

Vitamin E Regulates SMase Activity, GSH levels, and Inhibits Neuronal Death in Stroke-Prone Spontaneously Hypertensive Rats during Hypoxia and Reoxygenation

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Abstract

Stroke-prone spontaneously hypertensive rats (SHRSP/IZM) develop severe hypertension, and more than 95% of them die of cerebral stroke, making them widely used as models of experimental cerebral ischemia. The neurons of SHRSP/IZM are more susceptible to hypoxia and reoxygenation (H/R) than those of Wistar Kyoto (WKY/IZM) rats. In particular, cerebral ischemia strongly induces neuronal death in SHRSP/IZM. We examined the effect of high dose vitamin E on the levels of glutathione (GSH) and cell death during H/R in neurons isolated from SHRSP/IZM and WKY/IZM rats. The neurons of SHRSP/IZM were more vulnerable and lost more GSH than those of the WKY/IZM rats. High dose vitamin E induced the expression of gamma glutamylcystenyl synthase (γ -GCS) mRNA, increased GSH levels, reduced neutral sphingomyelinase (N-SMase) activity, and strongly prevented neuronal death. The level of GSH was significantly lower in SHRSP/IZM than WKY/IZM neurons following exposure to hypoxia and H/R. On the other hand, the activity of N-SMase was increased in SHRSP/IZM compared to the WKY/IZM neurons. These results suggest the decrease in GSH levels of SHRSP/IZM neurons to be associated with neuronal vulnerability and that GSH, production of which is induced by a high dose of vitamin E.

Keywords: Glutathione; SMase; Hypoxia/reoxygenation; Ischemic neuronal death; SHRSP.

1. Introduction

Cell injury or death after cerebral vascular occlusion has been postulated to result from a number of interacting pathophysiological factors, including the depolarization-induced entry of calcium via N-methyl-D-aspartate (NMDA) receptors, the generation of intracellular free radicals, damage to mitochondrial respiratory enzymes, and the induction of programmed cell death (Choi, 1995; Dalkara et al., 1996). It is suggested that calcium channel blockers and antioxidants effectively capture the hydroxyl radicals produced during hypoxia and reperfusion, thereby preventing neuronal damage (Mason et al.,

1999). Glutathione (GSH) is a major cellular antioxidant and as such plays an important neuroprotective role (Dringe, 2000). GSH reacts directly with radicals in nonenzymatic reactions (Chance et al., 1979) and is also an electron donor in the reduction of peroxides catalyzed by GSH peroxidases (Chance et al., 1979). Acute depletion of intracellular GSH can cause cellular damage (Meister, 1991), and cellular GSH levels are closely correlated with cell survival under adverse conditions (Bains and Shaw, 1997; Bridges et al., 1991). The first step of GSH synthesis is rate-limiting and catalyzed by gamma glutamylcystenyl synthase (γ -GCS) (Aoyama et al., 2008). On the other hand, neuronal cell death may involve the activation of magnesium-dependent neutral sphingomyelinase (N-SMase) caused by a drop in GSH levels, followed by a rapid rise in cellular ceramide levels (Liu et al., 1998). The expression of N-SMase closely linked to the initial production of ceramide was induced by the depletion of GSH (Lavrentiadou et al., 2001).

Stroke-prone spontaneously hypertensive rats/IZM (SHRSP/IZM) develop severe hypertension, and more than 95% die of cerebral stroke, making them

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widely used as models of experimental cerebral ischemia (Yamori *et al.*, 1974). Cerebral ischemia for 20 min in SHRSP induced a massive efflux of glutamate, causing delayed neuronal death in the hippocampal CA1 region, whereas the mother strain of SHRSP/IZM, Wistar Kyoto (WKY/IZM) rats, lacked these characteristics under the same conditions (Gemba *et al.*, 1992). We previously reported that the antioxidant vitamin E prevents apoptosis in neurons during cerebral ischemia and reperfusion in SHRSP/IZM rat (Tagami *et al.*, 1998). This suggest that GSH is also an important regulatory neuropeptide in the central nervous system (CNS) (Guo *et al.*, 1992). We speculated that because increased peroxide generation occurs during hypoxia and reoxygenation (H/R), intracellular GSH content and apoptic regulation might be affected by vitamin E. The purpose of this study was to determine whether vitamin E affects the levels of GSH, γ -GCS mRNA expression, N-SMase activity, and cell death in neurons of WKY/IZM and SHRSP/IZM rats during H/R.

2. Materials and methods

2.1. Cell culture and treatments

Primary cortical neurons were prepared from WKY/IZM rat and SHRSP/IZM (15 days of gestation) as previously described (Tagami *et al.*, 1998). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Ind, Ltd., Tokyo, Japan) with 5% fetal bovine serum (FBS, Sigma, Japan) + 5% horse serum (Sigma) at 37°C in a humidified atmosphere of 5% CO₂ and room air. When nerve fibers and dendrites were well-developed (day 5-6), the airtight incubator chamber (Bellco Glass, Inc, Vineland, NJ.) was continually supplied with 1% O₂ 90% N₂ 5% CO₂, and the cells were cultivated at 1% O₂ for 36 h (hypoxia). The cells were then maintained in 5% CO₂ and room air (21% O₂) for 1.5 h (reoxygenation). Vitamin E (alpha-D-L-tocopherol, Wako Pure Chemical Industries, Ltd., Japan) was dissolved in ethanol supplemented with 5% FBS + 5% horse serum with the final concentration of ethanol being less than 0.01%. Vitamin E concentration was selected as in previously report (Tagami *et al.*, 1998). Vitamin E (100 μ M) was added both before reoxygenation and before hypoxia as in a previously described report (Tagami *et al.*, 1998). In addition, mature neurons were cultivated in air (about 21 % O₂) and 5% CO₂ for the same amount of time to serve as a control.

2.2. Measurement of GSH

GSH was measured as in a previously described report by Baker *et al.*, (1990). Briefly, cells (0.5-1 x 10⁶) were washed twice with PBS and lysed with 0.4% Triton X-100. One volume of 5% sulfosalicylic acid was added, the precipitated protein was removed by centrifugation for 10 min at 8000 x g, and the supernatant was stored at -80°C. For the assay, 25- μ l aliquots of supernatant were added to well of 96-well microtiter plates containing 140 μ l of 100 mM NaHPO₄, 5 mM NaEDTA (pH7.5), 25 μ l of 6 mM 5,5'-dithio-bis-(2-nitrobenzoic acid), and 10 μ l of 100 units/ml GSH reductase (Type III, Sigma). The reaction was started by adding 50 μ l of 1.1 mM NADPH, and the change in absorbance at 405 nm was monitored for 10 min. Results were calibrated against known GSH standards that were treated in parallel with the cell extracts and normalized to the protein content measured.

2.3. Measurement of N-sphingomyelinase activity

N-SMase activity was measured according to the method of a previous report (Ochiai *et al.*, 2004). Briefly, cells (5 x 10⁶) were washed twice with PBS and lysed with 25mM Tris-HCl buffer (pH 7.5)/2mM EDTA/2mM EGTA/1mM phenylmethylsulfonyl fluoride (PMSF, Sigma)/20g/ml N-[N-(L-3-Trans-carboxirane -2-carbonyl)-L-leucyl]-agmatine (E-64, Roche). For the assay, 10- μ l aliquots of supernatant were added to well of 96-well microtiter plates containing 190 μ l of 15mM

2-hexadecanoylamino-4-nitrophenylphosphorylcholine (HNP, Sigma), 100mM Tris-HCl buffer (pH 7.4)/10mM MgCl₂. Incubation followed at 37°C for 60 min; the enzyme reaction was terminated by adding 400 μ l of 100mM glycine buffer (pH 10.5) and 700 μ l ethanol. The suspension was vortexed and centrifuged at 200 x g for 10 min. The absorbance of the supernatant solution was measured absorbance at 410 nm.

2.4. Evaluation of neuronal death

The cultures were terminated by fixing the cells with 1.25% glutaraldehyde and 1% paraformaldehyde in PBS for 30 min. The cells were washed overnight at 4°C in the same buffer, and post-fixed with 2% OsO₄ (Wako) buffered with PBS for 1 h. The cells were dehydrated in a graded series of ethanol concentrations and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate for electron microscopic examination. Viable and nonviable cells were counted under an electron microscope by two of the authors as previously reported (Tagami *et al.*, 1998). The criteria

were as follows: (1) cells lose their axons and dendrites and numerous lipid droplets appear in the cell bodies, although cell organelles remain intact (initial stage of apoptosis); (2) cells become round, small and electron-dense, and nuclei demonstrate prominent invaginations (second stage of apoptosis); (3) cells lose their cytoplasm and cell membrane, and nuclei become small and dark, then disappear (advanced stage of apoptosis); (4) cells become electron-lucent, the organelles decrease in number, and the nuclei demonstrate abnormal clusters of chromatin (primary or secondary necrosis). We performed at least 3 experiments per condition and examined at least 3 wells (diameter 10 mm) in each experiment. We examined over 3,000 neurons in each well. Consequently, we obtained data on 10,000 neurons per condition.

2.5. Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from cultured neurons using Trizol reagent (Gibco BRL, Tokyo, Japan). Quantitative PCR was carried out using real-time 'Taqman TM' technology (Gibson *et al.*, 1996), and the results were analyzed with a Model 7700 Sequence Detector System (Applied Biosystems, Foster City, CA.). Quantitative PCR was performed for γ -GCS and to monitor the expression of a 18s ribosomal RNA (TaqMan ribosomal RNA Control Reagents). Primers and the TaqMan probes were selected from Gene bank (Accession numbers: γ -GCS: J05181) and designed using the software Primer Express (Applied Biosystems). The forward primer chosen was 5'-TTGGCCACTATCTGCCCAA-3' for γ -GCS. The reverse primer was 5'-TCTGACACGTAGCCTCGGTAAA-3'. The sequence of the TaqMan probe was 5'-ATGGCTTTGAGTGCTGCATCGCCA -3' for γ -GCS.

2.6. Statistical analysis

Data are presented as the mean \pm SE. The significance was determined using Fisher's Protected Least Significance Difference (PLSD) method following an analysis of variance (ANOVA).

3. Results

3.1. Effects of vitamin E on the death of cultured SHRSP/IZM and WKY/IZM rat neurons during H/R

In the present study, primary dissociated neurons from WKY/IZM rat and SHRSP/IZM were prepared.

After hypoxia for 36 h, the percentage of dead neuronal cells from WKY/IZM rat and SHRSP/IZM was $8 \pm 4\%$ and $7 \pm 6\%$, respectively (without vitamin E) ($n=7$). After 1.5 h of reoxygenation following 36 h of hypoxia, the rate of death was $41 \pm 8\%$ and $78 \pm 8\%$ ($n=7$) (without vitamin E). Cell death was induced more extensively without vitamin E after exposure to 36 h of hypoxia and 1.5 h of reoxygenation in SHRSP neurons than WKY rat neurons (Fig. 1A). In contrast, both neuronal death was completely abrogated when a high dose of vitamin E (100 μ M) was added during reoxygenation and under all of the hypoxic conditions (Fig. 1B).

3.2. Electron microscopy

Furthermore, we examined the effects of vitamin E on the cell cultured neurons during H/R by electron microscopy. As shown in Fig. 2, SHRSP/IZM neurons, maintained in a hypoxic state for 36 h and then cultured in reoxygenated conditions for 1.5 h without vitamin E (B), were damaged and most cell organelles lost their original structure. Under the same conditions, the cells treated with vitamin E (A) were intact. Numerous cell organelles and normal nuclei were observed. With these results, the efficacy of vitamin E to prevent cell death was proved morphologically.

3.3. Effects of vitamin E on the levels of GSH in cultured neurons from SHRSP/IZM and WKY/IZM rat during H/R

We examined the levels of GSH in WKY/IZM rats and SHRSP/IZM neurons under normal, hypoxia and H/R conditions. Without vitamin E, the level was 18.3 ± 0.4 (nmol/mg protein) in WKY/IZM rats and 22.1 ± 0.7 (nmol/mg protein) in SHRSP/IZM ($n=4$) under normal conditions (Fig. 3). With vitamin E, the level was 17.3 ± 0.6 (nmol/mg protein) in WKY/IZM rats and 22.8 ± 0.5 (nmol/mg protein) in SHRSP/IZM ($n=4$) under normal conditions. After hypoxia for 36 h, the level was 17.2 ± 1.4 (WKY/IZM; nmol/mg protein) and 14.4 ± 0.6 (SHRSP/IZM; nmol/mg protein) without vitamin E, respectively ($n=4$). After hypoxia for 36 h, the level was 18.2 ± 0.4 (WKY/IZM; nmol/mg protein) and 17.4 ± 0.8 (SHRSP/IZM; nmol/mg protein) with vitamin E, respectively ($n=4$). With hypoxia, the GSH content of neural cells significantly decreased in SHRSP/IZM, but not WKY/IZM rats. After 1.5 h of reoxygenation following 36 h of hypoxia, the level was 9.8 ± 0.5 (nmol/mg protein) in WKY/IZM rats and 7.6 ± 0.7 (nmol/mg protein) in SHRSP/IZM ($n=4$) without vitamin E. With vitamin E, the GSH content of neurons during H/R was 16.8 ± 1.7 nmol/mg protein (WKY/IZM rat) and 17.0 ± 0.5 nmol/mg protein (SHRSP/IZM).

The GSH content decreased in SHRSP/IZM rat neurons under hypoxia and H/R compared with the control (normoxia). Furthermore, the addition of vitamin E prevented a decrease of GSH during H/R in WKY/IZM rats and SHRSP/IZM neurons. The level of GSH was significantly lower in SHRSP/IZM than WKY/IZM neurons following exposure to hypoxia and H/R without vitamin E.

3.4. Effects of vitamin E on the levels of γ -GCS mRNA in cultured neurons from SHRSP/IZM and WKY/IZM rat during H/R

Next, we examined the expression of γ -GCS mRNA in neurons from WKY/IZM and SHRSP/IZM rats during H/R and the effects of vitamin E by quantitative PCR. The expression was significantly decreased during hypoxia and H/R in WKY/IZM rats and SHRSP/IZM neurons. The addition of vitamin E (50, 100 μ M) significantly induced γ -GCS mRNA expression in WKY/IZM rat and SHRSP/IZM neurons during H/R (Fig. 4). However, the level of γ -GCS

mRNA was significantly lower in SHRSP/IZM than WKY/IZM neurons following exposure to hypoxia, H/R or H/R with 10 μ M of vitamin E.

3.5. Effects of vitamin E on the activity of N-sphingomyelinase in cultured neurons from SHRSP/IZM and WKY/IZM rat during H/R

Moreover, we examined the activity of N-SMase in neurons of WKY/IZM rats and SHRSP/IZM during H/R and the effects of vitamin E. The activity of N-SMase was enhanced during hypoxia compared with that observed during normoxia in neurons of WKY/IZM and SHRSP/IZM rats. On the other hand, the N-SMase activity in both strains strongly increased at 1.5 h reoxygenation. At 1.5 h of reoxygenation, the N-SMase activity was significantly greater in SHRSP/IZM than in the WKY/IZM neurons. However, the addition of a high dose of vitamin E (50 or 100 μ M) significantly reduced N-SMase activity in the neurons of the WKY/IZM rats and SHRSP/IZM rat during H/R (Fig. 5).

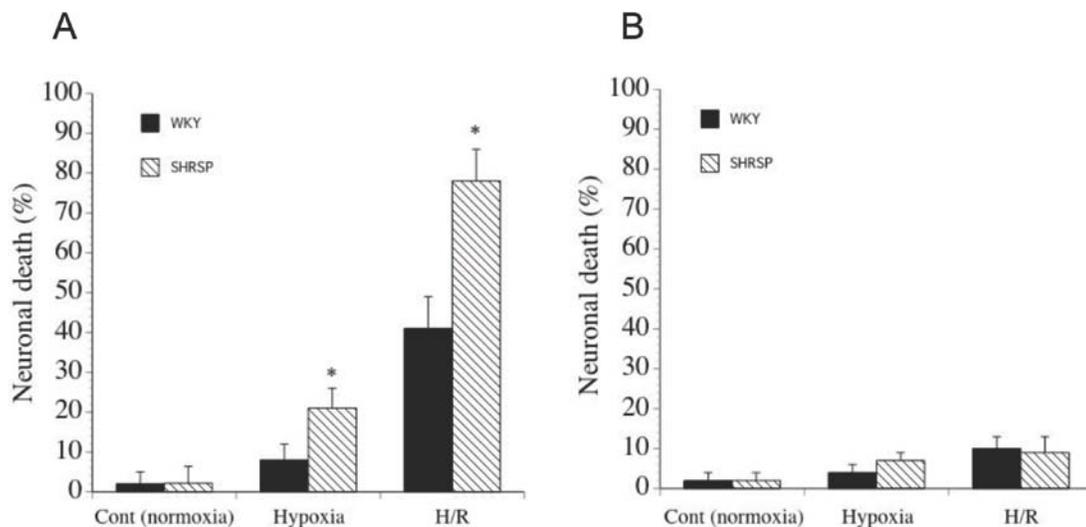


Fig. 1. Inhibition of cell death in cortical neurons by applying vitamin E during H/R in WKY/IZM rats and SHRSP/IZM. When neurons in culture matured, they were incubated in 1% O₂ for 36 h without (A) or with (B) vitamin E (100 μ M). After the hypoxic culture, they were maintained in 21% O₂ for 1.5 h with or without vitamin E (100 μ M). Vitamin E treatment was carried out as described in "experimental procedures" and in a previous report (Tagami *et al.*, 1998). Cont (normoxia) : 21% for 36 h, Hypoxia : 1% O₂ for 36 h. H/R : 1% O₂ for 36 h plus 21% O₂ for 1.5 h. Asterisk (*) : p < 0.001 compared with WKY/IZM rats. Values are the mean \pm SE (n=7).

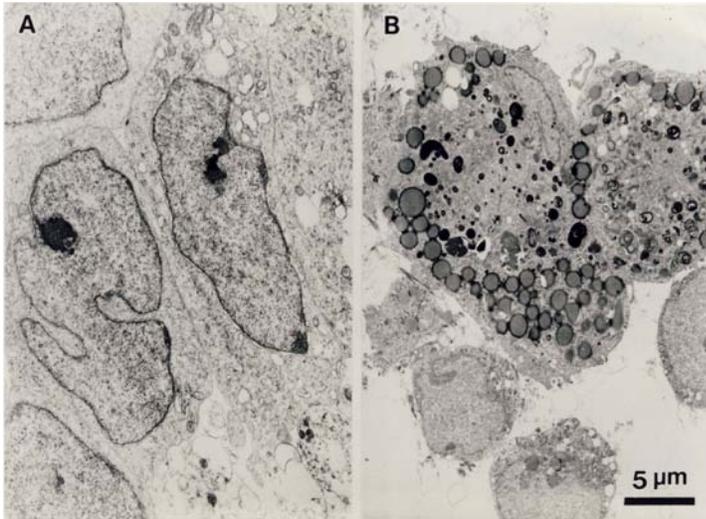


Fig. 2. Preventive effects on ischemic neuronal death of vitamin E in SHRSP/IZM neurons. When neurons in culture matured, they were incubated in 1% O₂ for 36 h and reoxygenated for 1.5 h with (A) or without (B) vitamin E (100 μM). Vitamin E treatment was carried out as described in “experimental procedures” and in a previous report (Tagami *et al.*, 1998).

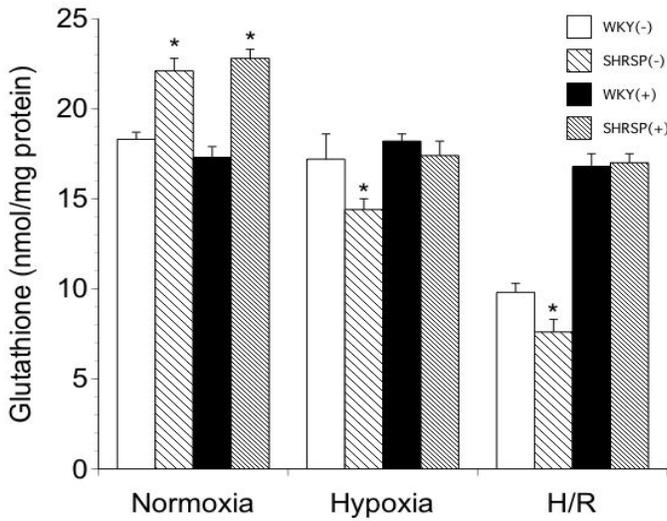


Fig. 3. Effects of vitamin E on levels of GSH in SHRSP/IZM neuron during H/R. When neurons in culture matured, they were incubated in 1% O₂ for 36 h without or with vitamin E (100 μM). Normoxia : 21% O₂ for 36 h. Hypoxia : 1% O₂ for 36 h. H/R: hypoxia and reoxygenation: 1% O₂ for 36 h plus 21% O₂ for 1.5 h. WKY (-) : without vitamin E, SHRSP (-) : without vitamin E (-), WKY (+) : with vitamin E, SHRSP (+) : with vitamin E. Values are the mean ± SE (n=4). Asterisk (*) : P < 0.05 compared with WKY/IZM and SHRSP/IZM without vitamin E under normoxia, hypoxia, H/R.

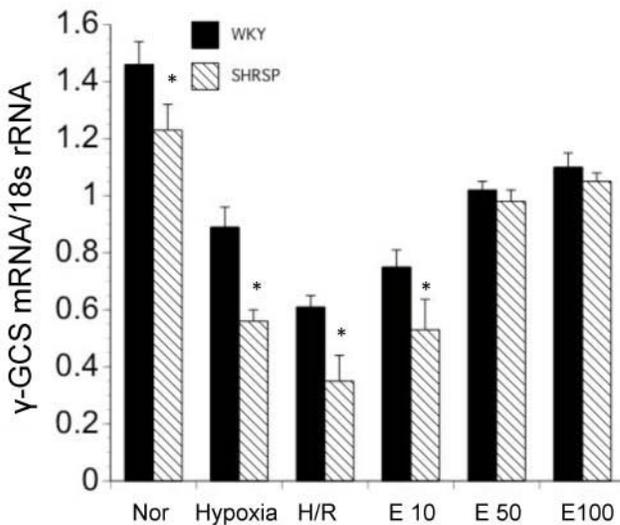


Fig. 4. Effects of vitamin E on expression of γ-GCS mRNA in WKY/IZM rats and SHRSP/IZM neurons. Nor; normoxia, 21% for 36 h, Hypoxia : 1% O₂ for 36 h. H/R : hypoxia and reoxygenation : 1% O₂ for 36 h plus 21% O₂ for 1.5 h. E 10 : H/R plus vitamin E (10 μM). E 50 : H/R plus vitamin E (50 μM). E 100 : H/R plus vitamin E (100 μM). Values are the mean ± SE (n=4). Asterisk (*) : P < 0.05 compared with WKY/IZM rats.

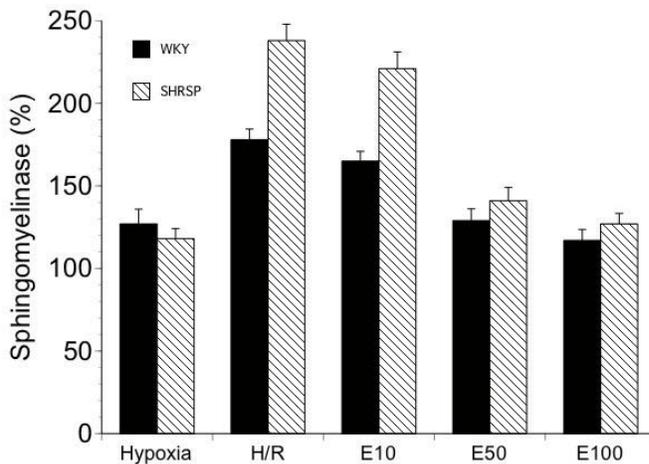


Fig. 5. Effects of vitamin E on N-SMase activity in neurons of WKY/IZM rats and SHRSP/IZM. When the neurons in culture had matured, they were incubated in 1% O₂ for 36 h without or with vitamin E (100 μM). Hypoxia : 1% O₂ for 36 h. H/R : hypoxia and reoxygenation, 1% O₂ for 36 h plus 21% O₂ for 1.5 h. E 10 : H/R plus vitamin E (10 μM). E 50; H/R plus vitamin E (50 μM). E 100 : H/R plus vitamin E (100 μM). Values are the mean ± SE (n=4). The N-SMase ratios were calculated with the value observed under normoxia as 100%; 21% O₂ for 36 h in WKY/IZM and SHRSP/IZM rats.

4. Discussion

We report here the effects on the levels of GSH, N-SMase activity, and cell death in neurons from WKY/IZM and SHRSP/IZM rats caused by exposure to vitamin E during H/R. We found the neurons of the SHRSP/IZM rats to be much more vulnerable than those of the WKY/IZM rats. The GSH content of the neurons was decreased in the SHRSP/IZM compared with the WKY/IZM rats during H/R. On the other hand, the activity of N-SMase was increased in the SHRSP/IZM rat during H/R. Furthermore, a high dose of vitamin E maintained GSH content, reduced N-SMase activity, and prevented neuronal death during H/R.

Cellular defenses against free radical and reactive oxygen species include enzymatic and nonenzymatic mechanisms. Cellular GSH levels are closely correlated with cell survival under adverse conditions (Bridges *et al.*, 1991). Lavrentiadou *et al* (2001) demonstrated the role of GSH in preventing epithelial cell apoptosis. GSH depressed both early before ceramide production and it after events leading to apoptosis. In addition, a report demonstrated that ceramide produced by the activation of N-SMase is an important mediator of ischemic neuronal death (Yoshimura *et al.*, 1999). SMase-formed ceramides can, themselves, activate additional proteases, including p15^{CDK} which cleaves poly (ADP-ribose) polymerase, a zinc-binding, nuclear DNA-dependent enzyme implicated in apoptosis (Dbaibo *et al.*, 1997). Neuronal cell death may involve the activation of magnesium-dependent N-SMase caused by a drop in GSH levels (Liu *et al.*, 1998). The ability of GSH to nonenzymatically scavenge both single oxygen atoms

and hydroxyl radicals provides the first line of antioxidative defense (Dringen, 2000; Chance *et al.*, 1979). We determined the levels of GSH in neurons of WKY/IZM rats and SHRSP/IZM under normal conditions. Cultured mouse neurons contained appreciable amounts of GSH (-33 nmol/mg protein) (Bridges *et al.*, 1991). This value is a little high in comparison with our result (WKY/IZM rat; 18.3 nmol/mg protein, SHRSP/IZM; 22.1 nmol/mg protein). The variability may reflect differences in the regions from which cells were obtained, species differences, differences in the age of the cells in culture, and differences in culture conditions. Moreover, in the present study, we demonstrated a decrease in intracellular GSH levels during H/R in WKY/IZM rats and SHRSP/IZM. In particular, the GSH content of SHRSP/IZM neurons (34.3%) was decreased compared with that of WKY/IZM rat neurons (53.5%) without vitamin E during H/R. In a previous study, the levels of nerve growth factor (NGF) protein were elevated in SHR (the spontaneously hypertensive rat; the mother strain of SHRSP) compared to WKY rats (Clemow *et al.*, 1998). At the control level, the decreased levels of GSH may be a common feature, passed on to the SHR strain. The attenuated expression of GSH by H/R may be related to the level of GCS gene transcription in SHRSP/IZM neurons during stroke status, although the precise mechanisms responsible for these changes remain unclear.

On the other hand, the activity of SMase was greater in SHRSP/IZM than WKY/IZM neurons. N-SMase is a key component of the signaling pathways in cytokine- and other stress-induced cellular responses. Furthermore, the effect of N-SMase promotes

nervous system cell death due to ischemia (Soeda *et al.*, 2004). It may also play a contributory role in the mechanism of nervous system cell death by H/R in SHRSP/Izm. Moreover, we demonstrated the addition of a high dose of vitamin E significantly reduced N-SMase activity in neurons of WKY/IZM rats and SHRSP/Izm during H/R. Report demonstrated that the vitamin E appears to inhibit this cascade in some experimental conditions. Lipopolysaccharide (LPS), Interferon (IFN)-gamma, and beta-amyloid treatment lead to the activation of the sphingomyelin-ceramide cascade with an increase in cellular ceramide and inducible nitric oxide synthase (iNOS) production (Ayasolla *et al* 2004). Vitamin E reduces beta-amyloid-induced iNOS and ceramide levels induced by the activities of C/EBP, NFkappaB, AP-1, and CREB in C6 glial cells. It appears that vitamin E inhibits neuronal cell death by some mechanism. In this study, we examined one of the effects of vitamin E.

GSH maintains the thiol redox potential in cells, keeping the sulfhydryl groups of cytosolic proteins in the reduced form (Dringen, 2000). Under normal conditions, a sufficient amount of GSH is present to maintain a redox state that allows for the prevention of cell death despite oxidative stress. However, acute depletion of intracellular GSH can cause cellular damage (Bains and Shaw, 1997). Oxidative toxicity due to the depletion of GSH rapidly impaired the mitochondrial function of motor neurons (Winterbourn and Metodiewa, 1994; Rizzardini *et al.*, 2003). We recently reported that ebselen, a GSH peroxidase mimic antioxidant, has a marked inhibitory effect on neuronal damage during H/R in SHRSP/IZM (Yamagata *et al.*, 2008). Our results indicated that alterations in GSH status and redox regulatory function are associated with ischemic neuronal death in SHRSP/IZM rats. Furthermore, in the present study, we demonstrated the effects of vitamin E on GSH levels and the expression of γ -GCS mRNA in cultured neurons during H/R. The amount of cysteine or cysteine precursors in the culture medium determines the GSH level in neurons (Dringen, 2000). However, cysteine is very unstable owing to its oxidation under aerobic conditions (Wang and Cynader, 2000). Likewise, GSH and other thiols will be oxidized to the corresponding disulfides (Wang and Cynader, 2000). Haloperidol (HP)-induced oxidative cell death was prevented by the pineal hormone melatonin, by its precursor N-acetyl serotonin, and most effectively by vitamin E (Post *et al.*, 1998). These antioxidants inhibited the intracellular accumulation of peroxide and stabilized the GSH content of mouse hippocampal HT22 cells after being challenged with

HP. Vitamin E may have a marked inhibitory effect on neuronal damage by quickly capturing the reactive oxygen and free radicals formed during H/R (Tagami *et al.*, 1998). Furthermore, we demonstrated that the N-SMase activity in both strains increased during reoxygenation. The activity of N-SMase was higher in the SHRSP/IZM than in the WKY/IZM neurons. A previous report demonstrated that the expression of N-SMase via the initial production of ceramide was induced by the depletion of GSH (Lavrentiadou *et al.*, 2001). Verheij *et al.*, (1996) reported that ceramide induces the stress-activated protein kinase/c-jun kinase (SAPK/JNK) signaling that precedes apoptosis. In addition, a report demonstrated a role for the JNK pathway in ceramide-stimulated cell death in parallel to neuronal apoptosis evoked by ceramide (Willaime-Morawek *et al.*, 2003). The cellular redox potential and reactive oxygen species, which is mainly regulated by GSH, are closely linked to the regulation of SMase activation (Won and Singh, 2006).

We conclude that the neurons of SHRSP/IZM are more vulnerable and lose more GSH than those of WKY/IZM rats during H/R. Simultaneously, vitamin E had the effect of maintaining GSH levels, inhibiting the death of SHRSP/IZM neurons. The level of GSH was significantly lower in neurons of SHRSP/IZM than WKY/IZM rats under H/R. Several reports suggest that cellular redox potential and reactive oxygen species, which is mainly regulated by GSH, are closely linked to the regulation of SMase activation (Won and Singh, 2006). We report here the effects on levels of GSH, N-SMase activity and cell death in neurons from SHRSP/IZM during H/R. These findings suggest that a decrease in GSH levels in SHRSP/IZM neurons is associated with neuronal vulnerability and that GSH production is induced by a high dose of vitamin E. Concomitantly, the altered GSH content of neurons during H/R might be involved in the neuronal vulnerability of SHRSP/IZM during stroke status.

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