Activation of GluR6-containing Kainate Receptors Induces Ubiquitin-dependent Bcl-2 Degradation via Denitrosylation in the Rat Hippocampus after Kainate Treatment*

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We previously showed that Bcl-2 (B-cell lymphoma 2) is down-regulated in a kainate (KA)-induced rat epileptic seizure model. The underlying mechanism had remained largely unknown, but we here report for the first time that denitrosylation and ubiquitination are involved. Our results show that the S-nitrosylation levels of Bcl-2 are down-regulated after KA injection and that the GluR6 (glutamate receptor 6) antagonist NS102 can inhibit the denitrosylation of Bcl-2. Moreover, the ubiquitin-dependent degradation of Bcl-2 was found to be promoted after KA treatment, which could be suppressed by the proteasome inhibitor MG132 and the NO donors, sodium nitroprusside and S-nitrosothiol. In addition, experiments based on siRNA transfections were performed in the human SH-SY5Y neuroblastoma cell line to verify that the stability of Bcl-2 is causal to neuronal survival. At the same time, it was found that the exogenous NO donor GSNO could protect neurons when Bcl-2 is targeted. Subsequently, these mechanisms were morphologically validated by immunohistochemistry, cresyl violet staining, and in situ TUNEL staining to analyze the expression of Bcl-2 as well as the survival of CA1 and CA3/DG pyramidal neurons. NS102, GSNO, sodium nitroprusside, and MG132 contribute to the survival of CA1 and CA3/DG pyramidal neurons by attenuating Bcl-2 denitrosylation. Taken together, our data reveal that Bcl-2 ubiquitin-dependent degradation is induced by Bcl-2 denitrosylation during neuronal apoptosis after KA treatment.

The agitation of glutamate receptors is thought to be the primary cause of epileptic seizures. Glutamate receptors are classified into metabolic glutamate receptors (GluRs) and ionic GluRs. Glutamate itself gates three types of ionotropic receptors: NMDA, AMPA, and kainate (KA) receptors. There are also five types of kainate receptor subunits: GluR5, GluR6, GluR7, KA1, and KA2 (1). These subunits can be arranged in different ways to form a tetramer. GluR5–7 can form homomers (for example, a receptor composed entirely of GluR5) and heteromers (for example, a receptor composed of both GluR5 and GluR6). However, KA1 and KA2 can only form functional receptors by combining with one of the GluR5–7 subunits (2). Kainic acid (KA) is a potent exogenous agonist of the KA receptors, and the systemic administration of KA produces epilepsy in rats or mice accompanied by neuronal damage, mainly in limbic structures such as the hippocampal pyramidal neurons in particular (3). KA-induced seizures in rodents have been widely used as a model of human temporal lobe epilepsy.

We have reported previously that the Bcl-2 (B-cell lymphoma gene 2) levels are down-regulated in a KA-induced rat epileptic seizure model (3). The Bcl-2 family proteins are mainly located in the mitochondrial outer membrane and are divided into pro-apoptotic and the anti-apoptotic groups (4–7). Several mechanisms have been reported to underlie the anti-apoptotic function of Bcl-2, including the regulation of Ca2+ homeostasis or action as an antioxidant. Furthermore, as a heterodimer, the pro-apoptotic protein Bax is attenuated by Bcl-2. Moreover, Bcl-2 prevents the release of cytochrome c from mitochondria and inhibits the activation of caspase-9 and caspase-3 (8).

It has been well established that the Bcl-2 protein levels are essential for its anti-apoptotic function. The regulation of these levels mainly occurs via post-translational modifications and degradation (9–14). More recently, protein S-nitrosylation has emerged as the principal post-translational modification by which nitric oxide exerts a myriad of biological effects. S-Nitrosylation, the covalent attachment of a NO group to a Cys thiol side chain, has been postulated to be a fundamental mechanism in cellular signal transduction. The stability of proteins, cleavage ofzymogens, and modification of active proteins can be controlled by this post-translational modification process (15, 16). More recently, nitrosylation has been found to be reversible. Nitrosylated Cys thiol groups can be reduced to free thiols, depending on the conditions, which is defined as denitrosylation and plays an important role in signal regulation in a broad range of diseases (17). Mutational analysis of Bcl-2 has shown that two cysteine residues in this...
protein (Cys-158 and Cys-229) are important for the S-nitrosylation process (18). Bcl-2 degradation is mainly mediated through ubiquitin-proteasome pathway. All four lysine residues in Bcl-2 (K17R, K22R, K218R, and K239R) target this protein for ubiquitin-dependent degradation (19). A more recent report has indicated that the S-nitrosylation of Bcl-2 prevents its ubiquitin-proteasomal degradation during the apoptotic cell death induced by chromium (VI) in lung cancer (18). Similarly, it has been shown that the mechanism of cisplatin resistance involves the up-regulation of Bcl-2 expression by NO, which occurs by preventing its ubiquitin-dependent degradation in human lung carcinoma H-460 cells (20).

In this study, we demonstrate that KA induces Bcl-2 denitrosylation through the GluR6-KA receptor pathway, which is proposed to facilitate Bcl-2 ubiquitination and finally down-regulate its protein level. The GluR6 antagonist NS102, NO donors S-nitrosoglutathione (GSNO), sodium nitroprusside (SNP), and the proteasome inhibitor MG132 prevent the denitrosylation and the degradation of Bcl-2, playing an important role in neuron protection in hippocampal CA1 and CA3/DG regions.

MATERIALS AND METHODS

Antibodies and Reagents—Anti-Bcl-2 (sc-492), anti-phospho-Bcl-2 (Ser-87, sc-16323-R), anti-Fas (sc-1023), and anti-Fasl (sc-6237) antibodies were purchased from Sigma (St. Louis, MO). Anti-β-actin (13E5), anti-cytochrome c (2472), anti-COXIV (4844), anti-pro-caspase-3 (9662), and anti-caspase-3 (9661) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-ubiquitin antibody (U5379), 6,7,8,9-tetrahydro-5-nitro-1H-benzo[g]indole-2,3-dione 3-oxime (NS102, N179), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801, M107), 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI-52466, G119), GSNO (N4148), DTT (43817), Z-Leu-Leu-Leu-al (MG132, C2211), N-acetyl-l-cysteine (NAC, A7250), neoepoxycine (N1501), methyl methimethionyl methyl sulfone (MMTS, 177954), (+)-sodium l-ascorbate (A7631), streptavidin-agarose (A1638), and Z-Leu-Leu-Glu β-naphthylamide (Z-Leu-Leu-Glu βNA, C0788) were purchased from Sigma-Aldrich (Poole, UK). Kainic acid (EA-123) was purchased from Enzo Life Sciences, Inc. (Plymouth Meeting, PA). (N-(6-(Biotinamido)hexyl)-3’-(2’-pyridylidithio)-propionamide (Biotin-HDHP, 21341) was purchased from Thermo Fisher Scientific (Bremen, Germany). Guava TUNEL kit (4500-0121) was purchased from Millipore Co. (Bedford, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (A0208), benzylxoxycarbonyl-Val-Ala-Asp fluoromethylketone Z-VAD-FMK (C1202), BeyoECL Plus (P0018), Kodak X-Omat film (FF057), PVDF membrane (FFN06), BCA kit (P0012), and methyl green staining solution (C0115) were purchased from Beyotime Co. (Beyotime, Jiangsu, China). SNP was purchased from a local traditional Chinese medical hospital. FasL antisense oligodeoxynucleotides (FasL AS-ODNs), FasL sense oligodeoxynucleotides (FasL S-ODNs), Bcl-2 antisense oligodeoxynucleotides (Bcl-2 AS-ODNs), Bcl-2 sense oligodeoxynucleotides (Bcl-2 S-ODNs), and β-actin primers were synthesized by Sangon Biotech (Shanghai, China) Co. Ltd. All other chemicals were obtained from Sigma unless indicated otherwise.

Drug Treatments—The rats were injected three times intraperitonally with SNP (5 mg/kg) dissolved in saline with an interval of 1.5 h; the first SNP administration was performed 30 min before KA infusion. NAC (150 mg/kg) dissolved in 0.9% NaCl was injected intraperitonally at 30 min before KA infusion. MK801 (3 mg/kg) was injected intraperitonally 100 min before KA infusion. KA (0.6 µg) dissolved in 10 µl of 0.9% NaCl (saline) was administered intracerebroventricularly (10 µl, bregma: 1.5 mm lateral, 0.8 mm posterior, 3.5 mm deep) to the rats. DTT (60 µg/kg) and GSNO (0.1 mg/kg) dissolved in 0.9% NaCl were administered (10 µl, intracerebroventricularly) 30 min before KA infusion. Z-Leu-Leu-Leu-al (MG132) (0.1 mg/kg) or z-VAD-FMK (0.6 µg/kg) dissolved in 0.2% dimethylsulfoxide was administered (10 µl, intracerebroventricularly) 30 min before KA infusion. NS102 or GYKI-52466 dissolved in dimethylsulfoxide at a concentration of 10 mM was administered (10 µl, intracerebroventricularly) 30 min before KA infusion. 10 nmol of FasL, or Bcl-2, antisense oligodeoxynucleotides (AS-ODNs) in 10 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) were given to the rats every 24 h for 3 days. The same dose of sense (S-ODNs) or vehicle (TE buffer) was used as a control. The sequence for FasL AS-ODNs was 5’-CTTCGAGATTCTGCAGCT-3’, and that for S-ODNs was 5’-AGCTGGCAGAATCCTCGAGAG-3’. The sequence for Bcl-2 AS-ODNs was 5’-TGTTCTC-CGGCTTGGCCAT-3’, and that for S-ODNs was 5’-ATGGCCGAACCGGGAGACA-3’.

Seizure Model—Adult male Sprague-Dawley rats weighing 250 ± 10 g were used. Seizures were induced by an intracerebroventricular injection of KA (0.6 µg/10 µl) dissolved in sterile saline. The animals were monitored behaviorally for seizures for at least 6 h after injection. The seizures were scored as follows: 1) behavioral arrest and staring spells, 2) head bobbing and gnawing, 3) unilateral forelimb clonus, 4) bilateral forelimb clonus, 5) severe seizures with loss of postural control, and 6) seizure-induced death (21). Only animals with stage 4 or 5 seizures were used in the analyses.

Sample Preparation—The rats subjected to KA for 3 or 6 h post-treatment were decapitated immediately, and the hippocampal CA1 or CA3/DG region was isolated from each animal and quickly frozen in liquid nitrogen. Tissues were homogenized in an ice-cold homogenization buffer containing 50 mM MOPS, pH 7.4, 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM MgCl₂, 0.2 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM p-nitrophenyl phosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml each of leupeptin, aprotinin, and pepstatin A. The homogenates were centrifuged at 800 × g for 10 min at 4 °C. Supernatants were collected, and protein concentrations were determined using the BCA method. The samples were stored at −80 °C until use.

S-Nitrosylation Assay—S-Nitrosylation was detected using the biotin switch method as described by Jaffrey et al. (22) in which the free thiols in S-nitrosylated proteins are blocked.
and the S-nitrosothiols are reduced by ascorbic acid sodium salt to yield free thiols, which can be covalently linked to bion- 
dinivatives and assayed using a biontin-based analysis. 
Briefly, the cells are lysed in HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Nonidet P-40, 150 
mM NaCl, 1 mM PMSF, protease inhibitor mixture), and the 
resulting lysates are mixed with an equal volume of MMTS 
buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μM neocu-
proine, 5% SDS, 20 mM MMTS) and incubated at 50 °C for 20 
min with frequent vortexing. After the free MMTS is removed 
by cold acetone precipitation, the precipitates are resus-
pended in HENS buffer (25 mM HEPES, pH 7.7, 0.1 mM 
EDTA, 10 μM neocuproine, 5% SDS, 1% Triton X-100), the samples 
are then modified with biontin in the following buffer (25 mM 
HEPES, pH 7.7, 0.1 mM EDTA, 1% SDS, 10 μM neocuproine, 
10 mM ascorbic acid sodium salt, and 0.2 mM biontin-HPDP). 
After free biontin-HPDP was removed by cold acetone precipi-
tion, biotinylated proteins are absorbed to streptavidin-aga-
rose. The streptavidin absorbates are then eluted by β-mer-
captoethanol (100 mM), separated by SDS-PAGE, and 
immunoblotted with an anti-Bcl-2 antibody.

**Immunoprecipitation and Western Blotting Analysis**—For 
immunoprecipitation, cytosolic fractions (each containing 
400 μg of proteins) were diluted 4-fold with HEPES buffer 
containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycer-
ol, 1% Triton X-100, and 1 mM each of EGTA, EDTA, 
PMSF, and Na4VO4. The samples were then preincubated for 
1 h with 20 μl of protein A-Sepharose CL-4B (Amersham Bio-
sciences) and centrifuged to remove any nonspecifically ad-
hered proteins from the protein A-Sepharose. The superna-
tant was then incubated with 2–5 μg of specific antibodies for 
4 h at 4 °C. After the addition of protein A-Sepharose, the 
mixture was incubated at 4 °C for an additional 2 h. The samples 
were triple washed with HEPES buffer and eluted by 
SDS-PAGE loading buffer then boiled at 100 °C for 5 min.

Western blot analysis was carried out following 12.5% SDS-
PAGE. Briefly, the proteins were electrotransferred onto 
PVDF filters (pore size, 0.45 μm). After blocking for 3 h in 
Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bo-
vine serum albumin, the membranes were incubated over-
night at 4 °C with primary antibodies in TBST containing 3% 
bovine serum albumin. The membranes were then washed 
and incubated with horseradish peroxidase-conjugated sec-
ondary antibodies in TBST for 30 min and visualized by the 
bioconjugated horseradish peroxidase for 1 h at 37 °C. To visu-
alize bound antibodies, the sections were incubated with a 3,3’-
diaminobenzidine peroxidase substrate kit and examined 
under a light microscope.

**Histology**—For histological analyses, rats subjected to KA 
post-treatment for 7 days were perfusion-fixed with 4% para-
formaldehyde in 0.1 M phosphate buffer (pH 7.4) under anes-
thesia. Paraffin-embedded brain sections (6 μm thick) were 
then prepared and stained with 0.1% (w/v) cresyl violet to assess 
necrosis in the hippocampus. The number of surviving 
hippocampal CA1 or CA3 pyramidal cells/mm was 
counted as the neuronal density.

**Cell Culture**—The human neuroblastoma cell SH-SY5Y 
was cultured in DMEM containing 10% fetal bovine serum at 
37 °C in humidified 8% CO2 atmosphere. For transfection 
experiments, the cells were seeded onto 6- or 24-well plates. 
Twenty-four h after inoculation, either Bcl-2-targeted or 
scrambled siRNAs (nonsilencing control) were transfected 
into the cells, which were at 40–50% confluence. The stock 
siRNA was diluted in reduced serum Opti-MEM to form 
complexes with Lipofectamine 2000 at a 1:2 ratio (3 μg of 
siRNA formulated with 6 μl of Lipofectamine 2000/well for

**Proteasome Activity Assay**—20 S proteasome activity was 
measured using an assay kit from Chemicon (Temecula, CA), 
in accordance with the manufacturer’s protocol but with some 
modifications. Briefly, tissues were homogenized in ice-
cold extract buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 
10% glycerol, 1 mM EGTA) and then exposed to ultrasound, 
followed by centrifugation at 800 × g for 15 min at 4 °C. The 
supernatants were collected and determined for protein con-
tent using the BCA method. 100 μg of proteins were incubated 
with 5 μM proteasome substrate LLVY-AMC in 1 ml of 
assay buffer at 37 °C for 2 h. The AMC fluorophore obtained 
after cleavage from the labeled substrate was quantified with 
Hitachi fluorescence spectrophotometer F-7000 at excitation 
and emission wavelengths of 335 and 460 nm, respectively 
(23).
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6-well plates; 0.75 µg of siRNA formulated with 1.5 µl of Lipofectamine 2000/well for 24-well plates). The mixtures were then incubated at room temperature for 20 min before transfection in a final volume of 2 ml/well in 6-well plates and 500 µl/well in 24-well plates. The final concentration of siRNA in the medium was 125 nM. The cells were cultured in Opti-MEM medium for 6 h without antibiotics during the transfection period. Then cells were cultured in DMEM containing 10% fetal bovine serum for 18 h and stimulated with 500 nM Bcl-2 siRNA pairs (siRNA-1, -2, -3, and -4) or 125 nM Bcl-2 siRNA-2, sense strand: 5'-GCAAGAACAUGGUGCUUCGTT-3' and antisense strand: 5'-ACAACUUUGGUUUGCUUCGTT-3'; siRNA-3, sense strand: 5'-UUCUCCGAGCGUGCUACGTT-3' and antisense strand: 5'-GUGGAUACUGAGAGUACGTT-3'; and siRNA-4, sense strand: 5'-ACACUUGGUUUGCUUCGTT-3' and antisense strand: 5'-ACACUUGGUUUGCUUCGTT-3'.

Transient Transfection of SH-SYSY Cells with Bcl-2 siRNA—SH-SYSY cells (2 × 10^6 cells/ml) were transfected with 125 nm Bcl-2 siRNA pairs (siRNA-1, -2, -3, and -4) or 125 nm scrambled control siRNAs (negative control, sense strand: 5'-UUCUCGACUGGCUACGTT-3' and antisense strand: 5'-ACUGACACGUCCGAGAATT-3') using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. The Bcl-2 siRNA pairs (siRNA-1, sense strand: 5'-GCAAGAACAUGGUGCUUCGTT-3' and antisense strand: 5'-UUUAUUGGAUGUUGCUUCGTT-3'; siRNA-2, sense strand: 5'-GUGGAUACUGAGAGUACGTT-3' and antisense strand: 5'-UUCUCCGAGCGUGCUACGTT-3'; siRNA-3, sense strand: 5'-GCAAGAACAUGGUGCUUCGTT-3' and antisense strand: 5'-ACACUUGGUUUGCUUCGTT-3'; and siRNA-4, sense strand: 5'-ACACUUGGUUUGCUUCGTT-3' and antisense strand: 5'-ACACUUGGUUUGCUUCGTT-3') were designed and synthesized by GenePharma (Shanghai GenePharma Co. Ltd). The cells were transfected with siRNA when 40–50% confluent and in the log growth phase. The transfection efficiency of FAM-conjugated siRNA-4 was determined by DAPI in 24-well plates. Total cell homogenates were extracted from 6-well plates for total protein quantification before Western blot analysis. Total RNA was also extracted from 6-well plates by TRIzol for RT-PCR analysis of Bcl-2.

RESULTS

The Degradation of Bcl-2 in the Hippocampal CA1 and CA3/DG Regions Is Induced by KA through the Activation of GluR6-containing Kainate Receptors—We previously reported that KA treatment through intracerebroventricular infusion or intraperitoneal injection down-regulates the Bcl-2 protein level gradually in hippocampal CA1 and CA3/DG regions in a concentration-dependent manner (3, 24). In our current experiments, we focused on both the KA concentration and time course. As shown in Fig. 1 (A and B), the treatment of rats with KA caused a dose-dependent decrease in Bcl-2, with the lowest level reached at a dose of 0.6 µg/10 µl. We also observed a time-dependent decrease, with this effect clearly noticeable at 6 h post-treatment and thereafter.

KA activates not only KA receptors but also AMPA receptors and even NMDA receptors at some higher doses. We therefore pretreated the rats with the AMPA receptor antagonist GYKI53655, NMDA receptor antagonist MK801 and GluR6 antagonist NS102 before administering a 0.6 µg/10 µl KA injection to investigate whether any of the corresponding receptors is involved in the degradation of Bcl-2 (25, 26). As shown in Fig. 1C, only pretreatment with NS102 could prevent the degradation of Bcl-2, indicating that KA at the dosage selected exerts its effects mainly through GluR6-KA receptors.

To confirm that the down-regulation of Bcl-2 by KA was at the post-translational level, we examined the Bcl-2 mRNA levels in the rat hippocampal CA1 and CA3/DG regions at 6 h post-treatment. As shown in Fig. 1D, KA has no affect on the Bcl-2 transcript levels that were equivalent to the saline control group.

GluR6-KA Receptors Mediate Bcl-2 Denitrosylation—It has been reported that Bcl-2 is denitrosylated in some circumstances (18). A few studies have also recently indicated that protein denitrosylation can be induced by membrane receptors (17). To test this potential function of KA receptors in our present study, we examined the Bcl-2 S-nitrosylation level using the biotin switch method at 3 h post-treatment when Bcl-2 degradation is not clearly evident. We also pretreated the animals with NS102 to block the activation of GluR6-KA receptors. As shown in Fig. 2A, KA infusions induce the denitrosylation of Bcl-2, which is prevented by NS102, suggesting that the Bcl-2 denitrosylation is indeed mediated by GluR6-KA receptors. We next administrated the exogenous NO donors SNP and GSNO by intracerebroventricular infusion prior to KA injection to suppress the denitrosylation of Bcl-2. As shown in Fig. 2B, both GSNO and SNP were found to be effective Bcl-2 NO donors under our experimental conditions, and Bcl-2 denitrosylation was thus rescued by pretreatment with these agents. This rescue effect was negated by co-administration with the reducing agent DTT.

The dephosphorylation of Bcl-2 Ser-87 after the stimulation of human endothelial cells with TNF-α has been shown to promote Bcl-2 ubiquitin-dependent degradation (19). It has also been elucidated that the down-regulation Bcl-2 Ser-87 phosphorylation by reactive oxygen species is critical for this ubiquitin-dependent degradation in manganese-superoxide...
dismutase antisense-transfected squamous cell carcinoma cell line OSC-4 (27). Our previous study also showed that the administration of KA could increase the production of reactive oxygen species (24). Consequently, in our present analyses we examined whether the Bcl-2 Ser-87 phosphorylation levels are influenced by KA and various NO modulators. The rats were treated with KA in the presence or absence of GSNO, SNP, DTT, and the antioxidant NAC to attenuate reactive oxygen species induction. The effects of these compounds on Bcl-2 phosphorylation were then determined by Western blot using a phospho-specific Bcl-2 (Ser-87) antibody. As shown in Fig. 2C, KA had minimal effects on Bcl-2 phosphorylation at 3 h post-treatment compared with the saline group or the groups pretreated with SNP, GSNO, DTT, or NAC. These results support the notion that KA induced Bcl-2 degradation is independent of Bcl-2 Ser-87 phosphorylation.

**KA Mediates Bcl-2 Ubiquitin-dependent Degradation**—To next investigate whether Bcl-2 degradation is ubiquitin-proteasome dependent, we pretreated rats with the proteasome inhibitor MG132 at 25 μg/10 μl by intracerebroventricular infusion at 30 min before KA injection. As shown in Fig. 3A, the degradation of Bcl-2 was completely rescued by MG132, implicating the ubiquitin-proteasome system in Bcl-2 degradation. Furthermore, immunoprecipitation with anti-ubiquitin antibody was performed to examine the Bcl-2 ubiquitin complex at 3 h post-treatment. The results showed that the up-regulation of Bcl-2 ubiquitination was promoted by KA but impaired by pretreatment with SNP or GSNO (Fig. 3B). DTT co-treatment negated the effects of these NO donors. These data suggest that the up-regulation of Bcl-2 nitrosylation impairs its ubiquitination and vice versa.

Further analyses were performed to examine the Bcl-2 levels in SNP- or GSNO-pretreated groups. As shown in Fig. 3C, both SNP and GSNO prevent the Bcl-2 degradation induced by KA. We have previously observed that caspases are activated after KA infusion, and given the finding that caspases cleave Bcl-2 in some cell lines (29), we pretreated the KA group with the caspase inhibitor z-VAD-FMK (150 ng/10 μl) (30). As shown in Fig. 3D, however, z-VAD-FMK did not attenuate the Bcl-2 degradation caused by KA, indicating that caspases are not involved in this pathway. To next investigate whether treatment with these compounds alone (i.e. in the absence of KA) had any effects on Bcl-2 S-nitrosylation and stability, the rats were treated with NS102, GSNO, SNP, or MG132, respectively. The Bcl-2 S-nitrosylation and relative expression levels were then determined. As shown in Fig. 3E, treatments with these compounds alone had no effect on Bcl-2 S-nitrosylation or stability (p > 0.05). The mechanism...
of Bcl-2 degradation by exposure to KA is therefore a stress response rather than a general Bcl-2 degradation pathway involved in homeostasis.

Immunohistochemical analysis further revealed the neuroprotective effects of NS102, GSNO, SNP, and MG132 upon Bcl-2 degradation at 6 h post-treatment. In the KA group (Fig. 3F, panels d–f), Bcl-2 immunoreactivity was barely detectable in the CA1 and CA3 pyramidal neurons but was significantly visible in the saline group (Fig. 3F, panels a–c). The protective effects of NS102, GSNO, SNP, and MG132 were also clearly evident from the stronger immunostaining (Fig. 3F, panels g–r), in contrast to the KA group (Fig. 3F). Our data thus suggest that NS102, GSNO, SNP, and MG132 cause an increase in the levels of Bcl-2 by blocking the degradation induced by KA in hippocampal CA1 and CA3 pyramidal neurons.

GluR6-KA Receptor Coupled Bcl-2 Denitrosylation Does Not Require Fas Activation—We have reported previously that FasL is up-regulated and that more Fas receptors are activated by JNK in a KA-induced rat epileptic seizure model (3). Moreover, it has been reported that the activation of Fas receptors gives rise to procaspase-3 denitrosylation, which facilitates cleavage to its active form (i.e. activated caspase-3) (31). To investigate whether the Bcl-2 denitrosylation triggered by KA injection is mediated by Fas activation, we administrated FasL antisense oligodeoxynucleotides to the rats prior to KA treatment. As shown in Fig. 4A, however, inhibition of the Fas pathway did not ameliorate the degradation of Bcl-2. This result indicates that the Fas pathway does not play a role in GluR6-KA receptor-coupled Bcl-2 denitrosylation.

It has been reported that the E3 ubiquitin ligase parkin is nitrosylated under conditions of NOS stress, which has a strong effect upon the ubiquitin-proteasome system (first promotes and then reduces its activity) (32). To test the possibility that ubiquitin-proteasome system activity affects Bcl-2 degradation, hippocampal homogenates were analyzed for proteasome activity by spectrofluorometry. As shown in Fig. 4B, however, KA and NO modulators did not interfere with proteasome activity.

Exogenous NO Donors and Proteasome Inhibitors Prevent the Activation of Caspase-3—A previous study has demonstrated that KA infusion down-regulates Bcl-2, which releases mitochondrial cytochrome c into the cytosol where it activates caspase-3 and induces neuronal apoptosis (3). In our present study, we have demonstrated that exogenous NO donors and the proteasome inhibitor MG132 can attenuate the Bcl-2 degradation induced by KA. To further investigate the possible neuroprotective effects of these agents, rats were pretreated with NO donor GSNO and MG132 by cerebroventricular injection 30 min prior to KA administration. The expression of cytochrome c in both the mitochondria and the corresponding cytosol was then examined by Western blotting. In addition, the activation of caspase-3 was determined by analyzing the procaspase-3 (32 kDa) and cleaved caspase-3 (17/19 kDa) levels. The results shown in Fig. 5 reveal that both GSNO and MG132 prevent the translocation of cytochrome c and the activation of caspase-3.
FIGURE 3. Effects of KA on Bcl-2 ubiquitin-dependent degradation. A, effect of MG132 on Bcl-2 expression in KA-treated CA1 and CA3/DG regions of the rat hippocampus. B, effects of NO modulators on KA-induced Bcl-2 ubiquitination at 3 h post-treatment. CA1 and CA3/DG regions were prepared and immunoprecipitated with anti-Bcl-2 antibody. The immune complexes were then analyzed using an anti-ubiquitin antibody. C, effects of NO donors upon Bcl-2 degradation. CA1 and CA3/DG regions were prepared and analyzed for Bcl-2 expression by Western blot. D, effects of the caspase inhibitor z-VAD-FMK (150 ng/10 μl) on Bcl-2 degradation induced by KA. The bands were scanned, and the intensities were determined by optical density measurement. The intensities are the fold changes versus the saline control. E, effects of pharmacological inhibitors and NO donors on Bcl-2 S-nitrosylation and stability in the absence of KA. The rats were treated separately with the GluR6 antagonist NS102, NO donor GSNO, SNP, or proteasome inhibitor MG132. The Bcl-2 S-nitrosylation and relative expression levels were then determined at 3 and 6 h post-treatment, respectively. F, immunohistochemical analysis revealing the mechanism underlying the degradation of Bcl-2. Coronal sections from rats injected with saline (panels a–c) or KA (panels d–f) or pretreated with NS102 (panels g–i), GSNO (panels j–l), SNP (panels m–o), or MG132 (panels p–r) were immunostained with anti-Bcl-2 antibodies. The results were obtained from six independent animals in each experimental group, and the results of a typical experiment are presented. The boxed areas in the left column are shown at higher magnification in the right columns. Scale bars, 200 μm (panel p) and 10 μm (panels q and r). The data are expressed as the means ± S.D. from three independent animals (n = 3). *, p < 0.05 versus the saline group; #, p < 0.05 versus the KA group or the respective DTT-pretreated group (n = 3).
Knockdown of Bcl-2 in SH-SY5Y Cells by siRNA and Anti-sense ODNs in Hippocampi Promotes Cellular Apoptosis—Experiments were performed to confirm whether the stability of Bcl-2 is causal to KA resistance in both cultured human neuroblastoma SH-SY5Y cells and hippocampal neurons in vivo. We suppressed Bcl-2 expression in SH-SY5Y cells using an RNA interference approach. Transfection of a scrambled siRNA control resulted in no gross morphological alterations under light microscopy (Fig. 6, A and B), and no toxicity was observed. Fluorescently labeled siRNA was successfully transfected, whereas no positive signal was evident in the non-transfection control group (Fig. 6, A'/H11032 and B'/H11032). Several siRNA sequences that were designed to target to Bcl-2 were next screened for their efficacy. We found that siRNA-3 and -4 effectively suppressed the expression of Bcl-2 (Fig. 6, C and D), whereas the siRNA-1 and -2 molecules had no effect (Fig. 6, C and D). After siRNA-4 transfection into cultured SH-SY5Y cells, cellular apoptosis was induced by KA stimulation (Fig. 6E). The effects of enhanced Bcl-2 stability was further confirmed in hippocampal neurons in vivo, in which the cell density of CA1 and CA3 pyramidal neurons was more reduced by Bcl-2 AS-ODNs administration in the presence of KA (Fig. 6G). Similar to the effects of siRNA-4, the down-regulation of Bcl-2 expression by Bcl-2 AS-ODNs also promoted the effects of KA on neuronal survival. Hence, Bcl-2 is vital to neuronal survival in our rat seizure model.

GSNO Protects Neurons Mainly by Enhancing Bcl-2 Stability—We investigated whether NO donors could up-regulate the S-nitrosylation of Bcl-2 and maintain its stability and also whether the down-regulation of Bcl-2 could promote KA-induced cellular apoptosis. To examine the role of NO donors in neuronal survival, in one test, we pretreated SH-

FIGURE 4. Effects of Fas on GluR6-KA receptor coupled Bcl-2 denitrosylation. A, 10 nmol of FasL AS, FasL missense oligonucleotide, or vehicle (TE) was administrated to the rats every 24 h for 3 days through a cerebral ventricular injection prior to KA treatment. At 6 h post-KA treatment, a portion of the samples was subjected to coimmunoprecipitation analysis of FasL with Fas followed by Western blotting with a FasL antibody. Other samples were immunoblotted with Fas, Bcl-2, and β-actin antibodies, respectively. The corresponding Western bands were scanned, and the optical density measurements are represented as the fold changes versus the saline control. The data are expressed as the means ± S.D. from three independent animals (n = 3). *, p < 0.05 versus the saline group; #, p < 0.05 versus the KA injection group. B, effects of KA in the presence or absence of NO modulators upon proteasome activity at 3 h post-treatment. Panel a, positive control curve. Different doses of 20 S proteasome positive control as indicated on the chart were treated with 5 μM of the LLVY-AMC proteasome substrate at 37 °C for 2 h and measured of 20 S activity. The relative fluorescent intensity was then measured by spectrophotometry. Panel b, AMC standard curve. Fluorophore AMC standards were serially diluted as indicated on the chart and quantified for fluorescent intensity. Panel c, 100-μg aliquots of protein samples were incubated with 5 μM of the proteasome substrate LLVY-AMC in 1 ml of assay buffer at 37 °C for 2 h. The fluorophore AMC obtained after cleavage of the labeled substrate was then quantified. The data are the means ± S.D. from three independent animals.
SY5Y cells with 100 μM GSNO at 30 min before KA stimulation. In another test, we performed siRNA knockdown of Bcl-2 before pretreatment with GSNO followed by KA treatment. According to the DAPI staining pattern, GSNO was found to protect neurons from KA-induced neuronal apoptosis (Fig. 6E, panels b and d). Significantly also, the down-regulation of Bcl-2 by siRNA attenuated the protective effects of GSNO (Fig. 6E, panels d and e), indicating that Bcl-2 is a key GSNO-targeted protein that protects neurons from KA stimulation. A similar experiment was performed in an animal seizure model system (Fig. 6G). Cresyl violet staining also indicated the neuroprotective effects of GSNO against KA-induced brain injury in rat hippocampal CA1 and CA3 regions, which was attenuated following Bcl-2 down-regulation by AS-ODNs. Taken together, these observations reveal that Bcl-2 stability is causal to KA resistance and underpins the mechanism by which NO functions.

The Protective Effects of Some Drugs against the Delayed Injury Induced by KA in Hippocampal CA1 and CA3 Pyramidal Neurons Are Exerted through the Prevention of Bcl-2 Degradation—To investigate whether NS102, GSNO, SNP, and MG132 play protective roles against KA-induced neuronal cell death, cresyl violet staining was performed to examine the survival of pyramidal neurons of the rat hippocampal CA1 and CA3 regions. Photomicrographs of cresyl violet-stained brain sections from the rats revealed the state of the neuronal cells. Normal neurons in the pyramidal layer of the hippocampi, as shown in the saline operation group, appear rounded with pale stained nuclei, whereas shrunken cells with pyknotic nuclei were considered dead. Samples from rats pretreated with NS102 (Fig. 7A, panels g–i), GSNO (Fig. 7A, panels j–l), SNP (Fig. 7A, panels m–o), or MG132 (Fig. 7A, panels p–r) were significantly protected compared with those administered with KA only. The numbers of viable cells were counted within a 1-mm length in the KA alone and saline—, NS102—, GSNO—, SNP—, and MG132-treated groups. TUNEL staining was also used to examine the level of apoptosis in CA1 and CA3 pyramidal cells in the hippocampus. As shown in Fig. 7B (panels d–f), a significant number of TUNEL-positive cells was observed on the seventh day post-treatment, with some of them showing characteristic appearances such as shrunken condensed nuclei and apoptotic bodies. Pretreatment with NS102, GSNO, SNP, and MG132 significantly reduced the number of TUNEL-positive cells (Fig. 7B, panels g–r). The numbers of viable TUNEL-positive cells were counted within a 1-mm length in the saline, KA, NS102, GSNO, SNP, and MG132 treatment groups. The above results suggest that NS102, GSNO, SNP, and MG132 protect rat hippocampal CA1 and CA3 pyramidal cells from apoptosis induced by KA infusion.

**DISCUSSION**

In our present report, we demonstrate that GluR6-KA receptor-mediated Bcl-2 denitrosylation facilitates Bcl-2 ubiquitination and proteasomal degradation during KA-induced neuronal apoptosis. Pretreatment with the GluR6 antagonist NS102, the NO donors GSNO and SNP, as well as the proteasome inhibitor MG132 exerted neuroprotective effects by attenuating Bcl-2 denitrosylation and ubiquitin-dependent degradation.

S-Nitrosylation and denitrosylation are crucial protein post-translation modifications that involve the regulation of protein function, such as phosphorylation and dephosphorylation, and that play important roles in the activity regulation of protein kinases under both physiological and pathological conditions.
FIGURE 6. Effects of Bcl-2 knockdown by siRNA in SH-SY5Y cells and by antisense administration in rat hippocampi. A and B, no gross morphological alterations are evident in SH-SY5Y cells after a control siRNA transfection, as seen in the nontransfected controls. A′ and B′, successful transfection of fluorescein-labeled scrambled control siRNA. Photomicrographs were captured under a fluorescence microscope at a 400× original magnification at the end of transfection period. BF, bright field; FAM, fluorescence from fluorescein-labeled scramble-siRNA. C and D, the relative protein expression and mRNA levels of Bcl-2 are decreased in the siRNA-3 and siRNA-4 groups at 18 h post-transfection period, compared with the nontransfected control. The results are the means ± S.D. of three separate experiments. *, statistical significance at the level of \( p < 0.05 \). E, DAPI staining at 42 h after siRNA-4 transfection. The indicated cells were treated with 500 \( \mu \)M KA at 18 h after the siRNA transfection period, in the presence or absence of 100 \( \mu \)M GSNO treatment 30 min ahead of time. Typical apoptotic cells with a condensed nucleus are indicated by arrowheads. Quantitative representations are expressed as a percentage of the total cells in 10 microscopic fields (×400) for DAPI staining. *, \( p < 0.05 \) versus the nontransfected control group; **, \( p < 0.05 \) versus the KA group and siRNA-4 plus KA stimulation group; #, \( p < 0.05 \) between GSNO plus KA group in the presence or absence of siRNA-4. F, effects of Bcl-2 sense and antisense oligonucleotides upon the expression of Bcl-2. The samples were subjected to immunoblotting analysis after treatment with vehicle (TE), antisense (AS), or sense oligonucleotides for 3 days. *, \( p < 0.05 \) versus the TE group. G, examples of cresyl violet-stained sections of rat hippocampi treated with TE buffer (panels a–c) or KA stimulation (panels d–o). Prior to KA stimulation for 4 days, some of the groups were pretreated with Bcl-2 sense oligodeoxynucleotides (panels d–f) or AS (panels g–i and m–o) for 3 days or with 0.1 mg/kg GSNO 30 min ahead of time in the absence (panels j–l) or presence (panels m–o) of AS administration. The data were obtained from six independent animals, and the results of a typical experiment are presented. The boxed areas in the left column are shown at higher magnification in the two right columns. The original magnifications were: panels a, d, and g, ×40; panels b, c, e, f, h, and i, ×400. Scale bars: left column, 200 \( \mu \)m; right columns, 10 \( \mu \)m. *, \( p < 0.05 \) versus TE group; **, \( p < 0.05 \) versus KA and AS plus KA stimulation groups; #, \( p < 0.05 \) between GSNO plus KA groups in the presence or absence of AS.
results were obtained from six independent animals in each experimental group, and the results of a typical experiment are shown. The

were injected with saline (h) or pretreatment with NS102 (g–i), GSNO (j–l), SNP (m–o), or MG132 (p–r). The results were obtained from six independent animals in each experimental group, and the results of a typical experiment are shown. The boxed areas in the left column are shown at higher magnification in the right columns. The original magnifications were: ×40 in panels a, d, g, j, m, and p; and ×400 in panels b, c, e, f, h, i, k, l, n, o, q, and r. Scale bars, left column, 200 μm; right columns, 10 μm. The numbers of viable cells were counted within a 1-mm region. *, p < 0.05 versus the saline group; #, p < 0.05 versus the KA injection group (n = 6). B, representative photomicrographs of TUNEL stained hippocampal samples counterstained with methyl green are shown. The rats were injected with saline (panels a–c), KA (panels d–f), or pretreated with NS102 (panels g–i), GSNO (panels j–l), SNP (panels m–o), or MG132 (panels p–r). The results were obtained from six independent animals in each experimental group, and the results of a typical experiment are shown. The boxed areas in the left column are shown at higher magnification in the right columns. The original magnifications were: ×40 in panels a, d, g, j, m, and p; and ×400 in panels b, c, e, f, h, i, k, l, n, o, q, and r. Scale bars, left column, 200 μm; right column, 10 μm. The viable TUNEL-positive cells were counted within a 1-mm area. *, p < 0.05 versus the saline group; #, p < 0.05 versus the KA injection group (n = 6).

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conditions. S-Nitrosylation can positively or negatively regulate the function of proteins, and denitrosylation has shown this same capacity (16). For example, the pro-apoptosis protease matrix metalloproteinase 9 is activated by S-nitrosylation (18). On the other hand, the denitrosylation impairs the anti-apoptotic function of cells and reduces their resistance to KA-induced neuronal apoptosis and cisplatin-induced cell death in the case of human lung carcinoma H-460 cells (20).

Degradation mechanisms also play important roles in the regulation of protein levels, cell apoptosis, and survival and signaling pathways. Among the different protein degradation systems, the ubiquitin-dependent proteasomal degradation pathway is the most well studied (37). The ubiquitination system functions in a wide variety of cellular processes, including antigen processing, apoptosis, the cell cycle, DNA repair, immune responses, the modulation of cell surface receptors, the response to stress, and extracellular modulators. Our findings here indicate that in response to KA stress, Bcl-2 denitrosylation initiates the ubiquitin-dependent degradation of Bcl-2, giving rise to the apoptosis of hippocampal neurons. When Bcl-2 expression was decreased by AS-ODNs or siRNA, the neuroprotection of exogenous NO donor GSNO was weak-
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ened (as shown in Fig. 6). As is well established, the ubiquitin-proteasome system recycles proteins with a short half-life. It would be interesting to measure whether the ubiquitin-proteasome system controls the basal Bcl-2 levels in homeostasis. However, based on our present results (Fig. 3E), the Bcl-2 levels should therefore be noticeably increased at the indicated time points in the presence of MG132. Although our present data do not exclude the possibility of this regulatory mechanism, they cannot confirm that the ubiquitin-proteasome system is a general regulator of Bcl-2 degradation or whether another pathway performs this function.

Although numerous theories have sought to explain the mechanism of protein S-nitrosylation, it remains largely unknown. Recently, two specific enzymatic systems of protein denitrosylation have been identified: the thioredoxin (Trx) system, which comprises Trx proteins, Trx reductase (TrxR) proteins, and NADPH; and the GSNO reductase system, which comprises GSH and GSNO reductase. Theoretically, the denitrosylation of target proteins is mediated either by catalyzing the conversion of reduced Trx-(SH)2 to oxidized Trx-S2 or by oxidizing glutathione (GSH). Moreover, candidate denitrosylases have been proposed, such as protein disulfide isomerase, xanthine oxidase, superoxide dismutase, glutathione peroxidase, and carbonyl reductase (17). Additionally, receptor-coupled denitrosylation mechanisms have been identified that are mediated by Fas receptors, TNFα receptors, VEGF receptors, insulin receptors, and β-adrenergic receptors. In addition, a rise in intracellular calcium has been proven to play a crucial role in protein denitrosylation (28). However, more studies are required to elucidate the mechanisms underlying GluR6-KA signaling. It will also be of great interest to determine the denitrosylase involved in Bcl-2 degradation and any other regulators that function in this process, such as calcium.

In summary, we propose from our findings that the intracerebroventricular infusion of KA results in ubiquitin-dependent Bcl-2 degradation, which is facilitated by GluR6-KA receptor coupled Bcl-2 denitrosylation and is independent of its phosphorylation. Moreover, NS102, GSNO, SNP, and MG132 exert neuroprotective effects by preventing Bcl-2 degradation through the suppression of denitrosylation or ubiquitination. Further studies are needed to elucidate the mechanisms underpinning GluR6-KA receptor coupled denitrosylation, which may lead to better therapeutic approaches to the future treatment of epileptic seizures.

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