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# Phosphorylation of p66shc mediates 6-hydroxydopamine cytotoxicity

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**Abstract**

6-Hydroxydopamine (6-OHDA) is a neurotoxin that has been widely used to generate Parkinson's disease (PD) models. Increased oxidative stress is suggested to play an important role in 6-OHDA-induced cell death. Given the lessened susceptibility to oxidative stress exhibited by mice lacking p66shc, this study investigated the role of p66shc in the cytotoxicity of 6-OHDA. 6-OHDA induced cell death and p66shc phosphorylation at Ser36 in SH-SY5Y cells. Pre-treatment with the protein kinase C $\beta$ (PKC$\beta$) inhibitor hispidin suppressed 6-OHDA-induced p66shc phosphorylation. Elimination of $H_2O_2$ by catalase reduced cell death and p66shc phosphorylation induced by 6-OHDA. Cells deficient in p66shc were more resistant to 6-OHDA-induced cell death than wild-type cells. Furthermore, reconstitution of wild-type p66shc, but not the S36A mutant, in p66shc-deficient cells increased susceptibility to 6-OHDA. These results indicate that $H_2O_2$ derived from 6-OHDA is an important mediator of cell death and p66shc phosphorylation induced by 6-OHDA and that p66shc phosphorylation at Ser36 is indispensable for the cytotoxicity of 6-OHDA.

**Keywords:** *Oxidative stress, cell death, 6-hydroxydopamine, Parkinson's disease, signal transduction*



## Introduction

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorders, affecting ~ 3% of the population over 65 years old [1]. The main hallmark of this disease is a selective loss of dopaminergic neurons in the substantia nigra. Although the aetiology of the disease is not completely understood, it is thought that oxidative stress is an important mediator in its pathogenesis [2,3]. Nigral dopaminergic neurons are rich in reactive oxygen species (ROS), such as superoxide, hydrogen peroxide ($H_2O_2$) and hydroxyl radicals, as a result of auto-oxidation of dopamine [4]. The levels of oxidation products of lipids, proteins and DNA are increased in the substantia nigra of PD patients [5]. Furthermore, it has also been reported that high intake of dietary antioxidants lowers the risk of PD [6]. Based on this evidence, excessive ROS production and consequent oxidative damage are believed to play a role in the pathogenesis of PD.

6-Hydroxydopamine (6-OHDA) is a selective catecholaminergic neurotoxin that has been widely used to generate PD models *in vitro* and *in vivo* [7]. It is known to induce toxicity that mimics the neuropathological and biochemical characteristics of PD. 6-OHDA has been demonstrated to induce cytotoxicity in a wide range of *in vitro* neuronal models such as PC12 and SH-SY5Y cells [8–10]. 6-OHDA is rapidly oxidized by molecular oxygen through auto-oxidation to yield superoxide, $H_2O_2$ and 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone (*p*-quinone) as follows [11]:

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$$\text{6-OHDA} + O_2 \rightarrow \textit{p}\text{-quinone} + H_2O_2$$

It is thought that ROS generated from 6-OHDA elicit cellular oxidative stress. However, 6-OHDA-induced cell death is also mediated by *p*-quinone [12]. Although these products generated from 6-OHDA have been suggested to promote cell death, the molecular mechanism underlying 6-OHDA-induced cytotoxicity has not been completely elucidated.

Shc (src homology containing) proteins were originally identified as adaptor molecules involved in signal transduction from receptor tyrosine kinases to the Ras-MAPK pathway [13,14]. ShcA, the most ubiquitously expressed shc, consists of three isoforms; p66shc, p52shc and p46shc. ShcA proteins are phosphorylated at tyrosine residues in response to stimulation by a variety of growth factors and cytokines [15–17]. The p52 and p46 isoforms transmit signals from receptor tyrosine kinases to the Ras-MAPK pathway by forming a stable complex involving Grb2 and Sos [18]. However, the p66shc isoform bears a unique N-terminal collagen homology 2 (CH2) domain and thereby appears to be functionally distinct from the p52/p46 isoforms. Mice deficient in all three shcA isoforms are embryonic lethal [19]. In contrast, mice selectively deficient for p66shc live 30% longer than control animals and cells derived from these mice are resistant to ROS-induced cell death [20]. Unlike other isoforms, p66shc undergoes serine phosphorylation mainly at Ser36 in cells exposed to oxidative stresses such as UV irradiation and $H_2O_2$ treatment [20–22]. Phosphorylation of p66shc at Ser36 is required to confer increased susceptibility to oxidative stress and is critical for the cell death response elicited by oxidative damage [23,24]. Therefore, this phosphorylation may be involved in the pathogenesis of diseases associated with oxidative damage. Although it is known that p66shc plays an important role in determining cellular susceptibility to oxidative stress in a variety of cell types, its role in neuronal cells has not been fully examined.

In this study we aimed to investigate the role of p66shc in 6-OHDA-induced neuronal cell death to gain insight into the molecular mechanisms underlying the cytotoxicity of 6-OHDA.

## Materials and methods

### *Materials*

6-OHDA, puromycin dihydrochloride and catalase were purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), $H_2O_2$ and polyvinylpyrrolidone were from Nacalai Tesque (Kyoto, Japan). Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12) and Dulbecco's modified Eagle medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). An anti-phospho-p66shc (Ser36) antibody, SP600125, PD98059, SB203580 and hispidin were purchased from Merck Biosciences (Darmstadt, Germany). Polybrene and Immobilon Western Chemiluminescent HRP Substrate were from Millipore (Billerica, MA). An anti-actin antibody and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-shc antibody was obtained from BD Biosciences (San Jose, CA). Plat-E cells [25] and pMXs-IP vector [26] were generous gifts from Dr Toshio Kitamura (University of Tokyo, Tokyo, Japan). Spontaneously immortalized p66shc(+/+) and p66shc(−/−) mouse embryonic fibroblasts (MEFs) were kindly provided by Dr Toren Finkel (National Heart, Lung, and Blood Institute, Bethesda, MD). Other reagents were obtained from Wako Pure Chemical Industries (Tokyo, Japan) unless otherwise stated.

### *Cell culture*

SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Manassas, VA) were grown in DMEM/F12 supplemented with 10% FBS (Hyclone) and antibiotics (0.05 U/ml penicillin, 0.05 μg/ml streptomycin; Invitrogen) at 37°C in 5% $CO_2$/95% air. Spontaneously immortalized p66shc(+/+) and p66shc (−/−) MEFs were maintained in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% $CO_2$/95% air.

### *Retroviral vectors*

To generate a pMXs-IP vector carrying wild-type human p66shc (p66 WT) and a p66shc mutant in which Ser36 was substituted into Ala (p66 S36A), cDNAs for p66 WT and p66 S36A were sub-cloned into the *Eco*RI site of a pMXs-IP plasmid. Constructs were sequenced to confirm their identities.

The day before transduction, Plat-E cells were seeded at $2 \times 10^6$ cells per 60 mm dish. On the next day, the pMXs-IP plasmid was introduced into Plat-E cells using Fugene 6 transfection reagent (Roche) according to the manufacturer's protocol. After adding transfection mixture, cells were then cultured overnight. Twenty-four hours after transfection, the culture medium was replaced with 4 ml of fresh DMEM and cells were cultured for another 24 h. The virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45 μm cellulose acetate filter and stored at –80°C.

### *Generation of stable polyclonal cell lines*

p66shc(–/–) MEFs were seeded at $6 \times 10^4$ cells/well in a 24-well plate and cultured overnight. Cells were incubated in the virus-containing supernatants

supplemented with 8 μg/ml polybrene for 6 h, followed by the addition of 0.5 ml of fresh DMEM. On the next day, the medium was replaced with 1.5 ml of fresh DMEM and cells were cultured overnight. After infection, the cells were then replated in a 100 mm dish. Three days after infection, 4 μg/ml puromycin was added to select cells bearing puromycin resistance.

### *Drug treatment*

After cells were serum-starved for 16–24 h, they were stimulated with either $H_2O_2$ or 6-OHDA as described elsewhere. Where indicated, the following kinase inhibitors were applied prior to 6-OHDA stimulation: SP600125 (a JNK inhibitor), PD98059 (an ERK inhibitor), SB203580 (a p38 MAPK inhibitor) and hispidin (a PKC$\beta$ inhibitor). In preliminary experiments, we evaluated the cytotoxicities of these inhibitors in SH-SY5Y cells by MTT assay and determined the treatment conditions that caused no reduction in cell viability by them. When catalase was utilized, SH-SY5Y cells were treated with 50 U/ml catalase for 10 min prior to either $H_2O_2$ or 6-OHDA treatment.

### *Cell viability*

For the determination of SH-SY5Y cell viability, MTT assay was conducted. Following treatment, cells were incubated with 0.5 mg/ml MTT in fresh medium at 37°C for 4 h. Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2, by volume) and it was mixed by pipetting until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a plate reader.

For MEFs, trypan blue exclusion was used to measure cell death after 6-OHDA exposure by counting the number of blue-stained cells in three random fields.

### *Western blotting*

Following treatment, cells were washed with PBS and suspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.5% Triton X-100, 1 mM $Na_3VO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 50 mM $\beta$-glycerophosphate disodium salt) for 30 min on ice. After centrifugation at 15 000 x g for 15 min at 4°C, supernatants were collected. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 0.5% casein/1% PEG 4000/1% polyvinylpyrrolidone in TBST (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 0.1% Tween 20) (for the phospho-p66shc antibody) or 5% non-fat dried milk in TBST (for other antibodies). After being probed with the appropriate primary and secondary antibodies diluted in 5% non-fat dried milk/TBST, the bound antibodies were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

### *Statistical analysis*

All results were expressed as the mean ± SD of at least three separate experiments. Statistical analyses were performed with Student's *t*-test or ANOVA using Tukey tests for multiple comparisons. The minimum level of significance was set at $p < 0.05$.

## Results

### *6-OHDA induces cell death in SH-SY5Y cells*

To determine the cytotoxicity of 6-OHDA in SH-SY5Y cells, we first measured cell viability after 6-OHDA treatment by MTT assay. As shown in Figure 1, cell viability was significantly reduced by 6-OHDA in a concentration-dependent manner. Decreases in cell viability were observed when cells were treated with 6-OHDA at concentrations greater than 50 μM, consistent with previous studies [8,9].

### *6-OHDA induces p66shc phosphorylation at serine 36*

Since various stresses including $H_2O_2$ have been shown to elicit p66shc phosphorylation at Ser36 in different cell lines [20,21,27], we first examined if SH-SY5Y cells carried p66shc that could be phosphorylated at Ser36 after stimulation. $H_2O_2$ treatment (500 μM, 15 min) caused a substantial increase in p66shc phosphorylation at Ser36 in SH-SY5Y cells (Figure 2A, left). When SH-SY5Y cells were treated with various concentrations of 6-OHDA, we found a

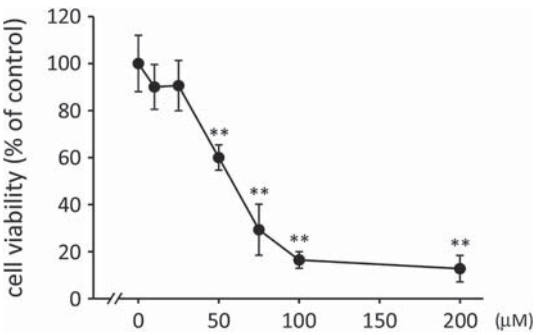


Figure 1. Effect of 6-OHDA on the viability of SH-SY5Y cells. After cells were serum-starved for 16 h, they were treated with various concentrations of 6-OHDA for 24 h. Cell viability was determined by MTT assay as described in the text. Data are expressed as means ± SD of nine experiments. $^{**}p < 0.01$ vs control (Tukey, ANOVA).

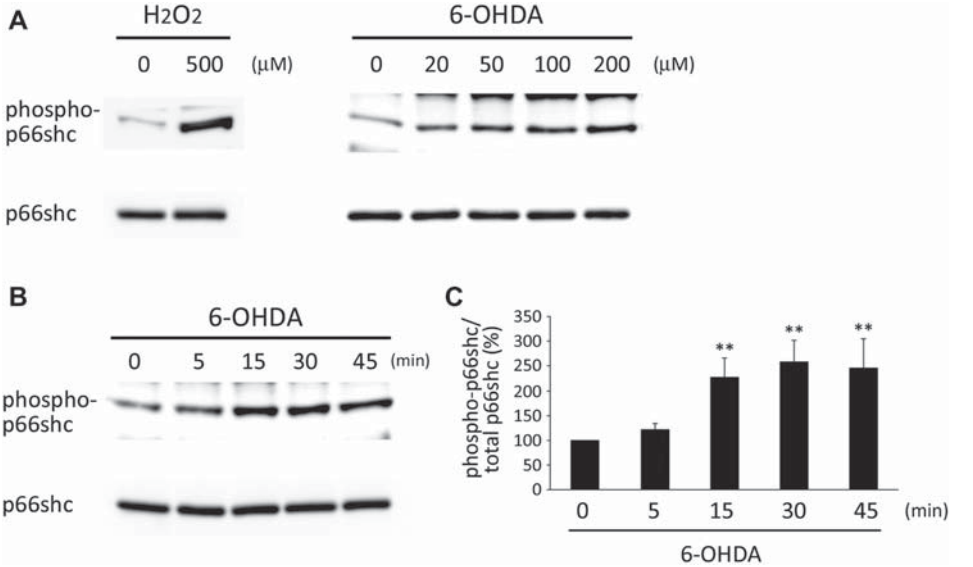


Figure 2. Phosphorylation of p66shc at Ser36 in SH-SY5Y cells. (A) SH-SY5Y cells were treated with 500 μM $H_2O_2$ (left) or 6-OHDA at various concentrations (right) for 15 min. Cells were harvested and cell lysates were subjected to Western blot analysis using antibodies against phospho-p66shc (Ser36) or shc. (B) SH-SY5Y cells were treated with 100 μM 6-OHDA for the indicated times. Cells were harvested and cell lysates were subjected to Western blot analysis using antibodies against phospho-p66shc (Ser36) or shc. The blot is representative of four experiments. (C) Bands corresponding to phospho-p66shc and total p66shc were quantified and intensities of phospho-p66shc bands were normalized to intensities of total p66shc bands. Data are expressed as means ± SD of four experiments. $^{**}p < 0.01$ vs 0 min (Student's *t*-test).

concentration-dependent increase of p66shc phosphorylation at Ser36 without any change in the p66shc expression level (Figure 2A, right). We next conducted a time-course study of p66shc phosphorylation by 6-OHDA in SH-SY5Y cells. After 6-OHDA treatment, significant phosphorylation was observed at 15 min and remained elevated until 45 min, whereas the expression level of p66shc was unaffected (Figures 2B and C). These results showed that 6-OHDA rapidly induced p66shc phosphorylation at Ser36 in SH-SY5Y cells.

### *PKCβ mediates 6-OHDA-induced p66shc phosphorylation*

Recently, several lines of evidence have indicated signalling proteins that regulate p66shc phosphorylation at Ser36, including protein kinases such as JNK, ERK, p38MAPK and PKC*β* [21,28–30]. To investigate whether these kinases are involved in p66shc phosphorylation after 6-OHDA treatment, we tested the effects of pharmacological kinase inhibitors on 6-OHDA-induced p66shc phosphorylation. Figure 3A shows the results for SH-SY5Y cells pre-treated with 5 μM SP600125, 50 μM PD98059, 20 μM SB203580 or vehicle (DMSO) in serum-free medium, followed by 100 μM 6-OHDA treatment for 15 min. Cells were harvested and cell lysates were subjected to Western blot analysis using antibodies against phospho-Ser36-p66shc and total shc. 6-OHDA-stimulated p66shc phosphorylation at Ser36 was not reduced by any of these pharmacological kinase inhibitors. We next evaluated the effect of hispidin, a specific inhibitor of the PKC*β* isoform [31], on 6-OHDA-induced p66shc phosphorylation. SH-SY5Y cells were pre-treated with vehicle (DMSO) or 20 μM hispidin prior to 100 μM 6-OHDA treatment for 15 min. As shown in Figure 3B, although hispidin did not alter the basal phosphorylation level of p66shc, it diminished p66shc phosphorylation induced by 6-OHDA. These results showed that PKC*β*, but not JNK, ERK or p38MAPK, regulated p66shc phosphorylation in response to 6-OHDA in SH-SY5Y cells.

### *$H_2O_2$ derived from 6-OHDA causes cell death and mediates 6-OHDA-induced p66shc phosphorylation in SH-SY5Y cells*

Previous studies have shown that 6-OHDA is readily oxidized in the presence of oxygen to form $H_2O_2$ and *p*-quinone and the cytotoxicity of 6-OHDA is mediated by $H_2O_2$-dependent and -independent mechanisms [7,8,10,12]. To clarify the involvement of $H_2O_2$ derived from 6-OHDA in neuronal cell death, we evaluated the effect of catalase, a $H_2O_2$-decomposing enzyme, on cell viability after the treatment of SH-SY5Y cells with 6-OHDA. As we expected, $H_2O_2$ treatment caused cell death in a concentration-dependent manner, which was prevented by catalase (Figure 4). We next examined the effect of catalase on the cytotoxicity of 6-OHDA in SH-SY5Y cells and found that catalase protected SH-SY5Y cells from 6-OHDA-induced cell death. These results indicated that $H_2O_2$

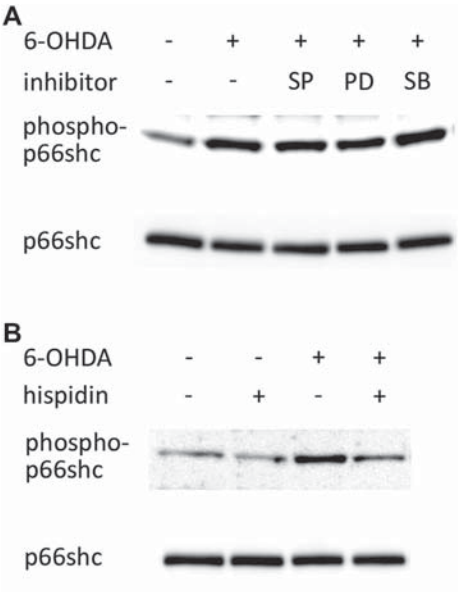


Figure 3. Effects of kinase inhibitors on the 6-OHDA-induced p66shc phosphorylation in SH-SY5Y cells. (A) SH-SY5Y cells were treated with vehicle (DMSO), 5 μM SP600125 (SP), 50 μM PD98059 (PD) or 20 μM SB203580 (SB) for 2 h in serum-free medium, followed by 100 μM 6-OHDA treatment for 15 min. Cells were harvested and cell lysates were subjected to Western blot analysis using antibodies against phospho-p66shc (Ser36) or shc. (B) SH-SY5Y cells were treated with or without 20 μM hispidin for 18 h in serum-free medium, followed by 100 μM 6-OHDA treatment for 15 min. Cells were harvested and cell lysates were subjected to Western blot analysis using antibodies against phospho-p66shc (Ser36) or shc. The blots are representative of three experiments.

derived from 6-OHDA was responsible for cell death in SH-SY5Y cells.

To determine if $H_2O_2$ derived from 6-OHDA was also responsible for p66shc phosphorylation after 6-OHDA treatment, we tested the effect of catalase

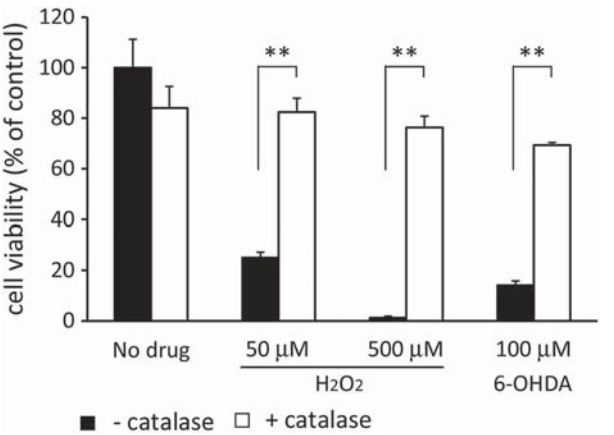


Figure 4. Effects of catalase on 6-OHDA and $H_2O_2$ cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were incubated with or without 50 U/ml catalase for 10 min in serum-free medium. Cells were then treated with $H_2O_2$ (50, 500 μM) or 6-OHDA (100 μM) for 24 h. Cell viability was determined by MTT assay as described in the text. Data are expressed as means ± SD of nine experiments. $^{**}p < 0.01$ (Tukey, ANOVA).

on p66shc phosphorylation at Ser36 after 6-OHDA stimulation. SH-SY5Y cells pre-incubated with 50 U/ml catalase were treated with 100 μM 6-OHDA or 50 μM $H_2O_2$ for 15 min. 6-OHDA, like $H_2O_2$, induced p66shc phosphorylation, which was diminished by catalase (Figures 5A and B). Therefore, $H_2O_2$ derived from 6-OHDA mediated p66shc Ser36 phosphorylation in 6-OHDA-treated SH-SY5Y cells.

### *p66shc is required for 6-OHDA-induced cell death*

Recent studies have shown that p66shc increases the susceptibility of cells to various stresses such as $H_2O_2$ treatment and UV irradiation [20,22,32,33]. We thus hypothesized that p66shc was involved in cell death induced by 6-OHDA. To test this hypothesis, we utilized mouse embryonic fibroblasts (MEFs) isolated from wild-type (WT) or p66shc-null (p66(–/–)) mice. Western blot analysis of cell extracts from WT MEFs and p66(–/–) MEFs confirmed the absence of p66shc in p66(–/–) MEFs although other shc isoforms (p52 and p46) were expressed in both types of MEFs (Figure 6A). After both types of MEFs were serum-starved and treated with 100 μM 6-OHDA, cell death was evaluated by the trypan blue exclusion test. As shown in Figure 6B, 6-OHDA caused cell

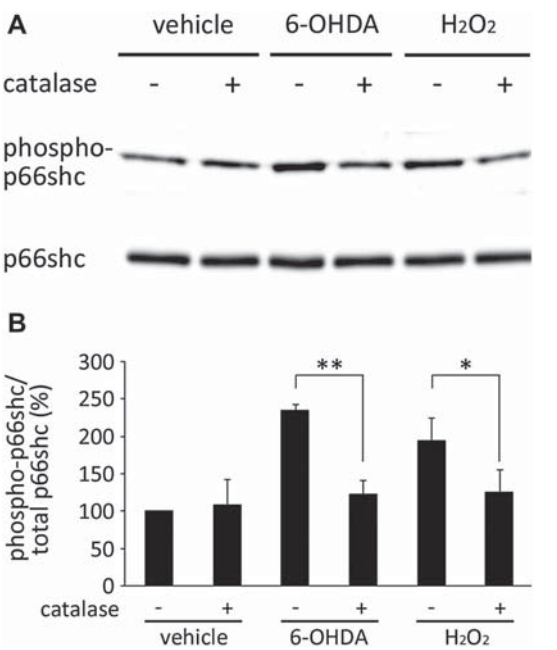


Figure 5. Effect of catalase on p66shc phosphorylation. SH-SY5Y cells were incubated with or without 50 U/ml catalase for 10 min in serum-free medium. Cells were then treated with 50 μM $H_2O_2$ or 100 μM 6-OHDA for 15 min. They were harvested and cell lysates were subjected to Western blot analysis using antibodies against phospho-p66shc (Ser36) or shc. (A) Representative blots of three separate experiments. (B) Bands corresponding to phospho-p66shc and total p66shc were quantified and intensities of phospho-p66shc bands were normalized to intensities of total p66shc bands. Data are expressed as means ± SD of three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ (Student's *t*-test).

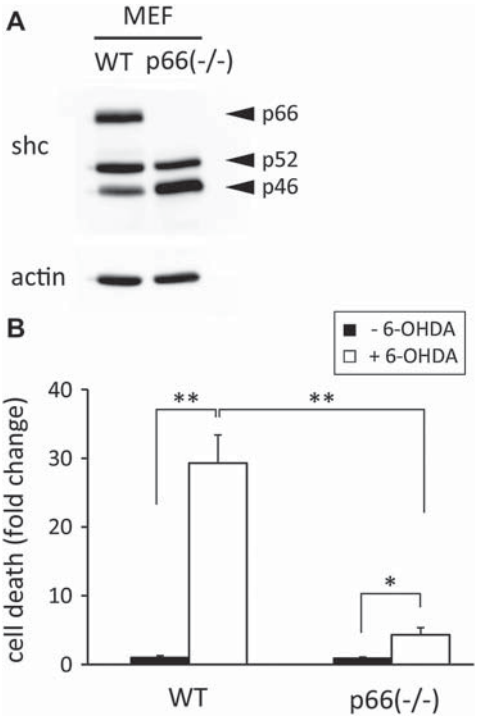


Figure 6. Effect of p66shc deletion on 6-OHDA-induced cell death. (A) Western blot analysis of shc isoform expression in spontaneously immortalized fibroblasts derived from wild-type mice (WT) or from p66shc-null mice (p66(–/–)). (B) Immortalized fibroblasts derived from wild-type mice (WT) or from p66shc-null mice (p66(–/–)) were treated with 100 μM 6-OHDA for 6 h after serum-starvation for 16 h. Cell death was evaluated by the trypan blue exclusion test. Data are expressed as means ± SD of six experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ (Tukey, ANOVA).

death in WT MEFs similar to SH-SY5Y cells. In contrast, p66(–/–) MEFs were resistant to its cytotoxicity. This showed that p66shc mediated 6-OHDA-induced cell death.

### *Phosphorylation of p66shc at serine 36 is indispensable for 6-OHDA-induced cytotoxicity*

6-OHDA induced p66shc phosphorylation at Ser36 in WT MEFs (data not shown) as well as in SH-SY5Y cells (Figure 2). To investigate the functional consequences of the p66shc phosphorylation at Ser36 by 6-OHDA, we established p66(–/–) MEFs over-expressing wild-type p66shc (p66shc WT) and a serine 36-to-alanine non-phosphorylatable mutant of p66shc (p66shc S36A) by transfecting p66shc WT and p66shc S36A into p66(–/–) MEFs, respectively. Western blot analysis of cell extracts from transfected p66(–/–) MEFs confirmed the expression of p66shc into p66(–/–) MEFs (Figure 7A). Transfected p66shc(–/–) MEFs were treated with 100 μM 6-OHDA for 16 h and cell death was evaluated by the trypan blue exclusion test. As shown in Figure 7B, p66(–/–) cells over-expressing p66shc WT were susceptible to 6-OHDA, whereas p66(–/–) cells over-expressing p66shc S36A, as well as an empty vector, were resistant to 6-OHDA-induced cell death. This result showed that p66shc phosphorylation at Ser36 was indispensable for 6-OHDA-induced cytotoxicity.

## Discussion

In this study, we showed that 6-OHDA induced cell death and p66shc phosphorylation at Ser36 through, at least in part, PKC$\beta$ activation. Elimination of $H_2O_2$ by catalase reduced cell death and p66shc phosphorylation induced by 6-OHDA. Cells deficient in p66shc were more resistant to 6-OHDA-induced cell death than wild-type cells. Furthermore, reconstitution of wild-type p66shc, but not the S36A non-phosphorylatable p66shc mutant, in p66shc-deficient cells increased the susceptibility to 6-OHDA. These results indicated that $H_2O_2$ derived from 6-OHDA was an important mediator of cell death and p66shc phosphorylation at Ser36 induced by 6-OHDA and that p66shc phosphorylation at Ser36 was indispensable for 6-OHDA-induced cytotoxicity.

Previous studies have shown that 6-OHDA causes cell death in $H_2O_2$-dependent and -independent manners [7,8,10,12]. 6-OHDA generates $H_2O_2$ and *p*-quinone through auto-oxidation and both are cytotoxic in

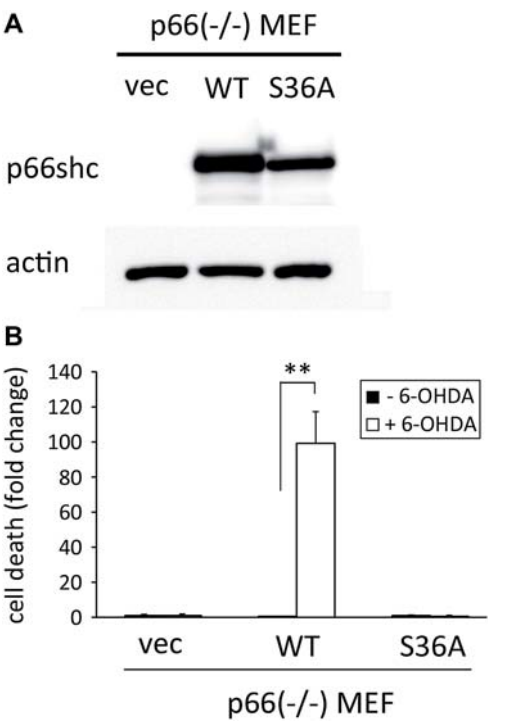


Figure 7. Effect of ectopic p66shc expression on 6-OHDA-induced cell death in p66shc(–/–) MEFs. (A) Western blot analysis of p66shc expression in transfected p66shc(–/–) MEFs. p66shc(–/–) MEFs were retrovirally transfected with vector (vec), wild-type human p66shc (WT) or a p66shc mutant in which Ser36 was substituted into Ala (S36A). (B) Transfected p66shc(–/–) MEFs were treated with 100 μM 6-OHDA for 16 h. Cell death was evaluated by the trypan blue exclusion test. Data are expressed as means ± SD of six experiments. $^{**}p < 0.01$ (Tukey, ANOVA).

various cells [7,8,10,12]. In this study, we showed that elimination of $H_2O_2$ by catalase effectively inhibited 6-OHDA-induced cell death and p66shc phosphorylation. Therefore, $H_2O_2$ was primarily responsible for the cytotoxic effect of 6-OHDA in SH-SY5Y cells.

There have been many studies that have established a role for p66shc in cell death. Migliaccio et al. [20] demonstrated that p66shc(–/–) MEFs were more resistant to the cell death induced by $H_2O_2$ treatment and UV irradiation than WT MEFs. The absence of p66shc prevents cell death after stresses such as acute ischemia, hyperglycaemia and angiotensin II infusion [34–36]. It is proposed that p66shc promotes the opening of permeability transition pores in mitochondria, reduction of mitochondrial membrane potential ($\Delta\Psi_m$) and release of apoptosis-inducing proteins such as cytochrome c into cytosol, resulting in apoptosis [24,37,38]. In addition, previous studies have shown that 6-OHDA activates mitochondrial apoptosis signals [8–10,39]. Considering these findings together with the data in this study, it seems likely that the absence of p66shc in p66(–/–) MEFs prevents apoptosis induced by 6-OHDA.

In the present study, 6-OHDA treatment resulted in p66shc phosphorylation at Ser36 in SH-SY5Y cells. We also observed that 6-OHDA induced p66shc phosphorylation in WT MEFs (data not shown). Various stresses, including $H_2O_2$ treatment, have been shown to cause p66shc phosphorylation at Ser36 [20–22]. Although the kinases that phosphorylate p66shc at Ser36 have not been exhaustively catalogued, the involvement of MAPKs (ERK, JNK and p38 MAPK) and PKC$\beta$ has been suggested [21,28–30]. To examine whether any of these kinases were responsible for 6-OHDA-induced p66shc phosphorylation, we tested the effects of kinase inhibitors (SP600125 for JNK, PD98059 for ERK, SB203580 for p38MAPK and hispidin for PKC$\beta$) on p66shc phosphorylation after 6-OHDA treatment. We found that only hispidin inhibited p66shc phosphorylation, suggesting that PKC$\beta$ played a role in 6-OHDA-induced p66shc phosphorylation.

Our data demonstrated that reconstitution of wild-type p66shc, but not the S36A non-phosphorylatable p66shc mutant, in p66shc-deficient cells increased susceptibility to 6-OHDA. Other studies have also reported that p66shc phosphorylation at Ser36 has an important role in determining the susceptibility of cells to oxidative stress [33,35,40]. However, it is still unclear how p66shc phosphorylation is translated into the cell death signal. Recent studies have suggested a functional link between p66shc phosphorylation and forkhead transcription factors. Foxo3a/FKHRL1 preferentially induces anti-oxidant and survival genes such as Mn-SOD, but this activity is diminished by its phosphorylation [41]. Ser36-phosphorylated p66shc promotes Foxo3a/FKHRL1 phosphorylation through Akt activation, indicating that p66shc phosphorylation at Ser36 results in the decline of cellular antioxidant and survival activities [42]. In addition, Pinton et al. [30] recently showed that p66shc phosphorylation at Ser36 led to translocation of p66shc to mitochondria. Since another study demonstrated that mitochondrial p66shc interacted with cytochrome c in the mitochondrial intermembrane space to produce $H_2O_2$, which can promote the opening of the mitochondrial permeability transition pores [37], p66shc phosphorylated at Ser36 could enhance cell death by controlling the mitochondrial oxidative stress level.

To date, the function of p66shc in neuronal cells remains elusive. Berry et al. [43] recently demonstrated that basal levels of brain-derived neurotrophic factor (BDNF) in the hippocampus in p66shc-knockout mice were elevated as compared to those of wild-type mice, whereas the level of 15-$F_{2t}$-isoprostane, a marker of oxidative stress, in the same brain area was reduced. This was associated with better cognitive performance in p66shc-knockout mice as well. In addition, Smith et al. [29] have indicated that p66shc is essential for cytotoxicity of amyloid $\beta$-peptide, which plays an important role in neuronal loss in Alzheimer's disease by promoting intracellular ROS production. They also suggested that p66shc phosphorylation at Ser36 played a critical role in this process. These findings, combined with our study, suggest that p66shc plays an important role in determining the oxidative stress levels and fate of neuronal cells. Therefore, it is possible that p66shc contributes to the pathology of neurodegenerative diseases such as PD and Alzheimer's disease by influencing the oxidative stress levels in neuronal cells.

In conclusion, the present study shows that $H_2O_2$ derived from 6-OHDA makes a significant contribution to 6-OHDA-induced neuronal cell death and reveals that 6-OHDA-induced phosphorylation of p66shc at Ser36 is indispensable for the cytotoxicity of 6-OHDA. Considering the critical role played by p66shc in the induction of oxidative stress-related responses in various models and the importance of oxidative stress in the process of dopaminergic neuronal cell death, which leads to PD *in vivo*, our results may imply a role for p66shc as a regulator of neuronal cell death in the pathogenesis of PD.

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## Declaration of interest

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