**PLANT BIOLOGY**


The authors note that on page 7255, Fig. 4 appeared incorrectly in part. The regulatory arrow between CCA1/LHY and TOC1 pointed in the opposite direction. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

![Corrected figure](image)

**NEUROSCIENCE**


The authors note that due to a printer’s error, on page 4904, the lower right corner of Fig. 5 was cut off. The corrected figure and its legend appear below.

![Corrected figure](image)
S-nitrosylation of XIAP compromises neuronal survival in Parkinson’s disease


Inhibitors of apoptosis (IAPs) are a family of highly-conserved proteins that regulate cell survival through binding to caspases, the final executors of apoptosis. X-linked IAP (XIAP) is the most widely expressed IAP and plays an important function in regulating cell survival. XIAP contains 3 baculoviral IAP repeats (BIRs) followed by a RING finger domain at the C terminal. The BIR domains of XIAP possess antiapoptase activities, whereas the RING finger domain enables XIAP to function as an E3 ubiquitin ligase in the ubiquitin and proteasomal system. Our previous study showed that parkin, a protein that is important for the survival of dopaminergic neurons in Parkinson’s disease (PD), is S-nitrosylated both in vitro and in vivo in PD patients. S-nitrosylation of parkin compromises its ubiquitin E3 ligase activity and its protective function, which suggests that nitrosative stress is an important factor in regulating neuronal survival during the pathogenesis of PD. In this study we show that XIAP is S-nitrosylated in vitro and in vivo in an animal model of PD and in PD patients. Nitric oxide modifies mainly cysteine residues within the BIR domains. In contrast to parkin, S-nitrosylation of XIAP does not affect its E3 ligase activity, but instead directly compromises its anti-caspase-3 and antiapoptotic function. Our results confirm that nitrosative stress contributes to PD pathogenesis through the impairment of prosurvival proteins such as parkin and XIAP through different mechanisms, indicating that abnormal S-nitrosylation plays an important role in the process of neurodegeneration. 

Results

BIR Domains of XIAP Are S-Nitrosylated by NO. To determine whether XIAP could be S-nitrosylated in vitro, HEK293 cells expressing myc-XIAP were treated with S-nitrosoglutathione (GSNO). These samples were then subjected to the biotin switch assay (Fig. 1A). HEK293 cells expressing myc-XIAP treated with GSNO were readily S-nitrosylated, but S-nitrosylation was not observed in samples treated with glutathione (GSH) (Fig. 1A). The S-nitrosylation of XIAP was specific as under the same conditions, α-synuclein, which contains no cysteines, was not S-nitrosylated as demonstrated (7) (Fig. 1B). To confirm that S-nitrosylation not only occurred through the treatment with an exogenous NO donor, we performed a similar experiment with the use of N2A cells, which have been shown to possess endogenous neuronal NO synthase (nNOS) activity (8). After the biotin switch assay on N2A cells transfected with XIAP, we found that XIAP was S-nitrosylated under basal conditions (Fig. 1C). S-nitrosylation of XIAP was abolished by treatment with nitro-L-arginine (N-Arg), a nNOS inhibitor, and ascorbate, which reverses the S-nitrosylation modification. These results suggest that S-nitrosylation of XIAP depended on the NO produced by the nNOS in the N2A cells (Fig. 1C).

To map the potential S-nitrosylation sites of XIAP, we constructed a series of XIAP truncation mutants and subjected them to the biotin switch assay (Fig. 1D–F). We found that the sites for S-nitrosylation were concentrated in the BIR domains (Fig. 1D–F), but not in the RING finger domain (Fig. 1D). All 3 BIR domains are individually S-nitrosylated (Fig. 1D–F). To further confirm that XIAP could be S-nitrosylated, we switched to the 2,3-diaminonaphthalene (DAN) fluorometric assay on recombinant GST-tagged truncated and full-length XIAP. The fluorometric assay showed that both truncated BIR1–3 and full-length XIAP were readily S-nitrosylated at a comparable level, suggesting that S-nitrosylation of XIAP was concentrated primarily in the BIR domains (Fig. 1G). To identify the sites for S-nitrosylation in XIAP, we incubated recombinant XIAP with nitrogen monoxide and purified XIAP. The authors declare no conflict of interest. 


This article is a PNAS Direct Submission.


This article is contains supporting information online at www.pnas.org/cgi/content/full/0810591106/DCSupplemental.

To whom correspondence may be addressed. E-mail: tdawson@jhu.edu or bkchung@ust.hk.

This article contains supporting information online at www.pnas.org/cgi/content/full/0810591106/DCSupplemental.
XIAP S-Nitrosylation Does Not Affect Its E3 Ligase Activity. Through its E3 ligase activity, XIAP targets a number of substrates for ubiquitination (3). XIAP also targets itself for autoubiquitination, which can be used as an indicator for its E3 ligase activity (3). To determine whether NO could affect XIAP’s E3 ligase activity, HEK293 cells transfected with myc-XIAP and HA-tagged ubiquitin (Ub) were treated with the NO donors GSNO and NOC18. Both treatments of GSNO and NOC18 had no effect on XIAP autoubiquitination, which indicated that the XIAP E3 ligase activity was not affected by XIAP S-nitrosylation (Fig. 2A and B).

XIAP is known to form dimers, and this dimerization is important for its physiological function and E3 ligase activity (2, 9, 10). To determine whether NO could disrupt the dimerization of XIAP, HEK293 cells transfected with myc-XIAP and HA-XIAP were treated with GSNO and NOC18 and then followed by anti-myc immunoprecipitation (IP). Treatment of GSNO and NOC18 had no effect on XIAP dimerization (Fig. 2C and D).

XIAP S-Nitrosylation Impairs Its Ability to Inhibit Caspase-3 Activity. A number of physiological functions of XIAP are associated with its BIR domains. For instance, the BIR2 domain of XIAP binds to caspase-3 and inhibits its caspase activity. We suspected that XIAP S-nitrosylation could affect its caspase-3 inhibition activity because one of the modified cysteines we identified by MS resides within the BIR2 domain and is close to the conserved residues of the IAP-binding motif (IBM) interacting groove (1). To test this hypothesis, we incubated recombinant GST-tagged XIAP and His-tagged caspase-3 together and monitored caspase-3 activity by measuring the fluorescence intensity generated by the cleavage of the caspase-3 fluorogenic substrate Ac-DEVD-AFC (Fig. 3). Incubation of XIAP with caspase-3 selectively inhibited its caspase activity (Fig. 3A and B). In contrast, treatment of XIAP with GSNO before incubation with caspase-3 resulted in loss of XIAP’s anticaspase-3 activity (Fig. 3A and B). This loss of XIAP’s anticaspase-3 activity by NO could be restored by the treatment of DTT, which suggests that the NO modification on XIAP was reversible (Fig. 3A and B).

Caspase-3 has a number of cellular substrates and one of them is poly(ADP-ribose) polymerase 1 (PARP-1). To confirm that XIAP S-nitrosylation could affect its caspase-3 inhibition activity, we decided to test whether S-nitrosylation of XIAP could block its inhibition on caspase-3 cleavage of PARP-1. We set up an in vitro caspase-3 activity assay by combining GST-XIAP, caspase-3, and HEK293 cell lysate, and then monitored the PARP-1 cleavage by using an antibody specific for the caspase-3-cleaved PARP-1 fragment. Incubation of caspase-3 with HEK293 lysate resulted in the cleavage of PARP-1 (Fig. 3C). In contrast, coincubation of XIAP and caspase-3 reduced the amount of caspase-3-generated PARP-1 cleaved fragment (Fig. 3C). However, treatment of XIAP with GSNO before the incubation with caspase-3 abolished this...
suggests that the NO modification on XIAP was reversible (Fig. 3). The interaction could be restored by the treatment of DTT, which suggests that the NO modification of XIAP was reversible. XIAP impairs its binding with caspase-3 and directly inhibits XIAP's caspase-3 inhibition activity (Fig. 3).

To determine whether XIAP S-nitrosylation may lead to loss in XIAP's anti-apoptotic activity, we transfected cells with XIAP and then treated cells with rotenone and dopamine. Treatment of cells with rotenone (50 μM) and dopamine (2 mM) induced a significant increase in cell death (Fig. 4B and C). In contrast, cells transfected with XIAP were resistant to rotenone or dopamine challenge (Fig. 4B and C). However, the protection offered by XIAP was abolished by pretreatment of cells with the NO donor, NOC18 (Fig. 4B and C).

The protection offered by XIAP was abolished by pretreatment of cells with the NO donor, NOC18 (Fig. 4B and C).

Proteasomal dysfunction and protein aggregation-induced toxicity have been considered as other major contributors in the pathogenesis of PD (17). Consistent with this hypothesis, neurons exposed to proteasomal inhibitors or proteins prone to aggregation are more vulnerable to cell death (18–20). To test whether S-nitrosylation of XIAP could compromise its ability to protect neurons against rotenone- and dopamine-induced toxicity, we transfected cells with XIAP and then treated cells with rotenone and dopamine. Treatment of cells with rotenone (50 μM) and dopamine (2 mM) induced a significant increase in cell death (Fig. 4B and C). In contrast, cells transfected with XIAP were resistant to rotenone or dopamine challenge (Fig. 4B and C). However, the protection offered by XIAP was abolished by pretreatment of cells with the NO donor, NOC18 (Fig. 4B and C). These results suggest that S-nitrosylation of XIAP can compromise the survival of dopaminergic neurons in the process of neurodegeneration in cellular models of PD.

**Fig. 2.** NO does not affect E3 ligase activity and dimerization of XIAP. (A and B) NO has no effect on XIAP E3 ligase activity. HEK293 cells transfected with myc-XIAP and HA-XIAP were incubated with either 100 μM GSNO for 6 h or 100 μM NOC-18 for 24 h. The E3 ligase activity of XIAP was assessed by IP with anti-myc antibody and analyzed by Western blot. (C and D) NO has no effect on XIAP dimerization. HEK293 cells transfected with myc-XIAP and HA-XIAP were treated with 100 μM GSNO for 6 h or 100 μM NOC18 for 24 h. Dimerization of XIAP was assessed by IP by anti-myc antibody and analyzed by Western blot. These results were replicated at least 3 times.

Different studies suggest that the XIAP's anticaspase-3 activity depends on the direct physical interaction of XIAP with caspase-3 (1). Thus, XIAP S-nitrosylation may lead to loss in XIAP's anti-caspase-3 activity by interfering with the direct interaction between XIAP and caspase-3. To determine whether XIAP S-nitrosylation could affect the interaction between XIAP and caspase-3, lysates from HEK293 cells transfected with myc-XIAP were incubated with recombinant caspase-3 followed by anti-myc IP. We found that XIAP specifically coimmunoprecipitated with caspase-3 (Fig. 3E). Treatment of cell lysate with GSNO before incubation with caspase-3 abolished this XIAP caspase-3 interaction (Fig. 3E). This interaction could be restored by the treatment of DTT, which suggests that the NO modification on XIAP was reversible (Fig. 3E). Taken together, these results showed that S-nitrosylation of XIAP impairs its binding with caspase-3 and directly inhibits XIAP's anticaspase-3 activity.

**XIAP's Antiapoptotic Function Is Impaired by S-Nitrosylation.** XIAP is well known to possess antiapoptotic activity against a variety of cell death paradigms (2, 11, 12). Because we found that S-nitrosylation of XIAP impaired its anticaspase-3 activity, we suspected that S-nitrosylation of XIAP could also impair its antiapoptotic activity in cells exposed to various cell death stimuli. To test this hypothesis, HEK293 cells transfected with and without XIAP were treated with 50 ng/mL of TNF-α and 0.1 μg/mL of actinomycin D for 24 h to induce apoptosis. Consistent with previous studies, treatment of HEK293 cells with TNF-α induced cell death, but expression of XIAP significantly attenuated the cell death induced by TNF-α (13) (Fig. 4F). This protection was abolished by the treatment of NOC18, but not with the NO-depleted NOC18 (Fig. 4F).

Because we suspected that nitrosative stress could compromise the protective effects of XIAP and possibly contribute to the development of PD, we tested whether NO could impair XIAP's antiapoptotic function by using PD cell-based models. Rotenone inhibits mitochondrial complex I and exposure to rotenone like herbicides may lead to degeneration of dopaminergic neurons (14). Similarly, the high propensity of dopamine to oxidize among catecholamines may account for why dopaminergic neurons are more susceptible to degeneration in PD (15, 16). To test whether S-nitrosylation of XIAP could compromise its ability to protect neurons against rotenone- and dopamine-induced toxicity, we transfected cells with XIAP and then treated cells with rotenone and dopamine. Treatment of cells with rotenone (50 μM) and dopamine (2 mM) induced a significant increase in cell death (Fig. 4B and C). In contrast, cells transfected with XIAP were resistant to rotenone or dopamine challenge (Fig. 4B and C). However, the protection offered by XIAP was abolished by pretreatment of cells with the NO donor, NOC18 (Fig. 4B and C).

**XIAP S-Nitrosylation Is Increased in an Animal Model of PD and in PD Patients.** Our previous study showed that S-nitrosylated proteins are significantly increased in animal model of PD and PD patients (7). In this study, our results suggest that S-nitrosylation of XIAP could compromise its protective function in cells. Thus, we hypothesized that during the pathogenesis of PD, XIAP S-nitrosylation is elevated, which could possibly compromise the survival of neurons in the process of neurodegeneration. To test this hypothesis, we first used the well-established MPTP animal model of PD to determine whether S-nitrosylation of XIAP is increased in this model. Administration of MPTP in animals selectively induces the degeneration of the nigrostriatal dopaminergic neurons as observed in PD (22). To determine whether XIAP S-nitrosylation was increased in nigrostriatal system in mice after MPTP treatment, mice were injected with MPTP as described (7, 23). After MPTP treatment, mice were killed at 2- and 48-h time points, their brains were harvested, and XIAP S-nitrosylation in the striatum was determined by the biotin switch assay. We selected these 2 time points because from our previous study we found that 2 and 48 h after MPTP treatment...
marked the highest levels of parkin S-nitrosylation in the brain (7). After MPTP treatment, XIAP S-nitrosylation was markedly increased in the striatum (Fig. 5A). This increase in XIAP S-nitrosylation was particularly significant 48 h after MPTP treatment (Fig. 5A and B).

We next wanted to address whether increased XIAP S-nitrosylation could also be observed in PD patients. To determine whether XIAP S-nitrosylation was increased in PD patients, we performed the biotin switch assay on postmortem brain tissue from caudate from normal control and PD patients. We found that the protein levels of XIAP were similar in normal control and PD patients (Fig. 5C). However, we observed a significant increase in S-nitrosylated XIAP in PD patients (Fig. 5C and D). Taken together, these results suggest that XIAP is S-nitrosylated in vivo and is selectively increased in the MPTP animal model of PD and in the postmortem brain tissues of PD patients.

**Discussion**

XIAP is an antiapoptotic protein that is known to be crucial for cell survival. However, overexpression of XIAP is commonly observed in tumors and is implicated in the development of different types of cancer (11, 24). In this study we show that XIAP can be S-nitrosylated at the BIR domains. XIAP S-nitrosylation compromised its antiapoptotic function by inhibiting its anticaspase-3 activity. We further found that a significant increase of S-nitrosylated XIAP can be observed in the MPTP animal model of PD and in PD patients. These results support our previous finding that nitrosative stress is an important contributor in the pathogenesis of PD.

We first reported S-nitrosylation of parkin can compromise its protective functions (7). The mechanism by which S-nitrosylation of parkin impairs its protective function appears to occur through inhibition of its E3 ubiquitin ligase activity (7, 25). This idea contrasts with the mechanism of impairment of XIAP antiapoptotic function by S-nitrosylation. XIAP antiapoptotic function is inhibited by S-nitrosylation through preventing XIAP’s binding to caspase-3. Several recent studies have also reported that other neuroprotective proteins such as peroxiredoxin and protein-disulphide isomerase are modified by S-nitrosylation, and this modification compromises their normal protective functions (26, 27). Thus, the repertoire by which S-nitrosylation can compromise cellular survival is diverse.

Apart from impairing neuroprotection proteins, S-nitrosylation is also involved in mediating cell death through GAPDH (28, 29). Recent studies showed that S-nitrosylation of GAPDH can mediate the translocation of GAPDH–Siah I protein complex to the nucleus and initiates apoptosis (29, 30). Our findings that S-nitrosylation of XIAP can affect its antiapoptotic function further suggest that nitrosative stress can affect the survival of neurons through targeting a number of pathways. Because we found that XIAP S-nitrosylation was increased in the MPTP animal model of PD and in PD patients, these results suggest that neurons would be more vulnerable to cell death in face of unfavorable conditions such as proteasomal dysfunction and protein aggregation-induced toxicity. Our findings also suggest that fully understanding how nitrosative stress can contribute to PD will help develop new therapeutic approaches for this disease.

**Materials and Methods**

**Chemicals and Cell Culture.** All chemicals were purchased from Sigma–Aldrich unless otherwise stated. HEK293T and N2a cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco) at 37 °C with 5% CO2. Transfection was performed with Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer’s instruction.

**Generation of Plasmids.** XIAP and procaspase-3 were cloned from the SuperScript human brain cDNA library (Invitrogen). Full-length XIAP was cloned into pRK5-myc and HA vectors for cell culture study and into pGEX-4T-2 vector for recombinant protein production. The truncated fragments of XIAP encoding amino acids 1–446 (BIR1–3), 1–450 (BIR1–2), 1–164 (BIR1), 95–265 (BIR2), 231–446 (BIR3), and 332–497 (RING) were generated by PCR with the full-length XIAP template and cloned into pRK5-myc vector. The XIAP fragment BIR1–3 was also cloned into...
Representative protein levels of myc-XIAP after treatment as indicated are shown. (8–E) NO inhibits XIAP’s antiapoptotic function against various cell death stimuli. HEK293 cells were transfected with 0.25 μg of myc-XIAP. Thirty hours after transfection, cells were preexposed with either 100 μM NOC-18 or depleted NOC-18 for 6 h and then treated with 50 ng/mL TNF-α and 0.1 μg/mL actinomycin D for an additional 24 h. Cell death was assayed by trypan blue exclusion method (***, P < 0.01; ns = nonsignificant). Representative protein levels of myc-XIAP after treatment as indicated are shown. (8–E) NO inhibits XIAP’s antiapoptotic function against various cell death stimuli. HEK293 was transfected and preexposed to NOC-18 as described in A. After that, cells were challenged with various cell death stimuli (50 μM rotenone for 16 h, 2 mM dopamine for 24 h, and 3 μM MG132 for 24 h), and cell death was analyzed by trypan blue exclusion assay. In GFPu experiment (E), 0.125 μg of myc-XIAP was cotransfected with either 0.125 μg of GFPu or control plasmid. 30 h after transfection, cells were exposed to 100 μM NOC-18 and cell death was assessed 60 h after transfection (***, P < 0.01; ***, P < 0.001; ns = nonsignificant). These results were replicated at least 3 times.

pGEX4T.2 vector for recombinant protein production. Procaspase-3 was cloned into pET28C vector for expression of recombinant proteins. The cDNAs of α-synuclein and ubiquitin were generated as described (7). Sequence integrity of all constructs was verified by sequencing.

Overexpression and Purification of Recombinant Proteins. Recombinant GST, GST-XIAP, and GST-BIR1–3 were expressed in Rosetta (DE3) pLys Escherichia coli (Novagen). Overexpression of bacterial culture in linear growing phase (0.6 OD)

Fluorometric Detection of S-Nitrosylated XIAP. Fluorometric assay was performed according to Cook et al. (32). In brief, GSNO- or GSH-treated GST-tagged recombinant proteins were immunoprecipitated with polyclonal anti-GST antibody. The pellet was then washed 5 times with TBST (1% Triton X-100 in TBS) buffer. After washing, 100-μL assay buffer containing 100 μM DANS and 100 μM HgCl2 in TBS was added to pellets and incubated for 2 h at room temperature in darkness. The fluorescence generated by the formation of fluorometric product 2,3-naphththyltrazole was then measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

XIAP Dimerization and Autoubiquitination. For dimerization assay, HEK293T cells were transfected with myc-XIAP and HA-XIAP. After 24 h, cells were treated with GSNO (100 μM) or NOC-18 (100 μM) and then harvested at the selected time points with IP buffer (1% Triton X-100, 10% glycerol, 1 mM aprotinin, 1 mM leupeptin, 1 mM benzamidine, 10 mM PMSF in TBS) at 4 °C for 1 h and clarified by centrifugation. The cell lysates were subjected to anti-myc IP by incubating with 0.5 μg of anti-myc antibody (Roche) together with 50 μL of protein A agarose (GE Healthcare) for 2 h at 4 °C with rotation. The immuno-complexes were washed 5 times with IP buffer and eluted by SDS sample buffer and subjected to Western blot analysis. For ubiquitination assay, 24 h after transfection, HEK293T cells overexpressed with myc-XIAP and HA-ubiquitin were treated with GSNO (100 μM) or NOC-18 (100 μM) as indicated. After treatment, cells were lysed by IP buffer and followed by anti-myc IP protocol as described.

were induced by 0.2 mM IPTG at 18 °C overnight, and the recombinant proteins were then purified by GSH-Sepharose (GE Healthcare). His-tagged recombinant active caspase-3 was produced according to Stennicke and Salvesen (31) and was purified by Ni-NTA Sepharose (GE Healthcare). Concentrations of the recombinant protein were quantified by SDS/PAGE with the use of BSA as standard.

Preparation of S-GSNO. S-GSNO was prepared according to Cook et al. (32). GSNO was prepared freshly at the day of each experiment.

In Vitro S-Nitrosylation Assay. The bain switch assay was performed according to Jaffrey and Snyder (33) with some modifications. Nitrosylated cell lysates or recombinant proteins in HENT buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM N-ethylmaleimide, 1% Triton X-100) were incubated with 10 μM methyl methanethiosulfonate (MMTS) (Thermo Scientific) at 50 °C for 20 min and then excess MMTS was removed by passing through the G25 Sephadex spin column 3 times. The samples were then incubated with 5 mM ascorbate and 0.4 mM biotin-HDPD (Thermo Scientific) for 1 h at room temperature with rotation. Unreacted biotin-HDPD was then removed by G25 Sephadex spin column and biotinylated samples were then incubated with 50 μL of Neutravidin-agarose (Thermo Scientific) for 1 h. Pellets were then washed 5 times with neutralization buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) with 0.6 M NaCl and eluted by SDS sample buffer and subjected to Western blot analysis.

Fig. 5. XIAP is S-nitrosylated in vivo. (A) The level of S-nitrosylated XIAP is increased at 2 and 48 h after MPTP injection as observed in 3 independent experiments. (B) The amount of S-nitrosylated XIAP was quantified by a densitometer, and a significant increase of XIAP S-nitrosylation was observed in MPTP-treated animals (***, P < 0.01). (C) A marked increase of S-nitrosylated XIAP in the caudate was observed in PD patients. (D) The amount of S-nitrosylated XIAP was quantified by a densitometer, and a significant increase of XIAP S-nitrosylation was observed in PD patients (*, P < 0.05). These results were replicated at least 3 times.
XIAP Caspase-3 Interaction. HEK293T lysates with or without expressing myc-XIAP were treated with GSH (500 μM), GSNO (500 μM), or DTT (1 mM) for 15 min at 37 °C as indicated. The lysates were then passing through G25 Sephadex spin column once and recombiant active caspase-3 (7.5 μg) was added to the lysates and co-IP protocol was then carried out as described. Caspase-3 was detected by anticleaved caspase-3 antibody (Sigma).

Caspase-3 Activity Assay. In caspase-3 activity assay, recombiant GST-XIAP and GST were first treated with 5 mM DTT for 20 min at room temperature, and DTT was then removed by a G25 Sephadex desalting column (GE Healthcare). After passing through the column, 0.2 μM recombinant proteins were treated with 500 μM GSH or GSNO for 15 min at 37 °C followed by passing the samples again through the Sephadex desalting column to remove GSH and GSNO. The samples were then incubated with 20 nM active caspase-3 for 10 min at room temperature with rotation. After incubation, 50 μM DEVD-AFC (Sigma) was added as substrate. Caspase-3 activity was monitored by the fluorescence generated at an excitation wavelength of 355 nm and an emission wavelength of 460 nm at 30 °C.

PARP Cleavage. Recombinant GST-XIAP and GST were first treated with DTT, GSH, and GSNO as in the caspase activity assay. Treated proteins were then incubated with 0.5 μg of active caspase-3 (1:10 molar ratio) for 10 min at room temperature with rotation. After 10 min, 10 μg of 293T cell lysate was added to the mixture and incubated at 37 °C for 30 min. The reaction was stopped by adding SDS sample buffer, and PARP cleavage was analyzed by Western blot with the anticleaved PARP antibody (BD Bioscience).

Cell Death Analysis. HEK293T cells were transfected with 0.25 μg of myc-XIAP or control vector. Thirty hours after transfection, cells were pretreated with 100 μM NO donor NOC-18 (Calbiochem) for 6 h. Cells were then treated with selected drugs as indicated and assayed as follows: 50 ng/mL TNF-α and 0.1 μM myc-XIAP, or 0.1 μM acLDL, or 50 μM GSH or GSNO for 15 min at 37 °C followed by passing the samples again through the Sephadex desalting column to remove GSH and GSNO. The samples were then incubated with 20 nM active caspase-3 for 10 min at room temperature with rotation. After incubation, 50 μM DEVD-AFC (Sigma) was added as substrate. Caspase-3 activity was monitored by the fluorescence generated at an excitation wavelength of 355 nm and an emission wavelength of 460 nm at 30 °C.

The anticleaved PARP antibody (BD Bioscience).

In Vivo S-Nitrosylation Assay of XIAP in Human Brain Tissue. The assay was basically the same as the in vitro assay except tissues were homogenized in HENS buffer (250 mM Hepes (pH 7.7), 1 mM EDTA, 0.1 mM neocuproine, and 1% DMSO) without the incubation with NO donors. S-nitrosylated protein was detected by using specific XIAP antibody (BD Bioscience).

Statistical Analysis. Data are expressed as mean ± SEM. Significance was determined by ANOVA or Student’s t-test.

ACKNOWLEDGMENTS. We thank Prof. Randy Y. C. Poon (Hong Kong University of Science and Technology) for providing the anticleaved PARP antibody and the Johns Hopkins Medical Institutions according to the Health Insurance Portability and Accountability Act regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race, or ethnicity. The Johns Hopkins Medical Institutions Joint Committee on Clinical Investigations decided that the studies in this proposal are exempt from human subjects approval because of Federal Register 46.101 exemption 4. Four age-matched control brains, 4 PD and/or DLBD brains, were used for S-nitrosylation of XIAP by in vivo S-nitrosylation assay (Table S2).