Journal of Neurochemistry, 2007, **102**, 1863–1874 doi:10.1111/j.1471-4159.2007.04651.x

A novel mechanism underlying the susceptibility of neuronal cells to nitric oxide: the occurrence and regulation of protein *S*-nitrosylation is the checkpoint

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Abstract

The susceptibility of neuronal cells to nitric oxide (NO) is a key issue in NO-mediated neurotoxicity. However, the underlying mechanism remains unclear. As a cyclic guanosine monophosphate (cGMP)-independent NO signaling pathway, *S*-nitrosylation (or *S*-nitrosation) has been suggested to occur as a post-translational modification in parallel with O-phosphorylation. The underlying mechanism of the involvement of protein *S*-nitrosylation in the susceptibility of neuronal cells to NO has been little investigated. In this study, we focused on the role of *S*-nitrosothiols (RSNO) in the susceptibility of a cerebellar cell line R2 to NO. Our results showed the following: (i) *S*-nitrosoglutathione (GSNO) induced a burst of RSNO in GSH-depleted R2 cells, the majority of which were primarily contributed by the *S*-nitrosylation of proteins (Pro-SNOs), and was followed by severe neuronal necrosis; (ii) the elevation in the level of Pro-SNOs resulted from a dysfunction of *S*-nitroglutathione reductase (GSNOR) as a result of its substrate, GSNO, being unavailable in GSH-depleted cells. In the meantime, the suppression of GSNOR increased NO-mediated neurotoxicity in R2 cells, as well as in cerebellar granule neurons; (iii) Our results also demonstrate that the burst of RSNO is the ''checkpoint'' of cell fate: if RSNO can be reduced to free thiol proteins, cells will survive; if they are further oxidized, cells will die; and (iv) GSH-ethyl ester and Vitamin C protected R2 cells against GSNO neurotoxicity through two distinct mechanisms: by inhibiting the elevation of Pro-SNOs and by reducing Pro-SNOs to free thiol proteins, respectively. A novel mechanism underlying the susceptibility of neuronal cells to NO is proposed and some potential strategies to prevent the NO-mediated neurotoxicity are discussed.

Keywords: glutathione, neurotoxicity, nitric oxide, *S*-nitrosoglutathione, *S*-nitrosoglutathione reductase (GSNOR), *S*-nitrosation, *S*-nitrosylation.

J. Neurochem. (2007) **102**, 1863–1874.

Nitric oxide (NO) is a messenger molecule involved in neuronal survival (Rauhala *et al.*, 1998), differentiation (Peunova and Enikolopov 1995), neurotransmitter regulation (Shimizu-Sasamata *et al.* 1998), synaptic plasticity (Holscher 1997) and regulation of cerebral blood flow (Izuta *et al.* 1995). However, excess NO can be produced by activated microglia in an uncontrolled inflammatory response (Bal-Price and Brown 2001) or by the activation of nitric oxide synthase in excitotoxicity (Dawson 1995). The excessive production of NO has been reported to participate in acute and chronic neurodegenerative diseases including stroke, multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Samdani *et al.* 1997; Heales *et al.* 1999). Therefore,

Received April 2, 2007; accepted April 5, 2007.

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Abbreviations used: BCA, bicinchoninic acid; biotin-HPDP, N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide; BME, basal modified Eagle's medium; BSO, D,L-buthionine-[s,r]-sulfoximine; cGMP, cyclic guanosine monophosphate; CGNs, cerebellar granule neurons; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5,5′-dithio-bis(2-nitrobenzoic) acid; DTT, dithiothreitol; GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; GSSG, oxidized glutahione; LDH, lactate dehydrogenase; MMTS, methyl methanethiolsulfonate; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; PBS, phosphate-buffered saline; Pro-SNOs, *S*-nitrosylated proteins; RSNO, *S*-nitrosothiols; RSNO, *S*-nitrosothiols;-SOH, cysteine sulfenic acid; SDS, sodium dodecyl sulfate; shRNAs, small-hairpin RNAs; $-SO_2H$, cysteine sulfinic acid; $-SO_3H$, cysteine sulfonic acid; –SSG, *S*-glutathionylation; γ-GT, gamma-glutamyltransferase.

the susceptibility of neuronal cells to NO is an important issue in NO-mediated neurotoxicity.

In recent years, *S*-nitrosylation of proteins by NO, as a cyclic guanosine monophosphate (cGMP)-independent pathway, has been widely investigated. *S*-nitrosylation is likely a prototypic redox-based signaling mechanism (Stamler *et al.* 2001) because the *S*-nitrosothiols (RSNO) can not only be reduced to form thiols, but can also be oxidized to form either *S*-glutathionylation (–SSG), or cysteine sulfenic acid (–SOH), or cysteine sulfinic acid ($–SO_2H$) or cysteine sulfonic acid ($–SO_3H$). The substantially different roles of these different types of modification are implicated in various physiological and pathological processes (Stamler and Hausladen 1998). Numerous *S*-nitrosylated proteins have been identified *in vivo* including serum albumin (Stamler *et al.* 1992), hemoglobin β-subunits (Gow and Stamler 1998), the ryanodine-sensitive calcium release channels (Xu *et al.* 1998), N-methyl-D-aspartate (NMDA) receptor (Choi and Lipton 2000), methionine adenosyl transferase (Peraz-Mato *et al.* 1999), caspase-3 (Mannick *et al.* 1999), and matrix metalloproteinases (Gu *et al.* 2002). However, little is known about the roles and the regulation of protein *S*-nitrosylation in the susceptibility of neuronal cells to NO. Beltran *et al.* (2000) have shown that *S*-nitrosylation takes place in the presence of oxidative stress, but neither the progression nor the reversibility of this process, as well as its relevance in terms of either cell survival or death, have been investigated. More recently, it was reported that glutathione (GSH) depletion resulting in selective mitochondrial complex I inhibition in dopaminergic cells is via an NO-mediated pathway not involving peroxynitrite (Hsu *et al.* 2005). However, there is no direct experimental evidence about the relationship of *S*-nitrosylation and cell death. Moreover, there is also no evidence about the molecular mechanism of the occurrence of *S*-nitrosylation.

S-nitrosoglutathione reductase (GSNOR) was recently identified and found to be conserved from bacteria to humans. This enzyme is capable of regulating the levels of intracellular *S*-nitrosothiols (RSNO) (Liu *et al.* 2001). It belongs to the alcohol dehydrogenase class III family of enzymes, also known as glutathione-dependent formaldehyde dehydrogenase, and is capable of catalyzing the NADH/NADPH-dependent degradation of *S*-nitrosoglutathione (GSNO) to glutathione sulphinamide, oxidized glutathione (GSSG) and ammonia (Jensen *et al.* 1998). GSNOR has been reported to play an essential role in the biological processes such as vascular homeostasis and endotoxic shock (Liu *et al.* 2004). Intriguingly, GSNOR is the sole ADH in the brain and is abundant in the hippocampus, midbrain and cerebellum, which implies that it may play a role in the nervous system (Galter *et al.* 2003).

In this study, we use the R2 cell line, a conditionally immortalized cerebellar neural line (Rabizadeh *et al.* 1993), to investigate the roles of protein *S*-nitrosylation in the susceptibility of neuronal cells to NO and the involvement of GSH and GSNOR in this process.

Materials and methods

Materials

Dulbecco's modified Eagle's medium and newborn calf serum were obtained from Hyclone (South Logan, UT, USA). Trypsine and penicillin–stretomycin were from Gibco (Rockville, MD, USA). T4 DNA ligase was from New England BioLabs (Ipswich, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), D,L-buthionine-[S,R]-sulfoximine (BSO), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), Vitamin C, dithiothreitol (DTT), *N*-ethymaleimide, neocuproine and NADH were from Sigma (St Louis, MO, USA). Diethylenentriaminepenta-acetic acid, phenylmethylsufonyl fluoride, bathocuproinedisulfonic acid, sulfanilamide, and *N*-(1-naphthyl) ethylenediamine were from Aldrich (St Louis, MO, USA). *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP), methyl methanethiolsulfonate (MMTS) were from Pierce (Rockford, IL, USA). Glutathione was from Roche Molecular Biochemicals (Indianapolis, IN, USA). RNAi-Ready pSIREN-Retro-ZsGreen vector was from Invitrogen (Carlsbad, CA, USA). The Determiner M–NH_3 Diagnostic Kit was from Shanghai Runny Science & Technology Co. Ltd. (Shanghai, China). The lactate dehydrogenase (LDH) kit was from Zhongsheng High-tech. Bioengineering Company (Beijing, China). All other chemicals were of the highest grade commercially available.

Cell culture and treatments

R2 cells (cerebellum neural cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified 5%CO_2/95% air. To deplete intracellular GSH pools, cells were pretreated with BSO (an inhibitor of γ-glutamylcysteine synthetase). BSO^+ indicates pre-treatment with 0.5 mmol/L BSO for 24 h and $GSNO^+$ indicates treatment with 0.5 mmol/L GSNO for the indicated time unless otherwise stated.

Primary cerebellar granule neurons (CGNs) were isolated from 8-day-old Sprague-Dawley rats as described previously (Novelli *et al.* 1988). Briefly, cells were cultured in 35 mm Petri-dishes with a 14 mm glass microwell (MatTek corporation, Ashland, MA, USA) and seeded at a density of 2×10^6 per mL in basal modified Eagle medium containing 10% fetal bovine serum, 25 mmol/L KCl, 2 mmol/L glutamine, and penicillin (100 U/mL)-streptomycin (100 μg/mL). Cytosine arabinoside (10 mmol/L) was added to the culture medium 22–24 h after plating to limit the growth of non-neuronal cells. CGNs were cultured for 8 days and then used for the experiments.

Synthesis of *S*-nitrosoglutathione

S-nitrosoglutathione was synthesized as described previously (Hart 1985). Briefly, GSH in 625 mmol/L HCl was reacted with the equimolar sodium nitrite at 4°C for 45 min. After the addition of 2.5 volumes of acetone, the mixture was stirred for another 20 min. GSNO was washed once with 80% acetone, twice with 100% acetone, twice with diethyl ether, and dried under vacuum. The yield

of GSNO was quantified by the absorbance of its S–NO moiety at 334 nm (ε_{334} = 800/mol/L^{-1} cm^{-1}) (Singh *et al.* 1996). Fresh GSNO was prepared just before each experiment.

Cell viability assay

S-nitrosoglutathione-induced neurotoxicity was evaluated with the MTT assay. In brief, after exposure to GSNO for the indicated time, cells were incubated with 0.5 mg/mL MTT for 3 h at 37°C and dimethylsulfoxide was added to solubilize the formazan product. After 30 min incubation, the product was analyzed at 595 nm using an automatic microtiter reader (Bio-Rad, Hercules, CA, USA). The optical density of the control sample was defined as 100% of the cell viability.

Analysis of necrosis

Necrotic cells were detected by using the index of LDH leakage from damaged cells and were expressed as a percentage of total cellular LDH in the cells. LDH activity was measured using a commercial assay kit.

Intracellular GSH measurement

Glutathione levels were measured as described previously (Tietze 1969) Briefly, after the exposure to 0.5 mmol/L GSNO for the indicated time, cell cultures were washed twice with phosphate-buffered saline (PBS), lysed in 3 : 1 (v/v) 8.3% sulfosalicylic acid and 0.2% Triton X-100 at 4°C for 30 min, centrifuged at 12 000 *g* for 10 min. The supernatant was reacted with an equal volume of 1 mmol/L DTNB at 20–25°C for 5 min and the reaction was monitored at 415 nm using an automatic microtiter reader (Bio-Rad). A standard curve was produced by the different concentrations of GSH and DTNB under the same conditions as above. The protein concentrations in the supernatant were determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce).

Total cellular *S*-nitrosothiols content assay

S-nitrosothiols content was measured using the Saville-Griess assay (Hoffmann *et al.* 2001). In brief, cells were lysed in Griess lysis buffer (50 mmol/L Tris–HCl, pH 8.0, 150 mmol/L KCl, 1% Nonidet-P40, 1 mmol/L phenylmethylsufonyl fluoride, 1 mmol/L bathocuproinedisulfonic acid, 1 mmol/L diethylenentriaminepenta-acetic acid and 10 mmol/L *N*-ethymaleimide). Then the cell lysate was incubated with 1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine in either the presence or the absence of 0.375 mmol/L $HgCl_2$ at 20–25°C for 20 min, and the RSNO content was measured photometrically at 550 nm. The quantity was calculated using defined GSNO concentrations as a standard. The determination of the protein concentrations in cell lysates was carried out using the BCA protein assay reagent kit (Pierce).

Intracellular *S*-nitrosylated proteins (Pro-SNOs) and low-weight-molecules assay

Intracellular Pro-SNOs and low weight molecules were analyzed as described previously (Hoffmann *et al.* 2001). Cell lysates were prepared as described above following the procedures of RSNO contents assay, and were passed through Hitrap desalting columns (Amersham) pre-equilibrated with Griess lysis buffer. Pro-SNOs were separated from low molecular weight RSNO by desalting with 150 mmol/L NaCl, then the RSNO contents of the proteins and the low weight molecules were performed by using the RSNO contents assay as described above. Data were calculated as nmol RSNO per mg proteins loaded on the column.

Detection of Pro-SNOs by the biotin-switch method and western blotting

The analysis of Pro-SNOs was described previously (Jaffrey and Snyder 2001; Sumbayev *et al.* 2003). In brief, after exposure to 0.5 mmol/L GSNO for 2 h, cells were washed three times with ice-cold PBS, and trypsinized from the plate. And then, cells were lysed in HEN buffer (250 mmol/L Hepes-NaOH pH 7.7, 1 mmol/L EDTA, 0.1 mmol/L Neocupeoine) containing 0.5% NP-40 for 30 min on ice and centrifuged at 10 000 *g* for 10 min. Four volumes of blocking buffer [9 volumes of HEN buffer plus one volume 25% sodium dodecyl sulfate (SDS), and 20 mmol/L MMTS] were incubated with one volume of the supernatant at 50°C for 20 min with frequently vortexing. MMTS was then removed by protein precipitation with 10 volumes pre-chilled acetone. Biotin-HPDP (2 mmol/L) and sodium ascorbate (1 mmol/L) were incubated with the samples at 25°C for 1 h. After SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer was added, the samples were resolved by SDS-PAGE and transferred for immunoblotting with streptravidin–horseradish peroxidase. *S*-nitrosylated bovine serum albumin was used as a positive control.

Measurement of cell-surface thiols

The quantity of cell-surface thiols was measured as described previously (Zai *et al.* 1999). In brief, 1×10^7 treated cells were washed twice with cold PBS, trypsinized in the plate and resuspended in PBS. The cells were then incubated with 200 μmol/L DTNB at 37°C for 1 h, and centrifuged at 200 *g* for 5 min. The supernatants were measured by the absorption at 412 nm and the determination of protein concentrations in the supernatants was carried out by BCA protein assay reagent kit (Pierce).

Measurement of the thiols of intracellular proteins

The quantity of protein thiols was measured by using DTNB (Youn and Kang 2000). In brief, after exposure to 0.5 mmol/L GSNO for the indicated time, the supernatants of cell extract were precipitated with 5% trichloroacetic acid and washed twice. The protein pellets were solubilized in 0.5mol/L Tris–HCl (pH 8.8) containing 5 mmol/L EDTA and 1% SDS and divided into two aliquots. One was treated with 5 mmol/L DTT at 37°C for 1 h, and the other was not. Then each aliquot was reacted with 0.25 mmol/L DTNB at 20–25°C for 5 min and the reaction was monitored at 412 nm by automatic microtiter reader (Bio-Rad). The aliquot treated with 25 mmol/L *N*-ethymaleimide was used as a negative control.

Decomposition of *S*-nitrosoglutathione

After replacing the medium with either fresh cell culture medium or Locke's solution, cells were treated with 0.5 mmol/L GSNO for the indicated time. The GSNO contents in the supernatants were assayed with the absorbance of GSNO at 334 nm.

Intracellular ammonia assay

The treated cells were washed with PBS twice, and lysed in the lysis buffer (20 mmol/L Tris–HCl, pH 8.0, 0.5 mmol/L EDTA, 0.1% Triton X-100) on ice for 15 min. After centrifuging at 12 000 *g* for 15 min, the supernatant were assayed for ammonia.

The intracellular ammonia was determined using an enzymic assay (the NADH-dependent conversion of 2-oxoglutarate plus NH_3 to glutamate catalysed by glutamate dehydrogense) by the Determiner M–NH_3 Diagnostic Kit (Shanghai Runny Science & Technology Co. Ltd, China).

Construction of siRNA for *S*-nitrosoglutathione reductase and transfection

Two small hairpin RNAs (shRNAs) were generated using the oligonucleotide DNA sequences (pSIREN-110, 5′AGGCTCA-TGAAGTTCGGAT3′; pSIREN-223, 5′GGTGCTGGAATTGTGG-AAA3′), which were then subcloned into the RNAi-Ready pSIREN-RetroQ-ZsGreen vector (Clontech, BD Biosciences, San Jose, CA, USA). Luciferase shRNA annealed oligonucleotide (0.5 pmol/μL) was used as negative control. R2 cells were transiently transfected by electroporating using a Gene Pulser II System (Bio-Rad) set at 280 V and 1050 μF in HEPES buffer (0.283mol/L NaCl, 1.5 mmol/L Na_2HPO_4, 0.023 mol/L HEPES, pH 7.05).

The GSNOR RNAi plasmid for rat was constructed with RNAi-Ready pSIREN-RetroQ-ZsGreen vector. The plasmid sequence is: pSIREN-1622, 5′-GAAGTTCGAATTAAGATCA-3′. Luciferase shRNA annealed oligonucleotide (0.5 pmol/μL) was used as negative control. CGNs were transfected using a calcium phosphate co-precipitation kit (Profection Mammalian Transfection System-Calcium phosphate, Promega (Madison, WI, USA) as described previously (Li *et al.* 2000). Briefly, the conditioned culture medium was saved and 4 μg of plasmids was used for each 35 mm dish to produce DNA-calcium phosphate precipitation. Reactions were kept at 37°C for 30 min, and then the transfection medium was aspirated and washed twice. The conditioned medium was added back to the cultures. Seventy two hours after transfection, the CGNs were treated with 30 μmol/L GSNO for 18 h, and then used for the analysis for apoptosis.

Analysis of cell apoptosis

Analysis of cell apoptosis was determined by the condensed chromatin staining with the fluorescent probe Hoechst 33342. In brief, 4% paraformaldehyde-fixed CGNs were stained with Hoechst 33342 (5 μg/mL) at 20–25°C for 10 min, and then incubated in Loceke's solution (154 mmol/L Nacl; 5.6 mmol/L KCl; 2.3 mmol/L $CaCl_2$; 3.6 mmol/L $NaHCO_3$; 5.6 mmol/L Glucose; 5.0 mmol/L Hepes, pH 7.4) for assay. The chromatin images were observed with a fluorescent microscope (IX71, Olympus, Shinjuku-ku, Tokyo, Japan) and taken by CCD camera system (C4742, HAMAMATSU, Hamamatsu City, Shizuoka, Japan) (excitation,350 nm; emission, 450 nm).

Measurement of *S*-nitrosoglutathione reductase activity

The GSNOR activity in R2 cells was measured as described previously (Liu *et al.* 2001). In brief, cells were lysed in a solution containing 20 mmol/L Tris–HCl (pH 8.0), 0.5 mmol/L EDTA, 0.1% NP-40 and 1 mmol/L phenylmethysulphonylfluoride. To detect GSNO-metabolizing activity, 0.85–1.0 mg/mL lysate was incubated with 100 μmol/L GSNO in reaction buffer (20 mmol/L Tris–HCl, pH 8.0, 0.5 mmol/L EDTA) with 0 or 200 μmol/L NADH at 20–25°C for various times. The GSNO reductive activity was measured by GSNO-dependent NADH consumption using the change in absorbance at 340 nm per minute per mg protein.

Statistical analysis

All results are expressed as the means ± SEM derived from three or more experiments. Statistical analysis of the results was determined by the student *t*-test. The difference between means was considered statistically significant when $p < 0.05$.

Results

A burst of production of global Pro-SNOs in GSH-depleted R2 cells followed by severe neuronal necrosis

The quantity of RSNO in GSH-depleted cells increased by six-fold after 2 h treatment with GSNO, whereas it did not change significantly in control cells (BSO^-) even after 48 h of treatment with GSNO (Fig. 1a). We also measured the *S*-nitrosylation of proteins and of low molecular weight species using the Saville-Griess assay. Fig. 1b shows that the elevated level of *S*-nitrosylation was mainly contributed by proteins rather than low-molecular-weight species (<5 kDa). Using the biotin-switch and western blotting methods we further confirmed that there was a great degree of protein *S*-nitrosylation in the $BSO^+/GSNO^+$ group compared with the $BSO^-/GSNO^+$ group (Fig. 1c).

We then investigated the effect of this biochemical variation on the cell fate. In the $BSO^+/GSNO^+$ group, the cell viability decreased sharply and LDH leakage increased rapidly with time, indicating cell necrosis. In the $BSO^-/GSNO^+$ group, the cell viability decreased slowly and the LDH leakage was low, indicating cell apoptosis (Figs 1d and e). BSO treatment had little effect on cell viability (95% viability of the control after treatment for 24 h). Observation of the morphology showed that although the level of *S*-nitrosylation burst into the maximum after 2–4 h, cells became necrotic after 8 h (Fig. 1f).

The relationship between RSNO and cell susceptibility to NO

It has been reported that RSNOs are selectively reduced with Vc to form thiols (Jaffrey and Snyder 2001). Therefore, we tried to use Vc to regulate the RSNO level and hence to examine the effect of RSNO levels on cell fate. In both the $(BSO^+/GSNO^+)/Vc^+$ group and the $(BSO^+/GSNO^+)/Vc^-$ group, the RSNO level first increased and then fell with time. Addition of Vc even accelerated the rate of the decrease (Fig. 2a). However, the cell response was completely different in the two groups. In the $(BSO^+/GSNO^+)/Vc^-$ group, with the decreasing of RSNO level, the levels of thiols in the cells decreased about 50%; and the cell viability decreased to 23% of the level of normal cells. In contrast in the $(BSO^+/GSNO^+)/Vc^+$ group, with the decrease in the RSNO level the thiol level recovered to about 80% and the cell viability recovered to about 75% (Figs 2b and c). Addition of Vc did not result in 100% recovery of cell

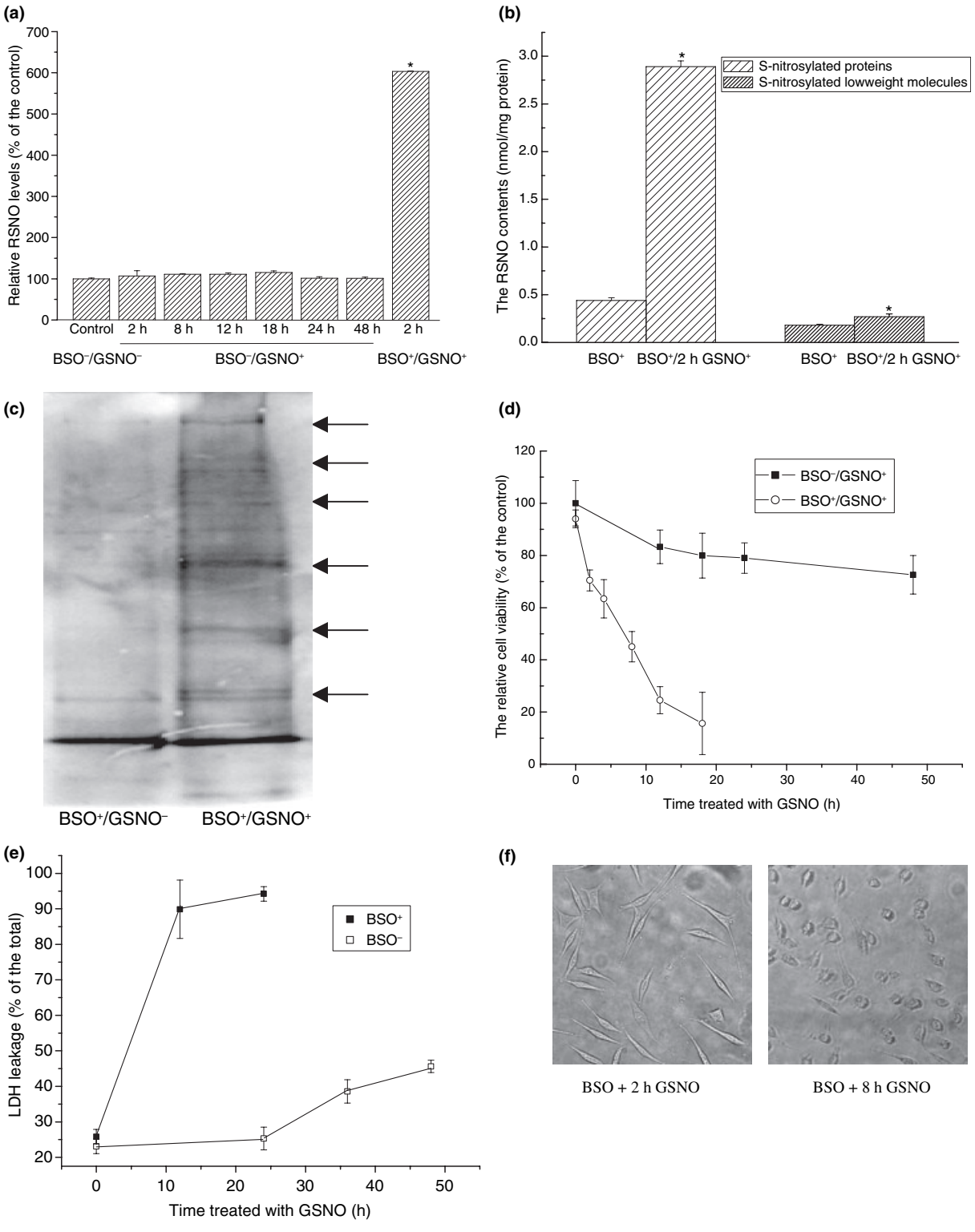

Fig. 1 The burst increase in *S*-nitrosylation levels and the effect on cell death. (a) The variation of *S*-nitrosothiols (RSNO) levels in cells treated with D,L-buthionine-[*S,R*]-sulfoximine (BSO) and *S*-nitrosoglutathione (GSNO). The indicated time represents the time of GSNO treatment. (b) The increase in levels of *S*-nitrosylated proteins and low molecule weight species (<5 kDa). $^*P < 0.05$ as compared with BSO^+ group. (c) Analysis of *S*-nitrosylated proteins by the Biotin-switch and western blotting method. (d) The relative cell viability varied with the time period of treatment with GSNO (■, $BSO^-/GSNO^+$; ○, $BSO^+/GSNO^+$). (e) Lactate dehydrogenase (LDH) leakage over the course of treatment with GSNO(■, BSO^+; ○, BSO^-). (f) Observation of R2 cells by microscope (amplified 400 ×).

viability, possibly because some *S*-nitrosylated proteins were oxidized to Pro-SSG or Pro-SOH, and Vc could not reduce them to form thiols. After addition of DTT, the level of protein thiols in both systems ($BSO^+/GSNO^+/Vc^+$, $BSO^+/GSNO^+/Vc^-$) recovered to the normal level. This suggests that the modification of proteins can be reversed by the addition of DTT, i.e., the thiols formed are –SSG and –SOH. Reducing RSNO to free thiols by Vc showed significant protection of cells against this nitrosative stress. RSNO was deduced to be the key factor in determining cell viability.

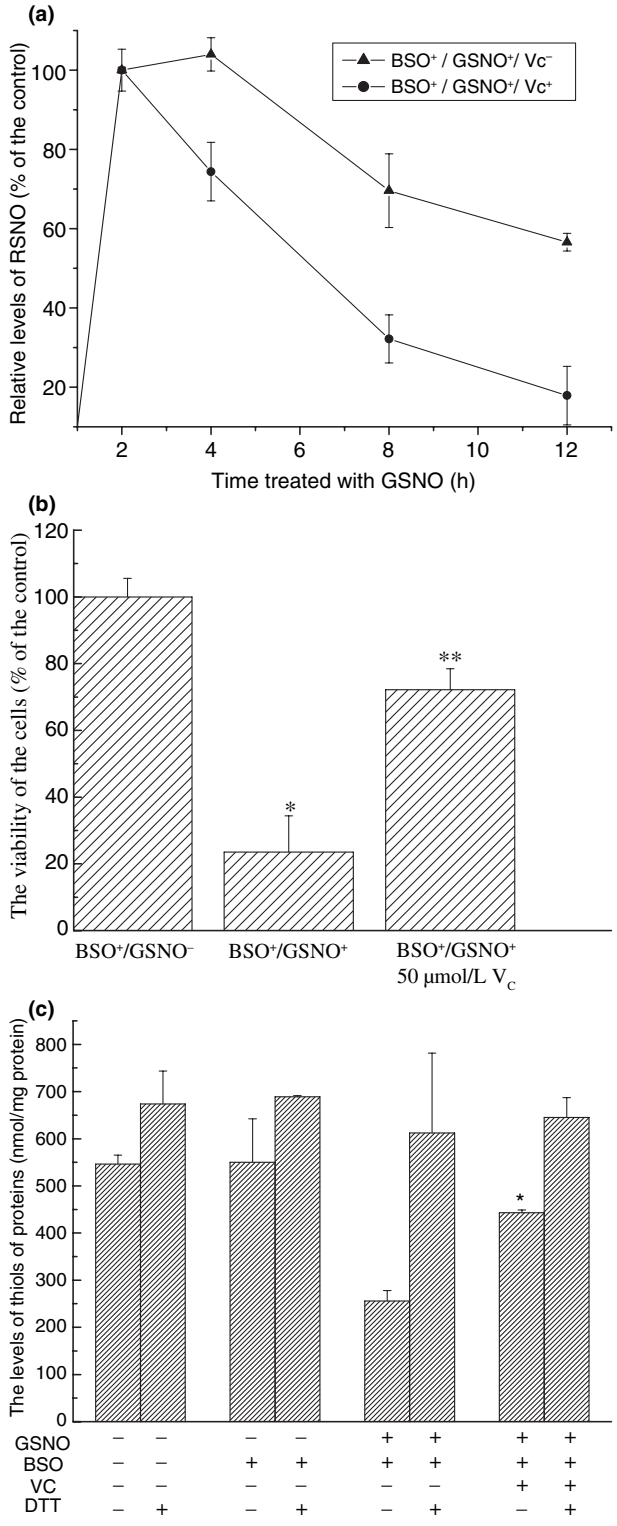

Fig. 2 The regulatory effects of Vitamin C (Vc) on levels of *S*-nitrosothiols (RSNO), cell viability and levels of free thiols. (a) The variation of the relative level of RSNO with time. Cells were treated with 50 μmol/L Vc in combination with 0.5 mmol/L GSNO for the indicated time (▲, $BSO^+/GSNO^+/Vc^-$; ●,$BSO^+/GSNO^+/Vc^+$). (b) Cell viability. Cells were treated with 50 μmol/L Vc in combination with 0.5 mmol/L GSNO for 24 h. $^*p < 0.05$ as compared with the control group $BSO^+/GSNO^-$; $^{**}p < 0.05$ as compared with the control groups $BSO^+/GSNO^+$. (c) Free thiols. The concerntrations of Vc and dithiothreitol (DTT) were 0.5 mmol/L and 2 mmol/L, respectively. $^*p < 0.05$ as compared with the group ($BSO^+/GSNO^+/Vc^-/DTT^-$). All data are represented as the mean ± SEM, $n = 3$.

The involvement of glutathione in the burst of *S*-nitrosothiols and in the cell susceptibility to nitric oxide

To investigate the reasons behind the burst of RSNO in BSO-treated cells, we firstly evaluated the effects of the cell culture medium, the cell itself and BSO treatment on GSNO decomposition. Fig. 3a shows that either BSO treatment or the presence of the cells themselves did not affect the decomposition of GSNO. However, cell culture medium greatly accelerated the decomposition of GSNO compared with Locke's solution. The culture medium is the same in both BSO^+ and BSO^- systems. Therefore, there must be another reason underlying the burst of *S*-nitrosylation.

Secondly, to elucidate what leaded to the elevation of RSNO, we tested a series of factors that were reported to regulate the metabolism of GSNO including the cell-surface thiols (Hart 1985) and the co-action of γ-glutamyltransferase (γ-GT) and peptidases (Dringen *et al.* 2000). The cell-surface thiols could decompose GSNO; therefore, the increase of cell surface thiols might enhance the effect of GSNO. However, our result showed that the levels of the cell-surface thiols in BSO-treated R2 cells decreased to 78.3 ± 0.8% of the control cells (BSO^-) (Fig. 3b), which suggested that the cell-surface thiols at least did not contribute to the elevation of RSNO. In the mean time, it has been reported that extracellular GSH and glutathione conjugates are substrates for the ectoenzyme γ-glutamyl transpeptidase. This enzyme catalyzes the transfer of a γ-glutamyl moiety from GSH or a glutathione conjugate onto an acceptor molecule. Therefore, it has been put forward that GSNO is likely to enter the cell by the co-action of γ-GT and peptidases (Taniguchi and Ikeda 1998). The addition of acivicin, an inhibitor of γ-GT, did not affect the increase of RSNO (Fig. 3c), which suggested

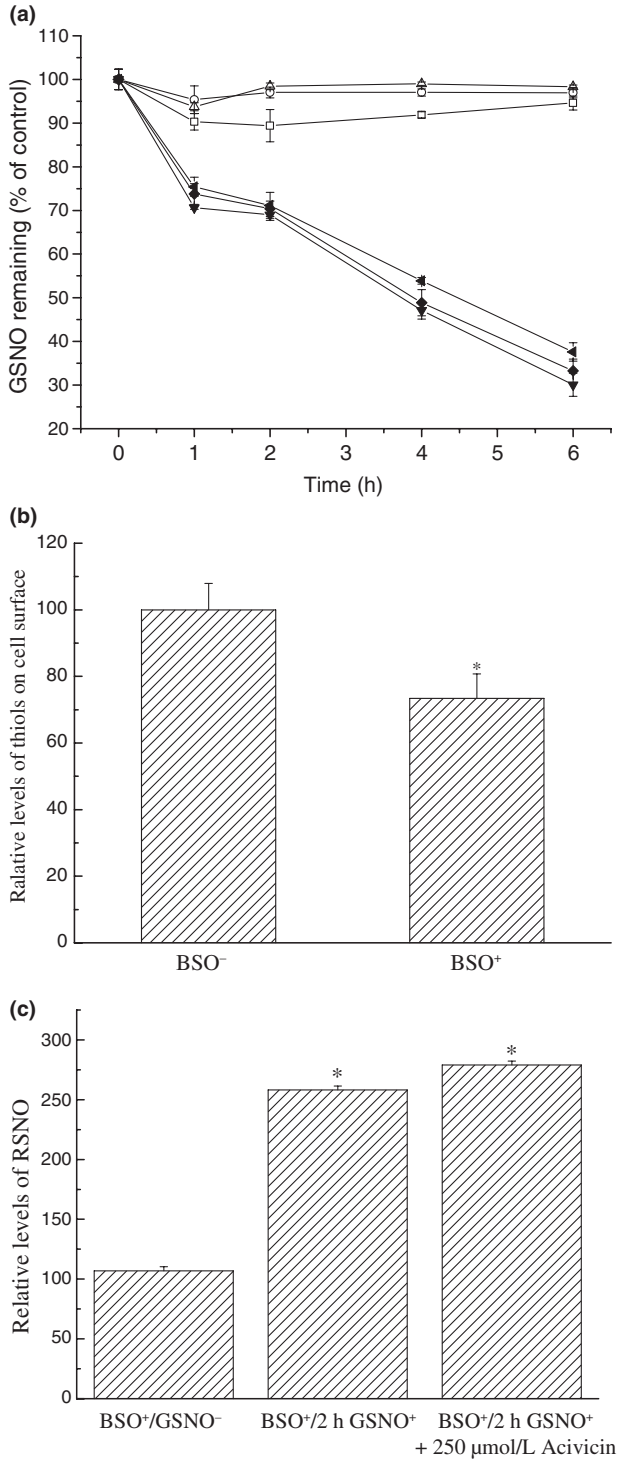

Fig. 3 The effects of culture medium and cell surface on the level of *S*-nitrosothiols (RSNO). (a) *S*-nitrosoglutathione (GSNO) decomposition under various conditions. The GSNO remaining in either the culture medium or the Lock's solution at the indicated time was monitored by the absorbance at 334 nm (□, Locke's$^+$/Cell$^-$/BSO$^-$; △, Locke's$^+$/Cell$^+$/BSO$^-$; ○, Locke's$^+$/Cell$^+$/BSO$^+$; ▼, medium$^+$/Cell$^-$/BSO$^-$; ◆, medium$^+$/Cell$^+$/BSO$^-$; ▲, medium$^+$/Cell$^+$/BSO$^+$). (b) The effects of BSO on the cell-surface thiols. *$p < 0.05$ as compared with the BSO$^-$ group. (c) The effects of the inhibition of the co-action of γ-glutamyltransferase (γ-GT) and peptidases by acivicin on the relative level of RSNO. The cells were treated with 250 μmol/L acivicin in combination with 0.5 mmol/L GSNO for 2 h. *$p < 0.05$ as compared with the control group (BSO$^+$/GSNO$^-$).

the co-action of γ-GT and peptidases was not involved in this event.

After excluding the effects of components of the culture medium and cell surface, we focused on the effect of BSO-induced GSH-depletion in the burst of RSNO. Different intracellular GSH levels were reduced by pre-treating cells with BSO for different times (Fig. 4a). RSNO levels increased sharply during 6–12 h of BSO treatment (Fig. 4b). These results show that intracellular RSNO is dependent on the GSH concentration and there is a threshold GSH level that leads to elevation of RSNO. We then added 2 mmol/L GSH-ethyl ester, a membrane permeable GSH analogue, to the cells together with GSNO. In the BSO$^+$/GSNO$^+$ cells, the initial RSNO level was 3.4 nmol/mg protein and the cell viability was 15% compared with normal cells. After the treatment with GSH-ethyl ester, the RSNO level was much lower than that in BSO$^+$/GSNO$^+$ cells (Fig. 4c) and the cell viability increased to 90% (Fig. 4d). These results indicate that endogenous GSH is able to regulate the intracellular RSNO level directly, and the inhibition of elevation of RSNO levels protects cells from cell death induced by GSNO.

The involvement of GSNOR in the burst of RSNO and in cell susceptibility to NO

R2 cells were transfected with RNA-interfering constructs (pSCI-110 and pSCI-223) and we verified the RNAi procedure by analyzing a green fluorescent protein image (Fig. 5a) and measuring the GSNOR activity. The activity of GSNOR in GSNOR$^-$R2 cells decreased about 50% (Fig. 5b). After the transfection, the GSNOR$^-$R2 cells were treated with GSNO and it was found that the RSNO content increased by nearly 50% (Fig. 5c) and the cell viability dropped about 40% (Fig. 5d). These results indicate that the loss of GSNOR renders R2 cells highly susceptible to NO because of the elevation of RSNO levels.

In addition to R2 cells, we further investigated the effect of GSNOR on the susceptibility of primary neurons to NO. GSNOR activity was abolished in CGNs using GSNOR interfering sequence. As shown in Figs 6a and b, the neurons transfected with either luciferase interfering sequences or GSNOR interfering sequences were recognized by bright GFP fluorescence. The apoptotic cells were recognized by Hoechst 33342 staining method. The condensed chromatin can be seen in most of GSNOR-interfering neurons after application of 30 μmol/L GSNO for 18 h, while not in the negative control (Figs 6c and d).

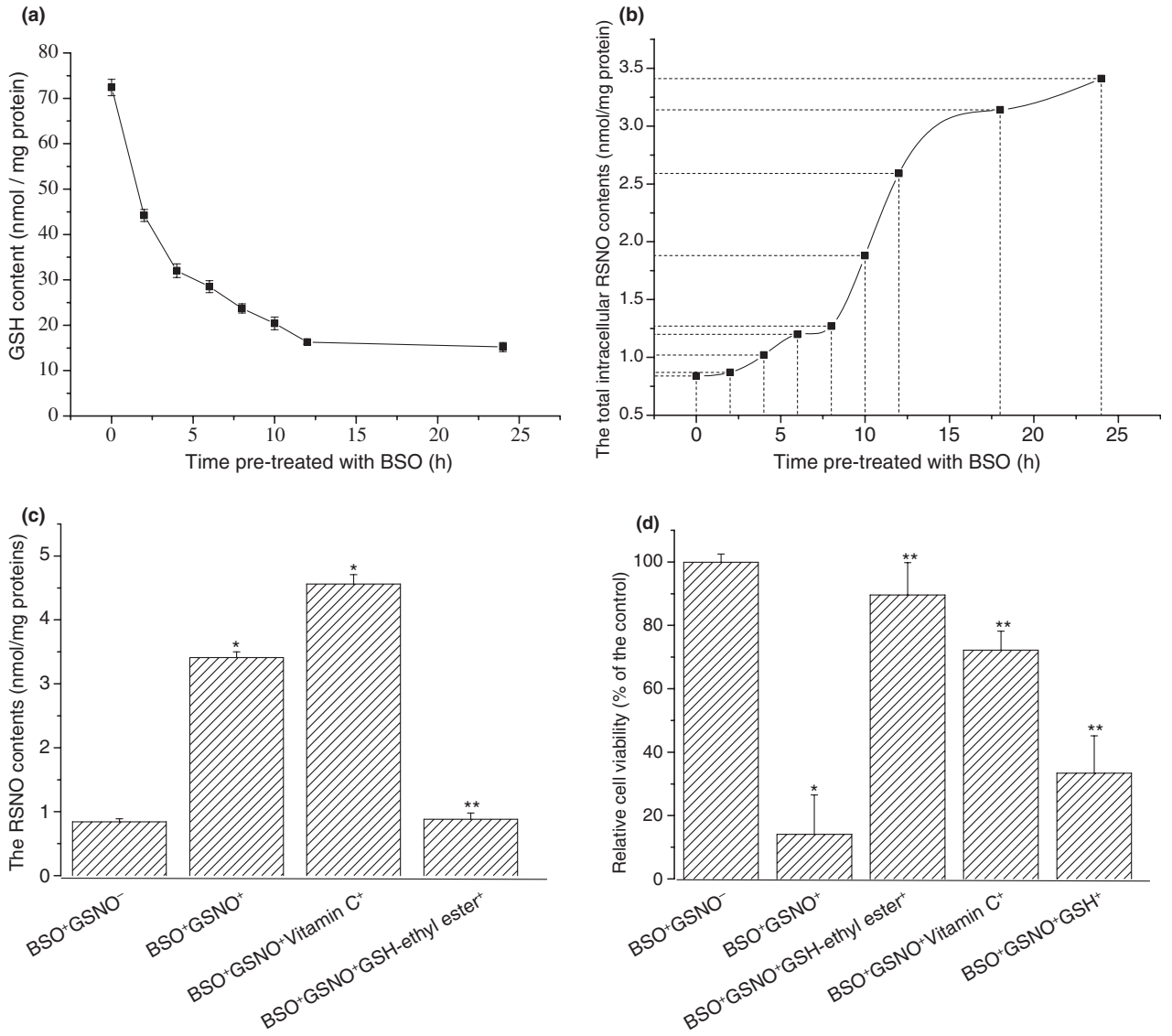

Fig. 4 The dependence of elevation of *S*-nitrosothiols (RSNO) levels on cellular glutathione (GSH) levels. (a) GSH levels in cells treated with D,L-buthionine-[*S, R*]-sulfoximine (BSO) for different times. (b) The intracellular RSNO content measured by the Saville-Griess assay after 2 h of treatment with GSNO. (c and d). The effects of GSH and Vitamin C on the RSNO levels and cell survival. BSO-pretreated R2 cells were treated with GSNO in combination with 2 mmol/L GSH, or 2 mmol/L GSH-ethyl ester, or 50 μmol/L Vitamin C. $^{*}P < 0.05$ as compared with $BSO^{+}GSNO^{-}$ group; $^{**}P < 0.5$ as compared with $BSO^{+}GSNO^{+}$ group.

The quantitative apoptosis of transfected CGNs was shown in Fig. 6e, which is about 20% apoptosis in the negtive control, about 40% apoptosis in the GSNOR RNAi group. This result indicates that RNAi treatment of GSNOR enhances primary neuron death, which is similar to the result in R2 cells.

Coupling of GSH and GSNOR in the metabolism of excess NO

In order to understand how GSH and GSNOR regulate the RSNO level, we first measured the GSNOR activity in GSH-depleted cells. The results indicated that the depletion of GSH has no effect on the activity of GSNOR itself (Fig. 7a), and also rules out a decrease in GSNOR activity as an explanation for the increase in RSNO levels in GSH-depleted cells.

Second, we measured the ammonia level, which is one of the main products of the metabolism of GSNO, in order to examine the metabolism of NO in BSO^{+} and BSO^{-} cells. In the BSO^{-} group, the ammonia level increased to about 100% after 2 h treatment with GSNO. In contrast, in the BSO^{+} group, the ammonia level increased only about 20% after 2 h of treatment with GSNO (Fig. 7b). These results indicate that the primary reason for the increase in nitrosylation of

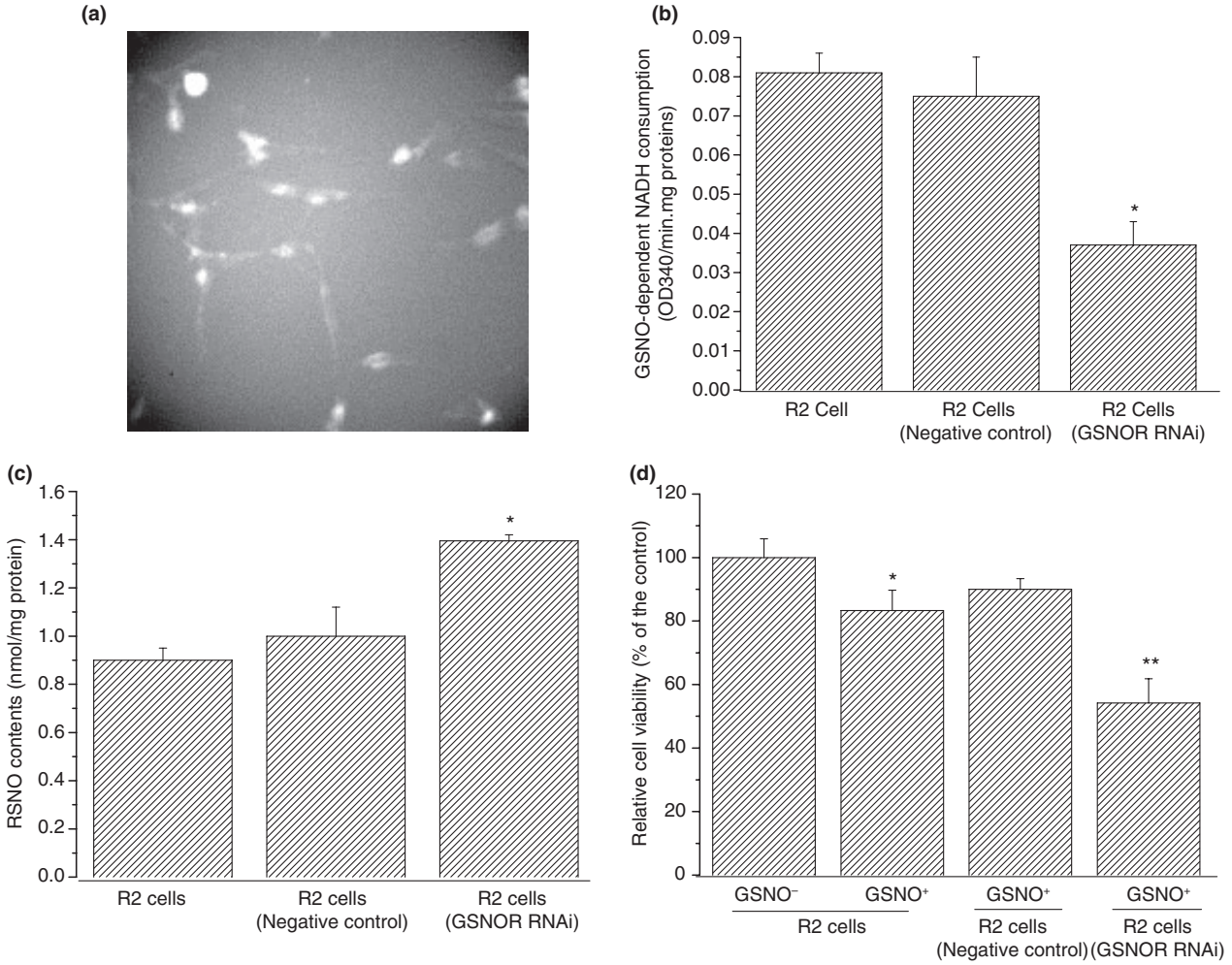

Fig. 5 The susceptibility of *S*-nitrosoglutathione reductase (GSNOR) -silenced R2 cells to *S*-nitrosoglutathione (GSNO). (a) Green fluorescent protein (GFP) image in R2 cells transfected with the interfering plasmid (amplified 400 ×). (b) GSNOR activity in GSNOR^{-}R2 cells measured by GSNO-dependent NADH consumption. R2 cells transfected with luciferase-interfering sequences are taken as the negative control. (c) GSNO induced the increase of endogenous *S*-nitrosothiols (RSNO) levels in GSNOR-silenced R2 cells. *$p < 0.05$ as compared with the negative control. (d) GSNO induced the decrease of cell survival in GSNOR-silenced R2 cells. *$p < 0.05$ as compared with the normal R2 cells. **$p < 0.05$ as compared with the negative control.

proteins is the impairment of NO metabolism through the GSH-GSNOR pathway

Discussion

From the elevation of Pro-SNOs, it is easy to think that GSH depletion switches *S*-nitrosylation from low to high molecular weight species. In this study, we emphasize that the key point of the elevation of PrO-SNOs is the consequence of the impairment of the metabolism of NO. In normal cells, GSNOR catalyzed the GSH-dependent reduction of excess NO to ammonia. There are two possible factors involved in this process: one is NADH, the coenzyme for GSNOR; the other is GSNO, the substrate for GSNOR. We found that the ratio of NADH/NAD^{+} was not decreased significantly in GSH-depleted R2 cells (data not shown). We therefore propose that when GSH is depleted, extra NO cannot be transformed to GSNO. Hence, GSNOR cannot function, and the excess NO results in *S*-nitrosylation of proteins. When GSNOR is down-regulated, although the quantity of GSH is sufficient to transform NO to the substrate GSNO, the catalytic reaction cannot be carried out in the absence of GSNOR, so the accumulated GSNO results in the elevation of Pro-SNOs. This mechanism showed a novel role of GSH in maintaining intracellular RSNO levels.

There is growing evidence supporting the notion that RSNO can function as an intermediate in NO signaling processes independent of the classical NO-guanylate cyclase signaling pathway. Our results suggest that *S*-nitrosylation of proteins is the checkpoint in susceptibility of neuronal R2 cells to NO, as well as in CGNs. The cell fate – either death or survival – is determined by how the formed Pro-SNOs are

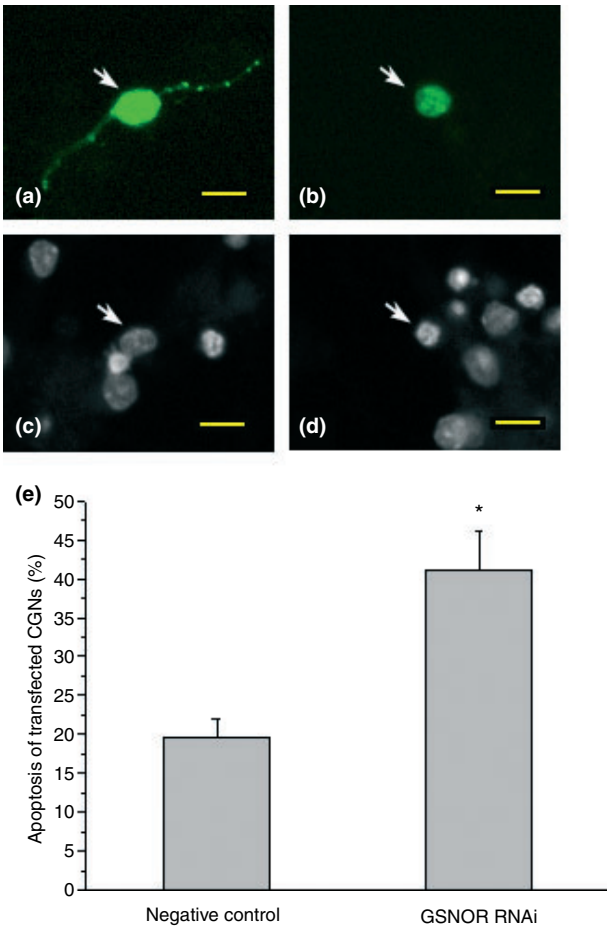

Fig. 6 The susceptibility of *S*-nitrosoglutathione reductase (GSNOR) RNAi-cerebellar granule neurons (CGNs) to *S*-nitrosoglutathione (GSNO). (a) and (b) Green fluorescent protein (GFP) imaging of CGNs transfected with negative control shRNA annealed oligonucleotide and GSNOR-interfering sequences, respectively. (c) and (d) Hoechst 33342 staining in negative control and GSNOR RNAi-CGNs, respectively. (e) The quantitative apoptosis of transfected CGNs. $^{*}p < 0.05$ as compared with the negative control. More than 200 cells were counted in each group. All data were shown as the means ± SD, $n = 3$. Bar = 5 μm.

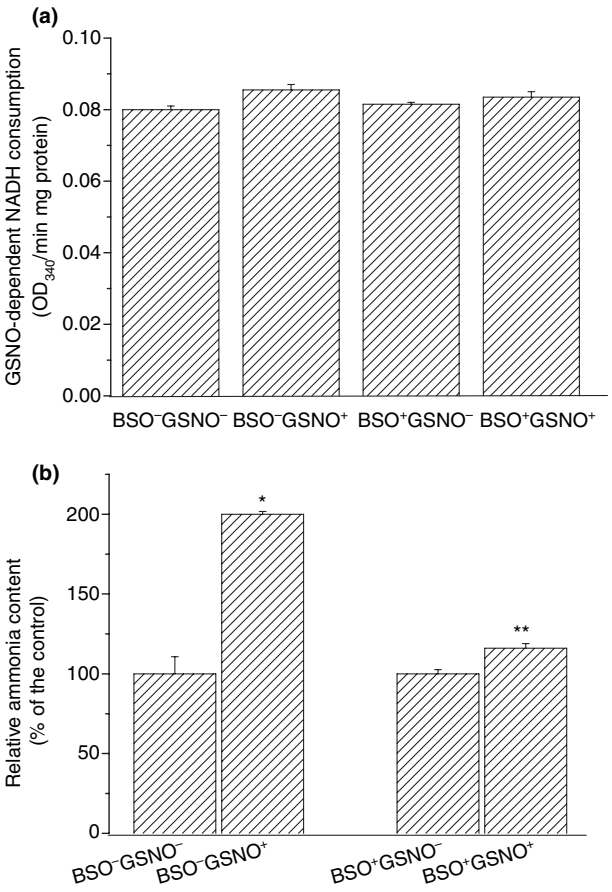

Fig. 7 The GSNO reductase activity (a) and the variation in the ammonia level (b) in cells after treatment with GSNO. The ammonia level before GSNO treatment was taken as 100%. $^{*}P < 0.05$ as compared with $BSO^{-}GSNO^{-}$ group; $^{**}P < 0.05$ as compared with $BSO^{+}GSNO^{-}$ group.

subsequently processed. In the $BSO^{+}/GSNO^{+}/Vc^{+}$ cells, RSNOs are reduced to thiols (–SH), and cells survive. On the other hand, in the $BSO^{+}/GSNO^{+}/Vc^{-}$ cells, RSNO levels continue to fall with increasing levels of reactive oxygen species (Data not shown), thereby leading to further oxidiation to form mixed disulfides (SSR) and cysteine sulfinic acid (SOH) (Fig. 2) and consequently cells die. These results are consistent with the previous report that formation of the S–NO bond is likely to accelerate disulfide formation (Arenelle and Stamler 1995). It has been reported that the glycolytic intermediate 3-phosphoglycerate decreases diethylenetriamine/NO -induced necrosis and increases apoptosis (Borutaite and Brown 2003). Comparing this with our work, down-regulation of Pro-SNOs with Vc rescued cells from necrosis to survival. We deduced that there are multiple pathways involved in neuronal cell death apart from the depletion of ATP as a result of the inhibition of glycolysis that was discussed in a previous report (Borutaite and Brown 2003). The burst in *S*-nitrosylation of cellular global proteins and its further oxidization results in the dysfunction of multiple cellular signaling pathways and leads to neuronal cell necrosis.

The proposed mechanism concerning the elevation of Pro-SNOs and its cellular effects is shown in Fig. 8.

We also noticed that GSH-ethyl ester and Vc both protected BSO-treated R2 cells against NO neurotoxicity (Figs. 2b, 4b). However, the underlying mechanism appears to be distinct for these two molecules: in Vc-treated cells, RSNO first increased and then decreased (Fig. 2a); whereas in GSH-ethyl ester-treated cells, RSNO did not increase at all (Fig. 4c). GSH-ethyl ester exerted its effect before RSNO was formed. In contrast, Vc assists the direct decomposion of RSNO after RSNO has been formed. The observation of two

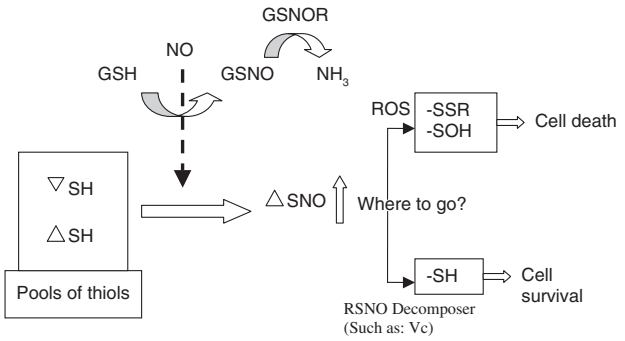

Fig. 8 The mechanism of increase in *S*-nitrosylation of proteins and the consequent cellular effects. ▽-SH: thiols of low molecular weight species, △–SH: thiols of proteins. When the cellular redox status is impaired or the *S*-nitrosoglutathione reductase (GSNOR) level is insufficient, nitric oxide (NO) cannot be metabolized to ammonia, resulting in a burst increase in nitrosylation of multiple proteins. In the presence of an –SNO decomposer, such as Vc, *S*-nitrosothiols (RSNO) are reduced to thiols (–SH), and cells survive. On the other hand, in the presence of ROS, RSNO are further oxidized to mixed disulfide (–SSR) and cysteine sulfenic acid (–SOH) and cells undergo necrosis.

mechanisms implies two distinct pharmacological strategies to regulate the susceptibility of neuronal cells to NO: either inhibiting the formation of RSNO or the direct decomposition of RSNO.

The loss of GSH is regarded as a marker in the aging process and several neurodegenerative diseases (Schulz *et al.* 2000). For example, in patients with Parkinson's disease, the levels of GSH are reduced in nigra dopaminergic neurons and glia cells (Pearce *et al.* 1997; McNaught and Jenner 1999; Mytilineou *et al.* 1999). In schizophnenic patients, the levels of GSH in the frontal cortex decreased 52% (Schulz *et al.* 2000). Our results will be helpful in understanding the details of the high susceptibility of neuronal cells to NO in these circumstances, particularly the biochemical variation and the cellular response. Either inhibition or reversal of the abnormal increased levels of nitrosylated proteins may provide a route for the therapy of neurodegenerative diseases and anti-aging. Screening natural products either for activity as anti-*S*-nitrosylation agents or for increasing the activity of GSNOR may be a useful approach to identify compounds of therapeutic value in these circumstances.

In conclusion, our results firstly demonstrate that the burst of *S*-nitrosylation is the "checkpoint" of cell fate. Secondly, we introduced a novel mechanism that explains why Pro-SNOs significantly increased in GSH-depleted neuronal cells. Here we emphasize that the key point of the elevation of Pro-SNOs is the consequence of the impairment of the metabolism of NO. At the same time, we found that GSH-ethyl ester and Vc play protective roles through totally different mechanisms. GSH-ethyl ester confers protection by inhibiting the elevation of Pro-SNOs, whereas Vc confers protection by reducing Pro-SNOs to free thiol proteins. Our evidence indicates a novel mechanism underlying the susceptibility of neuronal cells to NO, and suggests some potential strategies to prevent the NO-mediated neurotoxicity by the regulation of protein *S*-nitrosylation in the circumstance of cellular redox disturbance.

Acknowledgements

The authors would like to thank Dr. S. Perrett of this Institute for editing the manuscript and Dr. Jing Qu and Dr. Guang-Hui Liu for their helpful discussion. The work is supported by the National Natural Science Foundation of China (No. 30270352) and 973 program 2005CB522804 and 2006CB503900.

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