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Sulfhydration mediates neuroprotective actions of parkin

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Abstract

Increases in S-nitrosylation and inactivation of the neuroprotective ubiquitin E3 ligase, parkin, in the brains of patients with Parkinson's Disease (PD) are thought to be pathogenic and suggest a possible mechanism linking parkin to sporadic PD. Here we demonstrate that physiologic modification of parkin by hydrogen sulfide (H_2S), termed sulfhydration, enhances its catalytic activity. Sulfhydration sites are identified by mass spectrometry analysis and investigated by site directed mutagenesis. Parkin sulfhydration is markedly depleted in the brains of patients with PD, suggesting that this loss may be pathologic. This implies that H_2S donors may be therapeutic.

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Author Contributions:
M.S.V., B.D.P., and N.S. designed, performed, and analyzed experiments. R.X., F.R., H.S.K., S.K., and Y.I.L. assisted with experimental design and data analysis. M.S.V., B.D.P, A.M.S., generated plasmid constructs. V.L.D. and T.M.D. provided experimental support, data analysis, and edited the manuscript. M.S.V. and S.H.S. wrote the manuscript.

INTRODUCTION

Parkin is an E3 ubiquitin ligase that ubiquitinates diverse substrates[1]. Mutations in parkin which disrupt its catalytic activity are the most common cause of autosomal recessive Parkinson's Disease (PD), indicating that loss of parkin is neurotoxic, while its enhancement is neuroprotective[1,2]. Parkin may also participate in the pathophysiology of the much more common sporadic form of PD based on interactions with nitric oxide (NO). Dawson and associates[3] and Lipton and colleagues[4] reported that parkin is S-nitrosylated with greatly increased nitrosylation in brains of patients with PD. Nitrosylation of parkin inhibits its E3 ligase and neuroprotective activities, implying that the increased nitrosylation of parkin in PD is pathogenic. Recently, hydrogen sulfide (H_2S) has been appreciated as a gasotransmitter comparable to NO and CO[5–8]. H_2S signals primarily by attaching to SH groups of cysteines in proteins, a process termed sulfhydration[9]. Sulfhydration generally occurs on the same cysteines as nitrosylation so that the two processes may be reciprocal. This prompted us to explore relative roles of sulfhydration and nitrosylation in the function of parkin.

We report that parkin is physiologically sulfhydrated and that, whereas nitrosylation inactivates parkin, sulfhydration stimulates its activity. We identify major declines of parkin sulfhydration in the corpus striatum of PD patients. We also show that H_2S donors are cytoprotective in parkin-related models of neurotoxicity. Thus, diminished sulfhydration of parkin may be pathogenic in PD and selective H_2S donors may be therapeutic.

RESULTS

Parkin is physiologically sulfhydrated

The biotin switch method we developed[10] for monitoring nitrosylation can be modified to identify sulfhydration[9]. Utilizing the modified biotin switch assay, we demonstrate sulfhydration of overexpressed parkin in response to treatment with the H_2S donor NaHS (Fig. 1a). Recently we have developed improved methodology for monitoring sulfhydration employing fluorescently labeled derivatives of maleimide[11]. Virtues of the maleimide procedure include its greater specificity, the ability to quantify results readily, and the capacity of the technique to monitor nitrosylation and sulfhydration of the same samples[11]. Utilizing the maleimide procedure, we establish that under basal conditions parkin is robustly sulfhydrated in whole brain of mice and the striatum of rats (Fig. 1b).

To further substantiate the nature of parkin sulfhydration, we show that overexpression of cystathionine beta synthase (CBS), an H_2S biosynthetic enzyme, increases parkin sulfhydration more than 20-fold (Fig. 1c, Supplementary Fig. S1a). The neuronal cell line SH-SY5Y displays endogenous sulfhydration of parkin which is increased more than 5-fold by treatment with the H_2S donor GYY4137[12] (Fig. 1d, Supplementary Fig. S1b). Moreover, overexpressing CBS in SH-SY5Y cells increases parkin sulfhydration more than 8-fold (Fig. 1e, Supplementary Fig. S1c). There appears to be negligible basal nitrosylation of parkin present in brain tissue or cell lines, which is unchanged by treatment with H_2S donors or generating enzymes (Supplementary Fig. S1d–g).

H_2S enhances parkin E3 ligase activity via sulfhydration

Nitrosylation of parkin decreases its ubiquitination activity both exerted upon itself and on other substrates[3,4]. By contrast, in HEK293 cells the H_2S donor GYY4137 markedly augments parkin autoubiquitination whereas "old" GYY4137 (GYY4137 exposed to air overnight to eliminate any H_2S donating capacity), fails to influence ubiquitination (Fig. 2a). Treatment with GYY4137 results in an increase in parkin E3 ligase activity over time consistent with the activation of parkin via sulfhydration (Supplementary Fig. S2a). The stimulation by GYY4137 of parkin's ubiquitination capacity applies also to proteins that are implicated in the pathogenicity of the disease. AIMP2 expression is elevated in human postmortem brain from both sporadic and familial PD, consistent with the notion that parkin is inactivated in PD and that AIMP2 is neurotoxic[13,14]. GYY4137 substantially increases ubiquitination of AIMP2 by parkin (Fig. 2b) as well as ubiquitination by parkin of synphilin-1, another parkin substrate implicated in Parkinson's Disease (Fig. 2c).

To ascertain whether the influence of H_2S upon ubiquitination is exerted in a direct fashion, we conducted experiments *in vitro* comparing actions of the NO donor GSNO and the H_2S donor NaHS (Fig. 2d). As reported previously, GSNO substantially diminishes parkin's autoubiquitination,[3] whereas such autoubiquitination is markedly augmented by treatment with NaHS.

The contrasting actions of H_2S and NO upon parkin's ubiquitination activity suggest that the two gasotransmitters may exert reciprocal actions in the pathophysiology of PD. Sulfhydration and nitrosylation typically take place upon the same cysteines in proteins. This suggests that reciprocity between sulfhydration and nitrosylation of parkin impacts pathogenic features of PD. To examine this possibility, we monitored sulfhydration of parkin in brains of mice treated with the neurotoxin MPTP, which selectively damages dopamine neurons and is often employed as a model for PD (Fig. 2e, Supplementary Fig. S3a–c). We note increased sulfhydration of parkin 2–4 hours following MPTP treatment. In brains of mice with targeted deletion of inducible NO synthase (iNOS) or neuronal synthase (nNOS), in which parkin nitrosylation is lost,[3] its sulfhydration is increased 2.5–3 fold. This implies that sulfhydration and nitrosylation of parkin occur reciprocally, presumably on the same cysteines.

Parkin sulfhydration enhances it protective functions

To ascertain the pathophysiologic relevance of reciprocal nitrosylation/sulfhydration of parkin, we sought to identify the sites of sulfhydration. High resolution ESI-MS-MS technique was implemented, which can differentiate sulfhydration from sulfinic acid oxidation of the cysteine residues. Mass spectrometric analysis reveals five sites of parkin sulfhydration at cysteines 59, 95, 182, 212 and 377 (Fig. 3a, Supplementary Fig. S4). At least one of these sites, cysteine 95, has been identified as being nitrosylated (Harry Ischiropoulos, personal communication). In order to determine the relative importance of these sites in mediating regulation of parkin ubiquitination activity by H_2S, we performed systematic mutations of the various cysteines and assessed their activities (Fig. 3b). Parkin with C212S or C377S mutations fails to express or is unstable and could not be evaluated. Enhanced ubiquitination activity of parkin in response to GYY4137 is abolished with C95S

mutations, while substantial diminution of the enhancement of ubiquitination is evident with C59S and C182S mutations.

To determine whether sulfhydration of parkin regulates pathogenic events associated with PD, we monitored cell death using trypan blue exclusion as well as MTT assays in PC12 cells overexpressing AIMP2, whose ubiquitination and destruction are elicited by parkin in cellular models of PD[15] (Fig. 3c, Supplementary Fig. S5a). Overexpression of AIMP2 triples cell death, while parkin overexpression reverses this cytotoxicity. GYY4137 markedly reduces cell death in the parkin treated cells but not in those overexpressing AIMP2 in the absence of parkin. The selective action of GYY4137 indicates that its cytoprotective effects reflect modifications of parkin rather than some generalized antioxidant influence. This conclusion is supported by GYY4137's lack of cytoprotective influence in cells overexpressing AIMP2 along with catalytically inactive parkin-T240R.

Further evidence that GYY4137 protects by enhancing parkin sulfhydration comes from experiments in which the cytoprotective action of GYY4137 is lost in cells overexpressing parkin-C95S, which is not activated by sulfhydration. Furthermore, we utilized the MPP+ model of PD in PC12 cells and SH-SY5Y cells in which H_2S donors provide significant protection against MPP+ toxicity and inhibition of the H_2S producing enzymes results in enhanced toxicity of MPP+ which is relieved by administration of H_2S donors (Supplementary Fig. S5b,c). In order to resolve whether the anti-oxidant properties of H_2S releasing agents were partially responsible for the neuroprotective effects seen in the AIMP2 overexpressing cells, we determined ROS levels in these cells overexpressing vector or various parkin mutants (Supplementary Fig. 6). The various parkin cysteine-serine mutants exhibited increased ROS levels as described previously[16]. However, there was not a significant difference in the GYY4137 treated samples at the concentrations that were employed suggesting that this is not the primary mechanism of protection seen in the experiments described in Fig. 3c.

Decreased sulfhydration and increased nitrosylation in PD

The pronounced cytoprotective action of parkin sulfhydration as well as the reciprocal relationship of parkin's nitrosylation and sulfhydration suggest that alterations of sulfhydration participate in the pathophysiology of PD. Accordingly, we monitored sulfhydration and nitrosylation in the striatum of PD patients (Fig. 4, Supplementary Fig. 7). We confirm the increase of parkin nitrosylation in PD brain,[3] and also observe a 60% decrease in parkin sulfhydration in patient brain.

DISCUSSION

In the present study, we have demonstrated that parkin is physiologically sulfhydrated, a process that enhances its ubiquitination activity. This contrasts markedly with nitrosylation, which decreases such activity. Sulfhydration and nitrosylation of parkin appear to be reciprocal events. This may be related to the difference in chemical reactivity between the two modifications. Nitrosylated cysteines will present a distinctly different chemical group to the local environment than will sulfhydration. In patient striatum, we demonstrate major decreases in parkin sulfhydration, which are reciprocal to the increases in nitrosylation.

What might be the comparative roles of parkin nitrosylation and sulfhydration in the pathophysiology of PD? One possibility is that the cell stress of PD leads to increased generation of NO, which accounts for the augmented nitrosylation of parkin in patient brain. Cell stressors do lead to activation of both iNOS and nNOS, and increased S-nitrosylation of several proteins, such as parkin, protein-disulfide isomerase, and XIAP, has been speculated to impact PD[17–19]. However, increased NO formation in PD has not been directly demonstrated, and measurements of H_2S generation in PD are lacking. Nitrosylation and sulfhydration are sensitive and specific reflections of the presence of ambient NO and H_2S respectively, implying that altered nitrosylation/sulfhydration in PD brain reflects changes in levels of the two gasotransmitters.

Our experiments also establish cytoprotective actions of H_2S donors, which appear to reflect sulfhydration of parkin. Mutation of C95, a principal site of parkin sulfhydration, largely prevents the protective influences of H_2S donors indicating that the donors act via parkin sulfhydration to enhance its ubiquitination activity. C182 and C59 also appear to contribute to influences of H_2S donors upon parkin. We could not evaluate effects of H_2S on C212 and C377, as they did not express or were unstable in our cell lines. Interestingly, C95 occurs in human but not rodent parkin, while the other sulfhydrated cysteines are conserved in rodent and human species.

The beneficial effects of H_2S donors in PD models may have therapeutic implications. H_2S donors have already been noted to be beneficial in rodent models of PD[20–22], and an H_2S donating variant of L-DOPA has shown promising effects in cellular models of PD[23]. These influences had been speculated to reflect general antioxidant and anti-inflammatory actions of H_2S. However, numerous studies have failed to reveal a beneficial effect of antioxidants in PD[24,25]. Our findings provide a specific molecular mechanism whereby H_2S therapy may benefit PD and can explain the ineffectiveness of generalized antioxidant treatment. Modifications of parkin and other interactors of parkin may offer promise in the therapy of PD[15,26,27] so that H_2S donors selectively targeted to parkin may provide notable benefit.

Materials and Methods

Generation of plasmids

The full-length parkin cDNA was cloned into pRK5-myc and pCMV-FLAG (Stratagene) vectors between the *Sal*I and *Not*I. Full-length cDNAs of synphilin-1 and AIMP2 were cloned into pRK5-myc vector and pCMV-FLAG respectively between the *Sal*I and *Not*I sites. The cDNA of ubiquitin was cloned into pRK5-HA vector between the *Sal*I and *Not*I sites. The generation of C-S mutants was done using the pRK5-myc parkin construct and site-directed mutagenesis. The integrity of the constructs was confirmed by sequencing.

Modified biotin switch assay

A modified version of the modified biotin switch assay as described in[9] was used. In brief, overexpressed myc-parkin was transfected into HEK293 cells with polyfect (Qiagen) for 24 hours. Cells were treated with 100 μM NaHS (Sigma) as indicated and harvested in HEN buffer with 1% triton and spun down at 14,000 r.p.m. for 15 min. The supernatant is then

added to lysis buffer plus 2% SDS and 10 mM NEM for 1 h at 37°C while shaking. Blocked proteins are acetone precipitated with acetone (−20°C) to remove free NEM followed by 1 mM DTT treatment for 1 hour at 25°C while shaking. Another round of acetone precipitation was performed to remove the DTT and reduced S-NEM from sulfhydrated residues followed by treatment with 200 μM biotin-NEM for 1 hour at 25°C followed by precipitation with neutravidin (Thermo Scientific) beads and subsequent analysis via western blot with anti-myc antibody.

Maleimide Assay

Performed as described in[11]. In brief: cells or tissue were lysed in 20 mM Tris-HCl pH 7.5, 0.1% triton, 100 mM NaCl and immunoprecipitated with parkin antibody (Cell Signal) or appropriate antibody for 16 h at 4°C followed by washing 5 X with lysis buffer plus 300 mM NaCl (wash buffer). Beads were then incubated with 5 μM Red-Maleimide for 2 h at 4°C while rotating followed by 3 X wash with wash buffer. Beads were then divided equally between two tubes with one tube receiving 1 mM DTT and the other buffer and both rotated for 2 h at 4°C followed by 3 X wash with wash buffer. 50 μL of 2X LDS (Invitrogen) was then added, the beads boiled for 2 min, and proteins separated on SDS-PAGE and visualized on the LiCor fluorescent scanner. If the 2-color maleimide assay was used, an additional labeling step was included: after incubation with Red-Maleimide and subsequent wash, 1 mM ascorbate was added for 2 h followed by treatment with Green-Maleimide for 2 h and subsequent wash. Beads were then split equally again and treated with DTT as described above. Western blot analysis with the appropriate antibody was performed to ensure that any differences between bead allocations between tubes were taken into account for the experiment.

In vitro ubiquitination assay

Reactions were performed in a 20 μl mixture containing 50 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 1 mM ATP, 1 mM biotin-ubiquitin (BostonBiochem), 110 ng of E1 (BostonBiochem), 900 ng of UbcH7 (BostonBiochem), His6-Parkin (1.5 μg) that had been treated with either 100 μM of GSNO or NaHS (as indicated) for 20 min prior to the addition of buffer or 1 mM DTT (as indicated). After 30 min this was added to the main reaction which was carried out in darkness and devoid of any reducing agents such as DTT at 37 °C (except where specified). After 1 h, the reactions were terminated with an equal volume of 2X SDS sample buffer and the products were subject to Western-blot analysis with anti-ubiquitin antibody (Cell Signaling).

Ubiquitination assay

HEK293 cells were transfected with 4 μg of plasmids. After 24 h, the cells were treated with the MG132 (Sigma) followed by the selected drugs (as indicated) for the specified time course (3–9 h). If cells were transfected with CBS or CSE, 500 μM of L-Cysteine was added to the media as a supplement for H_2S generation. After 2–9 h all samples were harvested together to ensure that all samples were treated by MG132 for equal time and only the time treated with H_2S varied. The cells were harvested by washing with cold PBS and then lysed with immunoprecipitation buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 0.5% triton X-1000, 1mM EDTA, Roche Complete Protease Inhibitor Tablet). The lysates were then

sonicated at 4°C for 10 sec and rotated for 15 min at 4°C followed by centrifugation at 14,000 r.p.m. for 15 min. The supernatants were combined with 30 μL EZ-View anti-myc or anti-FLAG (Sigma) beads overnight at 4°C. The beads were pelleted and washed 5 times using immunoprecipitation buffer with 500 mM NaCl. The precipitates were resolved on SDS-PAGE gel and subjected to Western-blot analysis with antibodies against myc or HA (Roche). Bands were visualized with chemiluminescence (Pierce).

Animals and treatment

All experiments were approved and conformed to the guidelines set by the Institutional Animal Care Committee. Ten-week-old iNOS deficient mice and nNOS deficient mice (Jackson Laboratories) and their wild-type counterparts were used. Mice received four intraperitoneal injections of MPTP-HCl (20 mg/kg of free base; Sigma) in saline at 2 h intervals in 1 day, and were sacrificed at selected time points as indicated after the last injection. Control mice received saline only. The mouse brains were harvested and S-sulfhydration of parkin in the whole brain was determined by maleimide assay.

Human Tissue

Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson's Disease Research Center at Johns Hopkins Medical Institutions (JHMI) according to HIPAA regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race, or ethnicity. The JHMI 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 number 4. Seven age-matched control brains, and six PD and/or DLBD brains were utilized for the detection of S-sulfhydration and S-nitrosylation of parkin by the maleimide assay.

AIMP2-inducible cell lines

As described previously, PC12 cells were grown in DMEM containing 10% horse serum, 5% No-Tet FBS in a 5% CO2 atmosphere[15]. Tet-off cells (Clontech) were used to create PC12 cell lines expressing inducible AIMP2 as described previously. Differentiation was initiated by the addition of 100 ng/mL NGF to the culture medium. NGF was replenished daily for differentiation.

Cell-viability analysis

AIMP2-inducible PC12 cells were plated in a six-well plate for viability. Cells were transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were incubated with 100 μM GYY4137 or vehicle after 2 d of induction and differentiation with NGF. To assess cell viability in the PC12 cell experiments, we used the trypan blue exclusion assay. Cells were resuspended in plating medium with trypan blue stain for 5 minutes. We counted the percentage of blue-stained cells among total cells using the Cell Countess cell counter to determine a percentage of cell death in the trypan blue exclusion assay described previously[10]. The MTT assay was also used to determine cell viability. Briefly, cells were incubated with 3,-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide for 2 h after which the supernatant was removed and

centrifuged to collect any floating cells. 1 mL of DMSO was used to lyse the remaining cells after which any pelleted cells were lysed with 1 mL of DMSO and added to the original well from which it was taken. This was incubated at RT for 20 minutes while shaking. Absorbance measurements were then taken at 570 nm and 630 nm to determine cell viability.

Statistical Analysis

All data are expressed as mean ± s.e.m. Statistical significance between sample sets was analyzed by ANOVA analysis with post-hoc test where appropriate.

Mass Spectral analysis

Purified His6-Parkin (BostonBiochem) was purified to remove any trace DTT with spin column followed by incubation in 50 mM HEPES, 100 mM NaCl buffer with 100 μM NaHS for 1 h. These samples were then digested with tripsin and run on high resolution tandem mass spectrometry array for analysis. Database searching: Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by 1 version 3. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot) and Sequest. Mascot was set up to search the NCBInr_20080819 database (selected for Homo sapiens, 2, 133769 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 15 ppm. Oxidation of methionine, persulfide of cysteine, sulphur dioxide of cysteine (sulfination), sulfitolysis of cysteine, N-ethylmaleimide on cysteines of cysteine, N-ethylmaleimide hydrolysis of cysteine, NEM+S (NEM modified sulfhydration) of cysteine and NEM+S+H_2O of cysteine were specified in Mascot as variable modifications. Criteria for protein identification: Scaffold (version Scaffold_3.4.3, ProteomeSoftware Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm[28]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[29] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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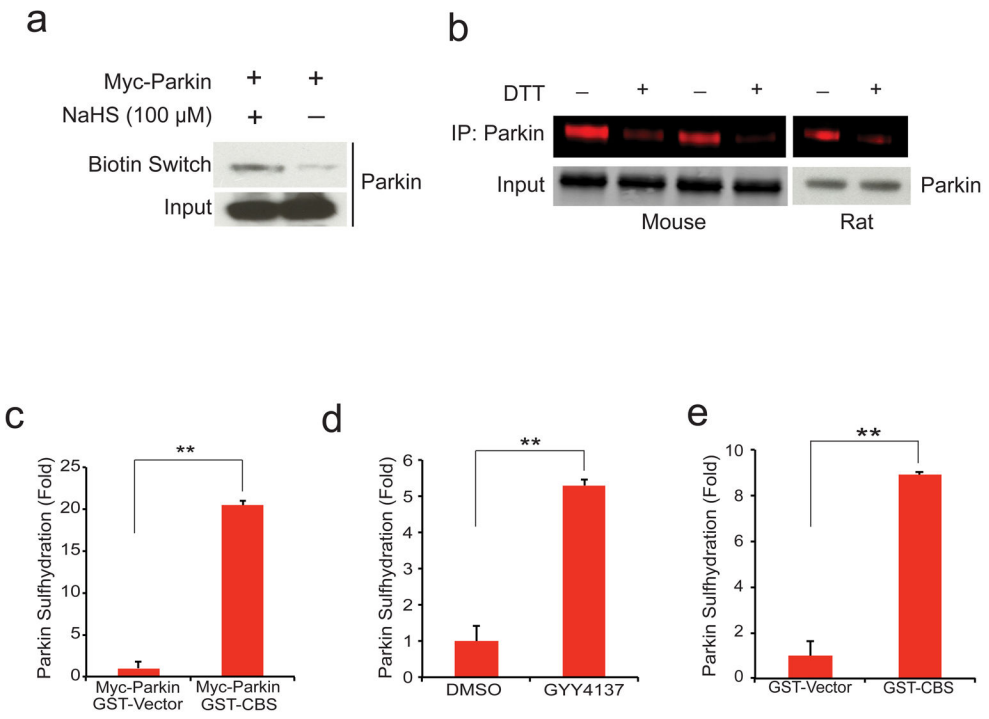

Figure 1. Parkin is physiologically sulfhydrated

(**a**) Parkin expressed in HEK293 cells is sulfhydrated by the H_2S donor NaHS as detected by the modified biotin switch method. (**b**) Endogenous parkin is basally sulfhydrated in both mouse brain and rat striatum as detected by the maleimide assay in which loss of red fluorescence signal following DTT treatment indicates sulfhydration of the protein. (**c**) Sulfhydration of myc-parkin overexpressed in HEK293 cells is enhanced almost 20 fold upon overexpression of GST-CBS, one of the principle H_2S producers in the brain. n=3, $P<0.01$ via one-way ANOVA. (**d**) Endogenous parkin sulfhydration in SH-SY5Y cells is enhanced over 5 fold upon treatment with 100 μM GYY4137, a hydrogen sulfide donor. n=3, $P<0.01$ via one-way ANOVA. (**e**) Endogenous parkin sulfhydration in SH-SY5Y cells is enhanced over 8 fold by overexpression of GST-CBS. n=3, $P<0.01$ via one-way ANOVA. All data expressed as mean ± s.e.m.

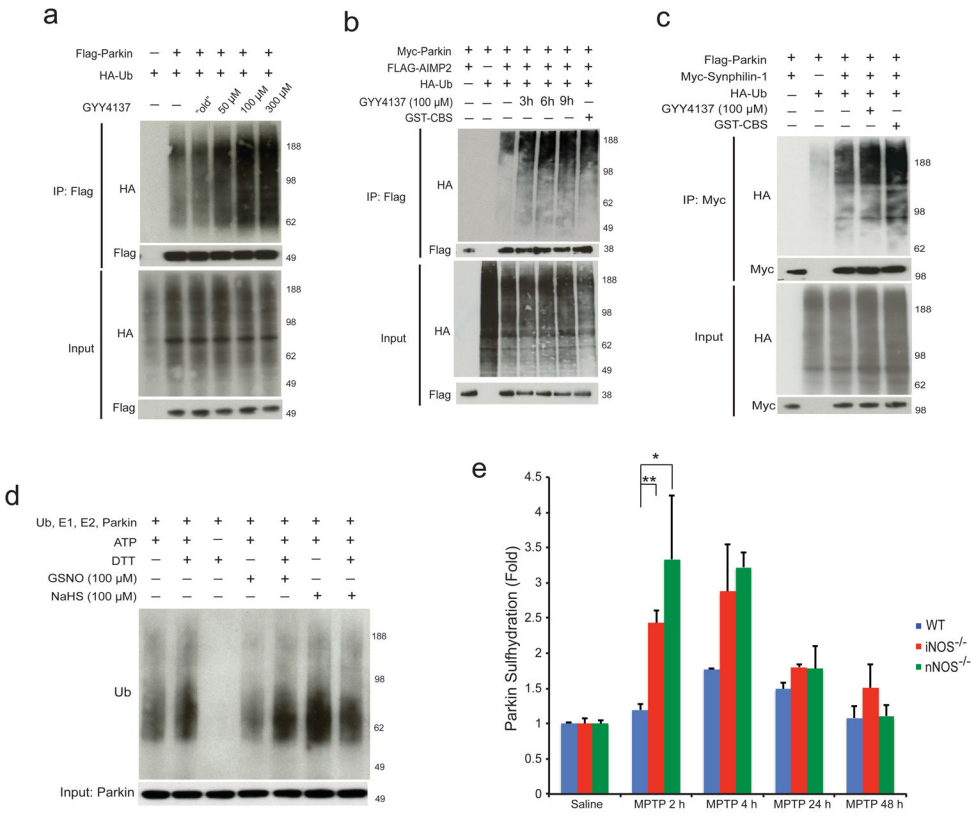

Figure 2. Hydrogen sulfide enhances parkin E3 ligase activity via sulfhydration

(**a**) Parkin E3 ligase activity in HEK293 cells is augmented by GYY4137 in a dose dependent manner, but not by "old" GYY4137, which was exposed to air overnight in PBS, and is subsequently unable to donate H_2S. (**b**) Parkin activity, measured by target ubiquitination of AIMP2, is stimulated by GYY4137 (100 μM) and by GST-CBS. (**c**) Ubiquitination by parkin of synphilin-1 is enhanced by treatment with GYY4137 or GST-CBS. (**d**) Parkin E3 ligase activity *in vitro* is increased by the addition of 100 μM NaHS and decreased by GSNO. DTT, a reducing agent, returns activity to baseline by reversing sulfhydration or nitrosylation. (**e**) MPTP influences parkin sulfhydration. Parkin sulfhydration was determined by the maleimide technique in WT, nNOS$^{-/-}$, and iNOS$^{-/-}$mice injected with saline or MPTP and sacrificed at 2 h, 4 h, 24 h, and 48 h after MPTP injection. Following MPTP treatment, parkin sulfhydration in WT mice increases by almost 2 fold at 4 h before returning to baseline. Sulfhydration levels are substantially greater at 2–4 h in iNOS and nNOS mice (n=3 mice for each data point). Statistical significance is as noted $^{**}P<0.01$ and $^{*}P<0.05$ by ANOVA analysis with Tukey HSD post-hoc test. All data expressed as mean ± s.e.m.

a

Sequence	Modification	Observed	Actual Mass	Charge	Cys Number
(R)NDWTVQN**C**DLDQQSIVHIVQRPWR(K)	Sulfhydration (+32)	994.8134	2,981.42	3	C59
(R) NAAGG**C**EREPQSLTR(V)	Sulfhydration (+32)	810.8728	1,619.73	2	C95
(R)QATLTLTQGPS**C**WDDVLIPNR(M)	Sulfhydration (+32)	1,180.58	2,359.15	2	C182
(K) **C**GAHPTSDKETPVALHLIATNSR(N)	Sulfhydration (+32)	613.3078	2,449.20	4	C212
(K)EAYHEGE**C**SAVFEASGTTTQAYR(V)	Sulfhydration (+32)	847.0281	2,538.06	3	C377

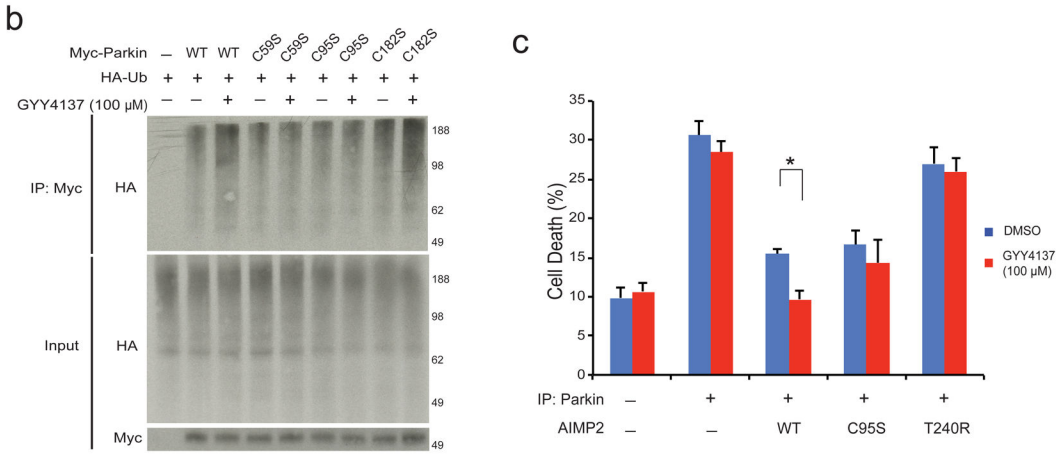

Figure 3. Parkin sulfhydration at specific cysteine residues enhances its protective functions

(**a**) Mass spectrometry detects probable parkin cysteines sulfhydrated by H_2S donor NaHS. (**b**) C59S, C95S, and C182S mutations all substantially reduce parkin activity stimulation by H_2S in HEK293 cells. (**c**) Cell death as monitored by trypan blue exclusion assay in tet-repressible AIMP2 expressing PC12 cells is prevented by parkin overexpression and by GYY4137, whose protective effect is not evident in the absence of parkin, with parkin-C95S or with the T240R catalytically inactive parkin mutant n=3–6 with **P*<0.01 by one-way ANOVA. All data expressed as mean ± s.e.m.

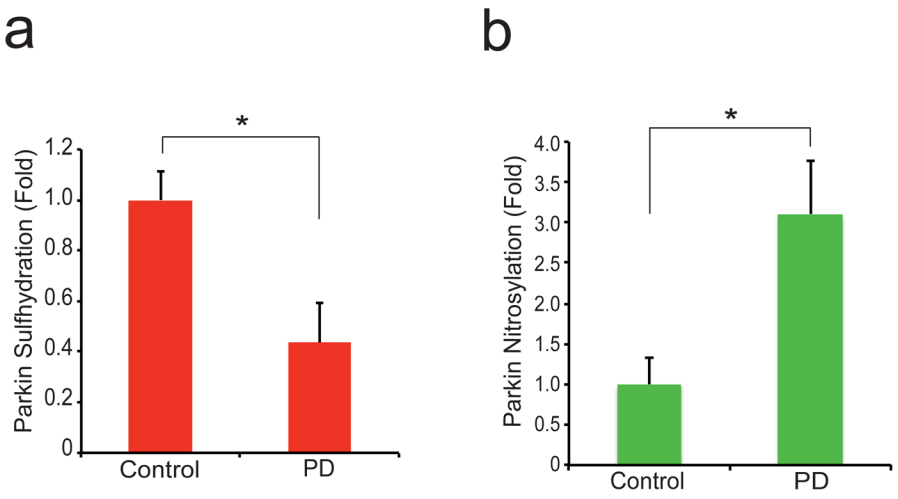

Figure 4. Parkin sulfhydration is decreased in PD brain while nitrosylation is increased in PD

(**a)** The maleimide technique demonstrates that parkin sulfhydration is depleted in the striatum of PD patients. (b) Nitrosylation of parkin is increased in PD patient brain. $P<0.02$ by one-way ANOVA (n=7 control and n=6 PD samples for (**a)**); $P<0.01$ by one-way ANOVA (n=5 each control and PD for (**b**)). All data expressed as mean ± s.e.m.