small vessels and result in providing more blood to the tissue. Very recently, it is reported that a connectivity exists between hemes and thiols in Hb, through which NO is dislodged from storage on the heme to form bioactive SNO-Hb (30). Thus, SNO-Hb can provide storage and a delivery route of bioactive NO to the tissues.

SNO-PEG-Hb, a new type of Hb derivative, has been developed as an artificial oxygen carrier (31, 32), which was modified with PEG to increase its molecular weight and to have longer half-life than non-PEG-Hb (SNO-Hb). SNO-PEG-Hb can also deliver oxygen and translocate NO to the SH moiety; and as a result, infusion of SNO-PEG-Hb (62.5 – 125 mg/kg) did not produce vasoconstriction, an undesirable effect of free Hb (31). Even at the supra-maximal dose of 250 mg/kg used here, there is only a small transient increase in blood pressure after infusion. We have also reported that SNO-PEG-Hb is a slow-releasing agent for NO in vivo (31). Indeed, plasma levels of oxidative NO metabolites (NOx) increased after treatment with *S*-nitrosylated-Hb (SNO-Hb or SNO-PEG-Hb; 125 mg/kg, i.v.), while they decreased after PEG-Hb injection in intact Wistar rats (H. Togashi, et al., unpublished observation), possibly indicating an in vivo property of SNO-PEG-Hb as an NO donor. Thus, SNO-PEG-Hb is likely to have a dual role as an NO scavenger and donor. This might contribute to the alleviating effects of SNO-PEG-Hb on 2VO-induced cerebral dysfunction, LTP impairment. It is possibly supported by our finding that non-*S*-nitrosylated PEG-Hb treatment on Day 0 (immediately after reperfusion) did not ameliorate but exacerbated the 2VO-induced LTP impairment. However, the NO species concerned with the temporal effects of SNO-PEG-Hb in the postischemic stages remain to be defined.

We demonstrated here that 2VO upregulated expression of all three NOS isoforms at mRNA and protein levels when examined 4 days after ischemia/reperfusion. SNO-PEG-Hb treatment on Day 1 downregulated iNOS expression and further upregulated eNOS and nNOS expressions, when compared to those of vehicle-treated 2VO rats. Accumulated evidence suggests differential roles of NOS isoforms or their temporal NO production in the pathogenesis for ischemic brain injury (9 – 12, 16, 17, 21). eNOS-derived NO is thought to be beneficial to promote collateral circulation and microvascular flow, whereas nNOS- and iNOS-derived NO are detrimental in the ischemic brain. Together with these beneficial/detrimental roles of three NOS-derived NO, the alleviating effects of SNO-PEG-Hb on 2VO-induced LTP impairment might be in part supported by our findings on hippocampal NOS features of upregulated eNOS and downregulated iNOS expressions.

The present results concerning nNOS features indicate that SNO-PEG-Hb further upregulated hippocampal nNOS expression can not explain the alleviating effects of SNO-PEG-Hb, since nNOS-derived NO is generally thought to have a detrimental role in the ischemic brain. However, Santizo et al. (20) reported that nNOS-derived NO plays a greater role than eNOS-derived NO in promoting vasodilation in the rat hippocampus, striatum, and cortex after forebrain ischemia, suggesting a beneficial role of nNOS-derived NO. It is plausible that nNOS-derived NO exerts beneficial and deleterious effects, dependent upon time and/or upon the tissue microenvironments after ischemia/reperfusion (22). Therefore, SNO-PEG-Hb-induced nNOS upregulation in 2VO rats might be contributable to its alleviating effects on LTP impairment.

It is known that some angiogenic molecules, including VEGF and bFGF and their receptors, increase after cerebral ischemia (40 – 43); however, there are few reports on global cerebral ischemia. The present study demonstrated that 2VO upregulated expression of angiogenic molecules, VEGF and bFGF, at mRNA and protein levels in the hippocampus on Day 4 (4 days after ischemia/reperfusion). SNO-PEG-Hb treatment on Day 0 or 1 downregulated both angiogenic molecules in 2VO rats. The treatment on Day 1 was more effective than that on Day 0, in accordance with the ameliorating effect on LTP. These changes in 2VO-induced expression of angiogenic molecules, VEGF and bFGF, might possibly indicate that SNO-PEG-Hb prevented the progression of ischemia-induced cerebrovascular remodeling in the hippocampus. Of note is that eNOS and VEGF expression patterns dissociated in the hippocampus of 2VO rats with SNO-PEG-Hb treatment, although the similarity of anatomical and temporal pattern of VEGF and eNOS induction has been reported in the rat brain with permanent ischemia (44). Very recently, reciprocal regulation between NO and VEGF has been reported (45 – 47): exogenous NO significantly enhanced angiogenesis in the ischemic brain with a concomitant increase in VEGF levels, while an excessive amount of NO negatively controlled VEGF synthesis. Dissociated expressions between eNOS and VEGF observed in the present study might be due to iNOS-derived excessive amount of NO after transient cerebral ischemia, which resulted in the downregulated VEGF expression in the hippocampus.

The precise mechanisms through which SNO-PEG-Hb alleviated 2VO-induced LTP impairment remain to

be elucidated. *S*-Nitrosylation is thought to be a biological process involved in some physiologic and pathophysiologic states such as cerebral ischemia (5 – 7). Translocating NO bioactivity from Hb can modulate these biological processes, where intracellular thiols might be a key element since the translocation is accelerated in the presence of low molecular weight thiols such as glutathione and a trace amount of copper ions (38, 48). A nitrosylated molecule (*S*-nitrosothiol) such as *S*-nitrosoglutathione (GSNO) is known to be present in a micromolar concentration in the rat brain and might serve as signaling molecules between endothelial cells and neurons (48, 49). However, little is known about whether ischemia/reperfusion can alter *S*-nitrosothiol levels in the hippocampus. SNO-PEG-Hb might exert a dual action as an NO scavenger/donor to modulate the regional NO levels dependent upon the tissue microenvironments after ischemia/reperfusion (31). Indeed, very recently, it was reported that there is a connectivity between hemes and thiols in Hb, through which NO is dislodged from storage on the heme to form bioactive SNO-Hb to regulate NO function (29). Alternatively, to elucidate the mechanisms underlying the ameliorating effects of SNO-PEG-Hb on the ischemic dysfunction is of importance for understanding the temporal role of NO in the pathophysiology of the postischemic neuronal damages and a novel intra- and/or intermolecular biochemistry of Hb.

In the present study, the therapeutic time window was observed for the ameliorating effects of SNO-PEG-Hb on 2VO-induced hippocampal LTP impairment. It is of note that the therapeutic window was different from that for a free radical scavenger, edaravone (50). Namely, the maximum effect of SNO-PEG-Hb was obtained by the treatment on Day 2. On the other hand, the significant effect of edaravone was observed when administered immediately after reperfusion, accompanied with abolishment of hydroxyl radical generation in the 2VO rat hippocampus (51). Out findings that temporal effects of SNO-PEG-Hb on 2VO-induced neuronal dysfunction were different from those of edaravone might reflect the temporal difference in target molecules and/or processes after ischemia /reperfusion. In other words, SNO-PEG-Hb, which has different mechanisms and therapeutic time windows from a free radical scavenger, might provide a new strategy for ischemic cerebral injury.

In summary, our data demonstrated that administration of SNO-PEG-Hb alleviated the hippocampal neuronal dysfunction after 2VO, with a relatively wide therapeutic window, accompanied with the changes in 2VO-induced molecular features of NOS isoforms and angiogenic molecules. These findings suggest that a Hb derivative, SNO-PEG-Hb, possesses protective effects on the brain from cerebral ischemic neuronal damages. A non-*S*-nitrosylated Hb derivative, PEG-Hb, exacerbated the 2VO-induced LTP impairment, suggesting that *S*-nitrosylation of Hb is important for this ameliorating effect. This might increase the therapeutic potentials of SNO-PEG-Hb as an artificial oxygen carrier for use in the area of oxygen therapy.

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