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S-nitrosylation of laforin inhibits its phosphatase activity and is implicated in Lafora disease

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Abstract

Recently, the relation between *S*-nitrosylation by nitric oxide (NO), which is overproduced under pathological conditions and neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, has become a focus of attention. Although most cases of Parkinson's disease are known to be caused by mutations in the Parkin gene, a recent finding has indicated that *S*-nitrosylation of Parkin affects its enzymatic activity and leads to the Parkinsonian phenotype. Therefore, it is important to understand the function of *S*-nitrosylated proteins in the pathogenesis of neurodegenerative diseases. Lafora disease (LD, OMIM 254780) is a neurodegenerative disease characterized by the accumulation of insoluble glucans called Lafora bodies (LBs). LD is caused by mutations in genes that encode the glucan phosphatase, Laforin, or the E3 ubiquitin ligase, Malin. In this study, we hypothesized that LD may be caused by *S*-nitrosylation of Laforin, which is similar to the finding that Parkinson's disease is caused by *S*-nitrosylation of Parkin. To test this hypothesis, we first determined whether Laforin was *S*-nitrosylated using a biotin switch assay, and compared the three main functions of unmodified and *S*-nitrosylated Laforin, namely glucan- and Malin-binding activity and phosphatase activity. Furthermore, we examined whether the numbers of LBs were changed by NO in the cells expressing wild-type Laforin. Here, we report for the first time that *S*-nitrosylation of Laforin inhibited its phosphatase activity and that LB formation was increased by an NO donor. Our results suggest a possible hypothesis for LD pathogenesis; that is, the decrease in phosphatase activity of Laforin by *S*-nitrosylation leads to increased LB formation. Therefore, LD may be caused not only by mutations in the Laforin or Malin genes, but also by the *S*-nitrosylation of Laforin.

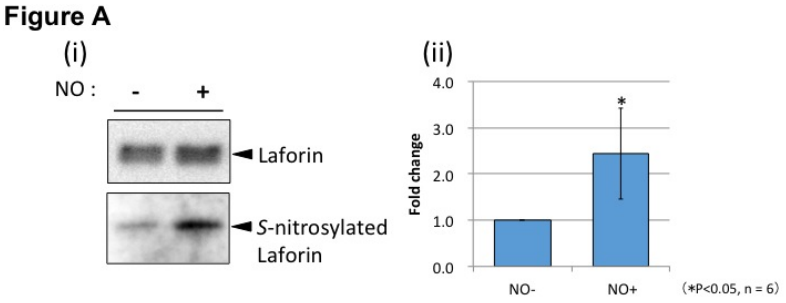
Objective

Lafora disease (LD, OMIM 254780) is a neurodegenerative disease characterized by the accumulation of insoluble glucans called Lafora bodies (LBs) and is caused by mutations in genes that encode the glucan phosphatase, Laforin, or the E3 ubiquitin ligase, Malin. We aimed to test whether *S*-nitrosylation of Laforin affects its phosphatase activity and is thus involved in the disease.

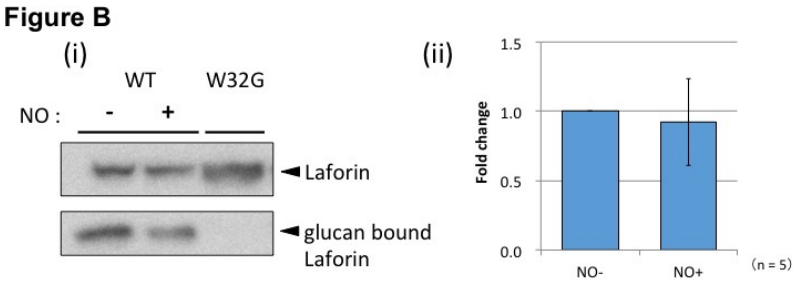
Introduction

A delicate redox state balance is maintained in cells by the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and the antioxidant system that detoxifies them. In a balanced redox state, a low concentration of ROS/RNS is maintained, and ROS/RNS can activate the specific signaling pathways that are required for diverse cellular functions, including cell growth and immune responses [1]. However, overproduction of ROS/RNS or decreased antioxidant capacity can disrupt the redox balance, causing oxidative/nitrosative stress [2]. ROS and RNS are highly reactive free radicals, and one such ROS/RNS is nitric oxide (NO). NO plays important roles in the regulation of neuronal, immune, and cardiovascular systems and is produced in many mammalian cells through a reaction catalyzed from L-arginine by a family of NO synthases [3]. NO has an unpaired electron in its pi molecular orbital, which can react with, for example, proteins, lipids, and DNA [4]. *S*-nitrosylation, a posttranslational modification of cysteine residues on specific proteins, regulates protein function and is the result of the reaction between NO and the protein residues [5] [6]. Protein *S*-nitrosylation has been found in the pathogenesis of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, and is known to contribute to the formation

of the intracellular inclusion bodies associated with neurodegenerative diseases [7] [8] [9]. For example, *S*-nitrosylation of Parkin was found to affect its enzymatic activity and to lead to the Parkinsonian phenotype [7] [8]. Lafora disease (LD, OMIM 254780) is a fatal autosomal recessive neurodegenerative disorder. LD initially manifests during the teenage years with generalized tonic-clonic seizures, myoclonus, absences, drop attacks, and visual hallucinations, and patients usually die within 10 years of the first symptoms [10] [11]. LD is caused by mutations in Epilepsy, Progressive Myoclonus Type 2A or 2B (EPM2A or EPM2B), which encode Laforin, a glucan phosphatase, or Malin, an E3 ubiquitin ligase, respectively [11] [12]. LD is characterized by the accumulation of insoluble glucans, like glycogen, called Lafora bodies (LBs) in the cytoplasm of cells from most tissues [13]. Mutations in EPM2A and EPM2B account for about 80% of the LD families that were screened for genetic lesions [12]. In the remaining families, the role for a third gene was suggested [12]. Because protein *S*-nitrosylation has been found in the pathogenesis of neurodegenerative diseases, *S*-nitrosylation of Laforin may be a third factor in LD pathogenesis. We hypothesized that *S*-nitrosylation of Laforin may affect its enzymatic activity and lead to LD; similar to the finding that *S*-nitrosylation of Parkin affects its enzymatic activity and leads to Parkinson's disease. To test this hypothesis, we first determined whether Laforin is *S*-nitrosylated, using a biotin switch assay, and whether the function of Laforin is affected by *S*-nitrosylation. Furthermore, we observed LB formation in cells expressing wild-type Laforin *S*-nitrosylated by an NO donor.



a



b

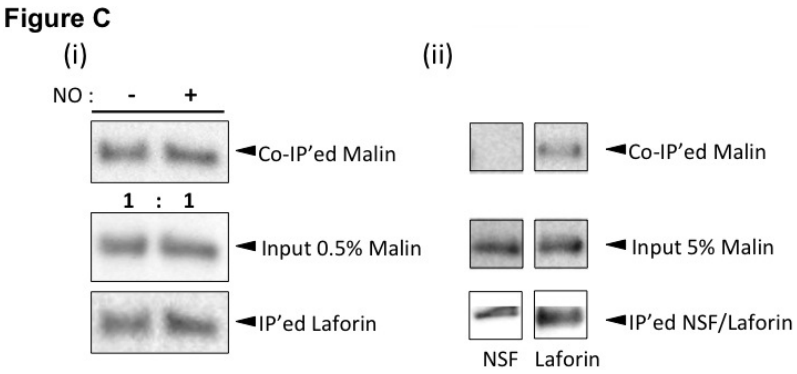


Figure C
(i)
(ii)
NO : - +
Co-IP'ed Malin
1 : 1
Input 0.5% Malin
IP'ed Laforin
Co-IP'ed Malin
Input 5% Malin
IP'ed NSF/Laforin
NSF Laforin

c

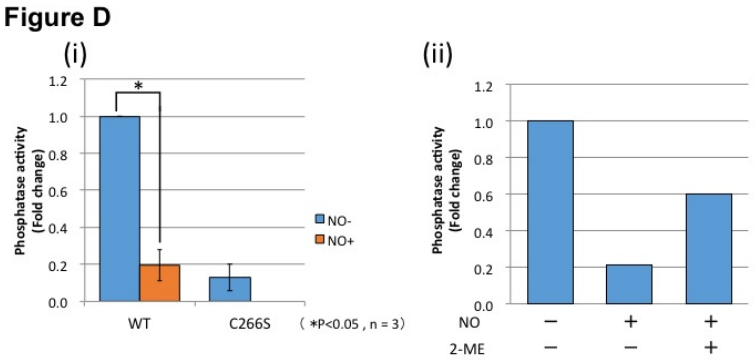


Figure D
(i)
(ii)
Phosphatase activity (Fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NO-
NO+
WT
C266S
(*P<0.05 , n = 3)
NO
2-ME

d

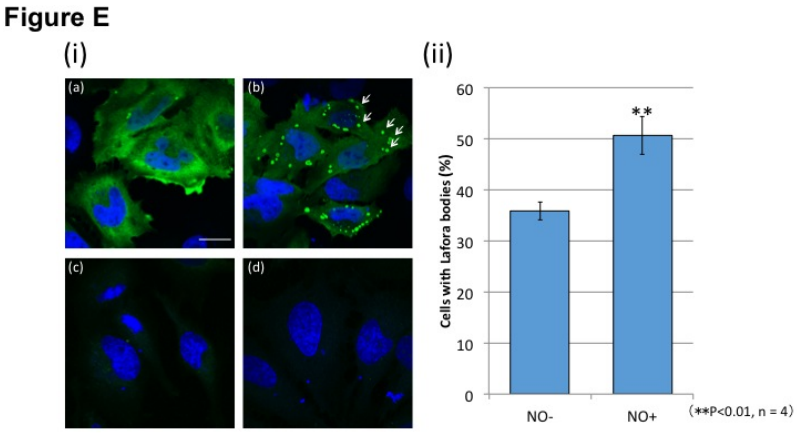
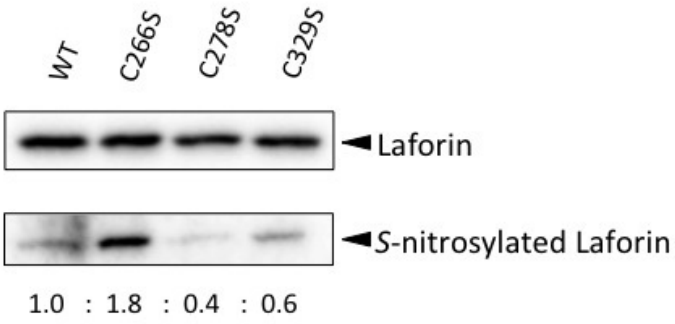


Figure E
(i)
(ii)
(a)
(b)
(c)
(d)
Cells with Lafora bodies (%)
60
50
40
30
20
10
0
**
NO-
NO+
(**P<0.01, n = 4)

e

Figure F



f

Figure Legend

Figure A: Laforin is *S*-nitrosylated by NO (i) Representative western blot of six independent experiments is shown. Upper panel is 0.7% aliquots of total cell lysates; lower panel is isolated *S*-nitrosylated Laforin. (ii) Intensities of the bands in the western blots were quantified. The levels of *S*-nitrosylated Laforin were normalized with those of Laforin. The graph shows the average fold changes with and without CysNO. Error bars, SD (n = 6). *P <0.05.

Figure B: Glucan-binding capacity of *S*-nitrosylated Laforin is maintained (i) Representative western blot of five independent experiments is shown. Upper panel is 1.7% aliquots of total cell lysates; lower panel is glucan-bound Laforin. (ii) Intensities of the bands in the western blot were quantified. The levels of glucan-bound Laforin were normalized with those of Laforin. The graph shows the average fold changes with and without CysNO. W32G is an unbound control. Error bars, SD (n = 5).

Figure C: Malin-binding capacity of *S*-nitrosylated Laforin is maintained (i) Representative western blot of two independent experiments is shown. The "Input 0.5% Malin" is a western blot of 0.5% aliquots of total cell lysates. Intensities of the bands in the western blot were quantified. The normalized ratio of Malin bound to Laforin is shown below the upper blot. (ii) Malin is specifically co-immuneprecipitated by Laforin but not NSF.

Figure D: *S*-nitrosylation of Laforin inhibits its phosphatase activity (i) The absorbance value was normalized with wild-type (WT) treated without CysNO and the C266S mutant treated with CysNO. WT (without CysNO) was set as 1 and C266S (with CysNO) was set as 0. The graph shows the average fold changes with and without CysNO. C266S is a negative control. Error bars, SEM (n = 3). *P <0.05. (ii) The absorbance value was normalized with WT without NO and 2-ME. WT without NO and 2-ME was set as 1. The graph shows the average fold changes.

Figure E: LB formation in cells expressing wild-type Laforin is increased by NO (i) Representative images of (a) untreated or (b) GSNO treated wild-type Laforin-expressing cells are shown. Green, cells stained for Laforin and LB; blue, nuclei. The arrows indicate LBs. (c, d) The same experiments to (a) were performed without primary or secondary antibodies, respectively. Bar, 20 μm. (ii) Numbers of cells expressing Laforin and numbers of cells with LBs were counted. The average of the percent cells with LBs from four independent experiments were calculated and shown in the graph. Error bars, SD (n = 4). **P <0.01.

Figure F: C266S Laforin mutant is highly *S*-nitrosylated by NO Intensities of the bands in the western blot were quantified. The levels of *S*-nitrosylated Laforin were normalized with those of Laforin. The normalized ratios of *S*-nitrosylated Laforin are shown below the blots. Wild-type (WT) was set as 1.0.

Results & Discussion

Results

Laforin is *S*-nitrosylated by an NO donor

We first tested whether Laforin was *S*-nitrosylated by an NO donor using a biotin switch assay. Lysates from HEK293 cells transfected with wild-type Laforin were treated with or without CysNO. After the treatment, free thiol groups were blocked and SNO groups were biotinylated. *S*-nitrosylated proteins were isolated on avidin beads. Both unmodified and *S*-nitrosylated Laforin were detected by western blotting. As shown in Figure A, Laforin was 2.4 times more *S*-nitrosylated in response to the NO donor, which indicates that Laforin was *S*-nitrosylated by NO. Error bars in the graph represent the standard deviation (SD) of 6 independent experiments.

Glucan-binding capacity of *S*-nitrosylated Laforin is maintained

We examined whether Laforin functions were affected by its *S*-nitrosylation under the same conditions used to test for *S*-nitrosylation by NO (see Figure A). The buildup of LBs is caused by loss of Laforin function. In normal glycogen metabolism, an enzymatic error of glycogen synthases can cause phosphate to be incorporated into glycogen. When phospho-glycogens are produced in this way, Laforin and other glycogen phosphorylases bind to glycogen and remove the phosphate. After the dephosphorylation reaction, Laforin dissociates from glycogen by binding to Malin and can then be degraded by a proteasomal system. Loss of function of Laforin and/or Malin can result in the accumulation of unremoved phosphates or Laforin, which disrupts the intricate spherical structure of glycogen and leads to a buildup of LBs [14] [15]. Therefore, glucan- and Malin-binding activity and the phosphatase activity of Laforin are all required to avoid LB accumulation. To examine whether any of these three functions of Laforin are affected by *S*-nitrosylation, we compared the functions of unmodified and *S*-nitrosylated Laforin. For glucan-binding, lysates from HEK293 cells transfected with wild-type Laforin or glucan-binding dead Laforin mutant (W32G) were treated with or without CysNO and incubated with glucan-conjugated beads. Glucan-bound Laforin was analyzed by western blotting. As shown in Figure B, similar amounts of both *S*-nitrosylated Laforin and unmodified Laforin were bound to the glucan. Error bars in the graph represent the SD of 5 independent experiments. These results suggest that the glucan-binding capacity of *S*-nitrosylated Laforin was similar to that of unmodified Laforin.

Malin-binding capacity of *S*-nitrosylated Laforin is maintained

For Malin-binding, lysates from HEK293 cells transfected with wild-type Laforin and wild-type Malin were treated with or without CysNO and co-immunoprecipitated. Malin-binding of Laforin was analyzed by western blotting. As shown in Figure C (i), similar amounts of both *S*-nitrosylated Laforin and unmodified Laforin bound to Malin. These results suggest that the Malin-binding capacity of *S*-nitrosylated Laforin is similar to that of unmodified Laforin. Importantly, *N*-ethylmaleimide-sensitive factor (NSF) did not co-immunopreciptate Malin, indicating that the assays were performed correctly (Figure C (ii)).

S-nitrosylation of Laforin inhibits its phosphatase activity

For phosphatase activity, GST-tagged wild-type Laforin and a phosphatase dead Laforin mutant (C266S; Cys is the catalytic residue) expressed in *E. coli* were purified and treated with or without CysNO. After treatment, phosphatase activity was measured using *p*NPP as a substrate. As shown in Figure D (i), the phosphatase activity of Laforin decreased by 80% in response to the NO donor. Error bars in the graph represent the standard error of the means (SEM) of three independent experiments in triplicates. To test whether the decreased phosphatase activity was caused by *S*-nitrosylation, we used a chemical-reducing agent. Previous reports suggested that protein *S*-nitrosylation was reversible, and the declined enzymatic activity caused by the NO donor could be reversed to the basal level by a chemical-reducing agent [16]. The declined phosphatase activity of wild-type Laforin was reversed to 60% of the basal level after incubation with the chemical-reducing agent 2-mercaptoethanol (2-ME), indicating that *S*-nitrosylation of Laforin was reversible (Figure D (ii)). These results suggest that *S*-nitrosylation of Laforin inhibits its phosphatase activity.

LB formation in cells expressing wild-type Laforin is increased by NO

We observed LB formation in cells expressing wild-type Laforin *S*-nitrosylated by the

NO donor. LBs are seen in most cell types. In this study, we used the HeLa cell line to observe LB formation. The HeLa cells expressing wild-type Laforin were prepared on coverslips and incubated in the presence or absence of GSNO. The number of cells expressing wild-type Laforin and the number of those cells with LB were counted. A representative image of untreated or treated wild-type Laforin-expressing cells is shown in Figure E (i). LBs are insoluble glucans and Laforin can bind to LBs because of its glucan-binding capacity. Inclusion bodies in Figure E (indicated by white arrows) are Laforin-binding aggregates and may be LBs. As shown in Figure E (ii), the cells treated with NO contained 1.4 times more LBs than the untreated cells. Error bars in the graph represent the SD of 4 independent experiments.

Discussion

We hypothesized that Lafora disease (LD) may be caused by *S*-nitrosylation of Laforin because we know that Parkinson's disease can be caused by *S*-nitrosylation of Parkin. To test this hypothesis, we first determined whether Laforin was *S*-nitrosylated using the biotin switch assay and whether the three main functions of Laforin were affected by *S*-nitrosylation. Furthermore, we observed LB formation in cells expressing wild-type Laforin *S*-nitrosylated by NO. For the first time, in this study, we showed that Laforin was *S*-nitrosylated by the NO donor and *S*-nitrosylation of Laforin inhibited its phosphatase activity. Furthermore, we observed that LB formation in cells expressing wild-type Laforin was increased by the NO donor and endogenous NO. LB formation is caused by loss of Laforin phosphatase activity [14]. Based on our results, we propose a possible hypothesis to describe LD pathogenesis; namely, the decrease in Laforin phosphatase activity by *S*-nitrosylation leads to increased LB formation. Laforin has nine cysteine residues that can be *S*-nitrosylated. We speculated that the *S*-nitrosylation site may be C266 (the catalytic residue) because *S*-nitrosylation of Laforin remarkably inhibited its phosphatase activity. To determine the *S*-nitrosylation sites, Laforin mutants with individual cysteine residues replaced with serine were constructed. These mutants were tested to determine whether the mutant Laforin could be *S*-nitrosylated (Supplementary Figure F). However, contrary to our expectation, the C266S mutant Laforin was more *S*-nitrosylated than the wild-type Laforin. This result suggests that replacement of C266 with serine generated artificial *S*-nitrosylation sites, which did not allow us to determine the *S*-nitrosylation site. Further work is needed to determine the Laforin *S*-nitrosylation sites. LD is a fatal autosomal recessive neurodegenerative disorder with generalized tonic-clonic seizures and myoclonus. LD is caused by mutations in EPM2A or EPM2B, which encode Laforin and Malin, respectively. Mutations in EPM2A and EPM2B account for about 80% of the LD families that have been screened for genetic lesions. In the remaining families, the role for a third gene has been suggested [15]. Our results suggest that LD is caused not only by mutations in EPM2A and EPM2B, but also by the *S*-nitrosylation of Laforin. We propose that *S*-nitrosylation of Laforin could be a third factor for LD.

Our data show that the phosphatase activity of Laforin is reduced by nitric oxide, suggesting that LD phenotypes can be caused by nitrosative stress.
Cell culture experiments should be repeated in animal models or neurons.
Test if endogenous NO would affect LB formation
Test if NO treatment would affect degradation of Laforin-Malin complex
Test if NO treatment would affect ubiquitination of Laforin

Additional Information

Methods and Supplementary Material

Please see https://sciencematters.io/articles/201606000014.

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Ethics Statement

Not applicable

Citations

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