trans-(−)-ε-Viniferin Increases Mitochondrial Sirtuin 3 (SIRT3), Activates AMP-activated Protein Kinase (AMPK), and Protects Cells in Models of Huntington Disease*

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Background: Mitochondrial dysfunction is a key event mediating mutant Htt-induced neurotoxicity.
Results: trans-(−)-ε-Viniferin attenuates mutant Htt-induced SIRT3 depletion, activates AMPK, and preserves mitochondrial function.
Conclusion: Increasing SIRT3 protects cells in HD.
Significance: The result suggests a promising new target for development of HD therapeutics.

Huntington disease (HD) is an inherited neurodegenerative disorder caused by an abnormal polyglutamine expansion in the protein huntingtin (Htt). Currently, no cure is available for HD. The mechanisms by which mutant Htt causes neuronal dysfunction and degeneration remain to be fully elucidated. Nevertheless, mitochondrial dysfunction has been suggested as a key event mediating mutant Htt-induced neurotoxicity because neurons are energy-demanding and particularly susceptible to energy deficits and oxidative stress. SIRT3, a member of sirtuin family, is localized to mitochondria and has been implicated in energy metabolism. Notably, we found that cells expressing mutant Htt displayed reduced SIRT3 levels. trans-(−)-ε-Viniferin (viniferin), a natural product among our 22 collected naturally occurring and semisynthetic stilbene compounds, significantly attenuated mutant Htt-induced depletion of SIRT3 and protected cells from mutant Htt. We demonstrate that viniferin decreases levels of reactive oxygen species and prevents loss of mitochondrial membrane potential in cells expressing mutant Htt. Expression of mutant Htt results in decreased deacetylase activity of SIRT3 and further leads to reduction in cellular NAD+ levels and mitochondrial biogenesis in cells. Viniferin activates AMP-activated kinase and enhances mitochondrial biogenesis. Knockdown of SIRT3 significantly inhibited viniferin-mediated AMP-activated kinase activation and diminished the neuroprotective effects of viniferin, suggesting that SIRT3 mediates the neuroprotection of viniferin. In conclusion, we establish a novel role for mitochondrial SIRT3 in HD pathogenesis and discovered a natural product that has potent neuroprotection in HD models. Our results suggest that increasing mitochondrial SIRT3 might be considered as a new therapeutic approach to counteract HD, as well as other neurodegenerative diseases with similar mechanisms.

Huntington disease (HD)3 is an autosomal dominant, progressive neurodegenerative disorder characterized by psychiatric manifestations, cognitive decline, and movement abnormalities (1). The causative gene mutation for HD is an unstable CAG trinucleotide repeat sequence encoding a polyglutamine tract in the huntingtin (Htt) protein resulting in neuronal dysfunction and neuronal death predominantly in the striatum and cortex (2). Neither the normal function of Htt nor the mechanism whereby polyglutamine expansion results in selective loss of striatal neurons is fully understood, although impaired energy metabolism (3), oxidative stress (4), excitotoxicity (5), and transcriptional dysregulation (6) are implicated. Cells expressing polyglutamine-expanded full-length or N-terminal fragments of Htt exhibited increased oxidative stress and mitochondrial dysfunction (7). Abnormalities in mitochondrial function have been observed in postmortem HD brains (8–10) and cells from HD patients (11, 12). In addition, mitochondrial respiration and ATP production are significantly impaired in striatal cells expressing mutant Htt (13). More recent data suggest that mitochondrial dysfunction is a key event mediating mutant Htt-induced neurotoxicity (14).

SIRT3, a sirtuin family member, is located to mitochondria and is highly expressed in metabolically active tissues including brain (15). Consistent with its expression pattern and mitochondrial localization, deletion of SIRT3 in mice leads to strik-
ing hyperacetylation of mitochondrial proteins (16). These hyperacetylated proteins include key energy metabolic enzymes as well as antioxidant enzymes, such as Mn-SOD. The hyperacetylation results in dysfunction of these enzymes, eventually leading to mitochondrial dysfunction. Therefore, compounds that increase mitochondrial SIRT3 would improve mitochondrial function and might have therapeutic potential for HD.

Natural products and their synthetic derivatives are vital resources for the discovery of biologically active small organic molecules, and they exhibit numerous biophysical attributes that make them outstanding candidates for development of new drugs (17). Moreover, natural products are unique in terms of their chemical diversity when compared with most combinatorial chemical libraries (18, 19). Stilbenoids, constituting a class of resveratrol and its derivatives, naturally occur in several plant families, such as Cyperaceae, Dipterocarpaceae, Gnetaceae, and Vitaceae, and have recently been characterized chemically (20, 21). Among available stilbenoids, resveratrol has shown promise in treatment and prevention of neurodegenerative disorders, including Huntington disease (22–24). However, recent work in HD transgenic mice demonstrated that resveratrol protected against peripheral deficits, but was not effective in the central nervous system in HD mice, probably due to instability of resveratrol (25). Moreover, concentrations of resveratrol required for neuroprotective actions were in the range of 10–100 μM (26).

To develop more potent neuroprotective agents from natural products, we have screened a unique collection of 22 stilbenic compounds that consists of naturally occurring resveratrol monomers and oligomers, as well as semisynthetic resveratrol derivatives. Here we demonstrate that one compound, trans-(−)-e-viniferin (viniferin), preserves mitochondrial membrane potential and reduces reactive oxygen species (ROS) levels induced by mutant Htt. Moreover, viniferin increases mitochondrial SIRT3 levels, activates AMP-activated protein kinase (AMPK), and replenishes cellular NAD+ levels. The increased NAD+ leads to activation of SIRT3 deacetylase activity and enhances the antioxidant activity of target substrate Mn-SOD. SIRT3 is required for the neuroprotection of viniferin as inhibition of SIRT3 abolished the protection.

**EXPERIMENTAL PROCEDURES**

*Compounds and Reagents—*We purified a collection of 22 resveratrol monomers and oligomers from wild and cultivated Vitis spp. All compounds were characterized by comparison with their published electrospray mass spectrometry and 1H and 13C NMR data. Cell culture medium and supplements including DMEM, Neurobasal medium, fetal bovine serum (FBS), B-27, and N2 supplement were obtained from Invitrogen; tetramethyl rhodamine methyl ester (TRME), 5-(and-6)-chloromethyl-2’-7’-dichlorodihydrofluorescein diacetate, and acetyl ester (CM-H2DCFDA) were obtained from Molecular Probes (Eugene, OR); SsoFast™ EvaGreen® supermix was purchased from Bio-Rad; and nicotinamide and trichostatin A were obtained from Sigma. Antibodies were obtained from the following commercial sources: anti-active caspase-3 pAb (Promega, G7481); rabbit anti-Sirt3 (C terminus, Millipore, 07-1596); mouse β-actin (Sigma, A5441); mouse AMPK α1+α2 (Abcam, ab80039); phospho-AMPK (Cell Signaling, 2535); mouse anti-LKB1 (Santa Cruz Biotechnology, mouse mAb, Sc-32245); rabbit anti-LKB1 (Cell Signaling, 3047); phospho-LKB1 (Ser-428, Cell signaling, 3482); rabbit anti-Mn-SOD (Millipore, 06-984); mouse anti-Mn-SOD (Santa Cruz Biotechnology, Sc133254); and anti-acetyl lysine (Immunechem, IC0380).

*Cell Culture—*Immortalized striatal precursor cells expressing normal Htt (SThdhQ7/Q7) or mutant Htt (SThdhQ111/Q111) were kindly provided by Dr. Marcy MacDonald and were prepared as described previously (27). The cells were maintained at 33 °C in DMEM containing 10% FBS, 400 μg/ml G418 (Invitrogen), in a humidified atmosphere of 95% air and 5% CO2.

*Primary cortical neurons were prepared from embryonic day 18 pregnant C57/BL6 mice. Neurons were cultured in Neurobasal medium supplemented with B-27. Myc-tagged mutant Htt (N63-148Q) were maintained as described previously (28). Mutant Htt was inducibly expressed when doxycycline was removed from the culture medium. Cells were differentiated in the presence of nerve growth factor (NGF, 50 ng/ml).

Neuroblastoma N2a cells were obtained from ATCC and cultured in DMEM+10% FBS medium. Mutant Htt (N63-148Q) was transfected by Lipofectamine 2000 (Invitrogen), and cell toxicity was determined by flow cytometry (FACSCalibur, BD Biosciences) 48 h after transfection.

Primary cortical neurons were prepared from embryonic day 18 pregnant C57/BL6 mice. Neurons were cultured in Neurobasal medium supplemented with B-27. Myc-tagged mutant Htt (N63-148Q) or normal htt (N63-16Q) was transfected by Lipofectamine 2000 (Invitrogen) at days in vitro 5; the transfection efficacy was ~5%. Neurons were fixed and stained for transgene expression by anti-Myc antibody and chromatin condensation by the dye Hoechst 33342 to determine the neuronal toxicity induced by mutant Htt.

*Caspase3/7 Assay—*Caspase3/7 activity was detected by a luminescence assay in 96-well plates. Cells were dissolved with 50 μl of Caspase-Glo® 3/7 Reagent (Promega, G8092) and gently mixed using a plate shaker at 300–500 rpm for 1 h. Then 100 μl of the mixture was transferred to a white-walled 96-well plate, 100 μl of medium with a corresponding concentration of compounds was used as a blank, and the luminescence intensity of each sample was measured in a luminometer (Fluoroskan Ascent FL, Thermo Scientific).

*Intracellular ROS Measurements—*Striatal cells were incubated with the fluorescent probe CM-H2DCFDA (1 μM) for 30 min in Krebs-Ringer-Hepes buffer supplemented with 5 mM glucose. Samples were analyzed using a flow cytometer (FACSCalibur). The mean fluorescence intensity of 10,000 cells was analyzed in each sample and corrected for autofluorescence from unlabeled cells.

*Mitochondrial Potential Determination in Live Cells—*Mitochondrial membrane potential was determined by using the fluorescent probe TRME. Striatal cells were incubated with TMRE for 1 h. Samples were analyzed by using a flow cytometer (FACSCalibur). The mean fluorescence intensity of 10,000 cells was analyzed in each sample and corrected for autofluorescence from unlabeled cells.

*NAD+/NADH Assay—*Intracellular NAD+ and NADH levels in striatal cells were measured with an NAD+ /NADH assay kit...
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(Abcam) according to the manufacturer’s instructions. Briefly, 5 × 10⁵ striatal cells were cultured in serum-free medium with or without viniferin for 24 h, and then the cells were washed with cold PBS and extracted with NADH/NAD extraction buffer by two freeze/thaw cycles (20 min on dry ice and then 10 min at room temperature). Total NAD (NADt) and NADH levels were detected in a 96-well plate, and color was developed and read at 450 nm. NAD+/NADH ratio was calculated as: NADt = NADH)/NADH.

Mitochondrial Biogenesis—The mitochondrial copy numbers were calculated from the ratio of 12 S rRNA/18 S rRNA and Cox-2/cyclophilin A. The PCR primers for detecting the 12 S rRNA and Cox-2 gene of murine mitochondrial genome were designed on the basis of the GenBank™ nucleotide sequence. Each tube contained 15 ng of total DNA from the same extract as well as 10 μl of reaction mixture consisting of 2 × SsoFast™ EvaGreen® supermix (1× final) and other pairs of the primers directed against either 18 S RNA or cyclophilin A (nuclear-encoding gene), with 0.5 μM primer in each case.

Quantitative RT-PCR—The levels of PGC-1α mRNA and SIRT3 mRNA were detected by quantitative RT-PCR as follows. Total RNA from cultured cells was extracted with an RNeasy mini kit (Qiagen). cDNA was then reverse-transcribed and amplified by PCR with a Transcriptor reverse transcriptase kit (Applied Biosystems). Quantitative RT-PCR was carried out with the SsoFast™ EvaGreen® supermix in ABI 9700, and the results were normalized to β-actin.

Knockdown of SIRT3 in Striatal Cells—Stealth RNAi duplexes (Invitrogen) were designed to target mouse SIRT3 (NM_001177804.1; NM_022433.2). RNAi treatment of cells was as follows. 1 × 10⁷ cells were collected and electroporated with 400 pm siRNA at 450 V, 600 microfarads. Then 10⁶ cells/well were placed in 6-well plates. After 24 h of recovery in maintenance medium, cells were maintained in serum-free medium for 24 h and then assayed for toxicity or analyzed by Western blotting.

Immunoprecipitation—Cell extracts were prepared by resuspending PBS-washed cell pellets in 1 ml of Nonidet P-40 extraction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40) supplemented with EDTA-free protease inhibitor mixture tablets (Roche Applied Science) with 10 mM nicotinamide and 5 μM trichostatin A. After incubation for 30 min on ice, nonextractable material was removed by centrifugation at 17,000 × g for 10 min at 4 °C, and the cleared supernatant fractions were subjected to immunoprecipitation.

Statistical Analysis—Data are expressed as means of triplicates ± S.D. from at least three independent experiments. Statistical significance was determined using a Student’s t test, accepting a significance level of p < 0.05.

RESULTS

Viniferin Protects Neurons against Mutant Htt-induced Cell Toxicity—To develop small molecular compounds that have high potential to treat HD, we screened our unique collection of 22 stilbenic natural products and their semisynthetic derivatives in an inducible PC12 cell model that we have successfully used for compound screening (28). Among these 22 compounds, we identified six compounds that had protective activity in an inducible PC12 HD cell model. The EC50 ranged from 30 nM to 10 μM (Table 1). Viniferin was the most potent compound that exhibited consistent protection in different cell models of HD, including the transient transfection of mutant Htt N-terminal truncated form in N2a cells (Fig. 1A), immortalized striatal precursor cells expressing full-length huntingtin (Fig. 1B), as well as a primary neuronal model (Fig. 1, C and D). The neuroprotective effect of viniferin is specific to mutant Htt as viniferin had no effect on cell death in untransfected neurons (20 ± 4% in DMSO-untransfected neurons versus 15.6 ± 1.6% in viniferin-treated untransfected neurons).

Viniferin Reduces Mutant Htt-mediated Oxidative Stress in Striatal Cells—The mitochondrion is the major site of superoxide formation, and the accumulation of superoxide is believed to contribute to oxidative damage associated with neurodegenerative disease (29). Cells expressing mutant Htt exhibited higher levels of ROS in response to withdrawal of trophic factors (serum deprivation). To determine whether viniferin could attenuate mutant Htt-induced accumulation of ROS, striatal cells were incubated in the absence or presence of 1 μM viniferin for 24 h prior to measurement of ROS levels. Intracellular ROS production was measured by fluorescence-activated cell sorting (FACS) with use of the fluorescent probe CM-H₂DCFDA (30). Fig. 2 shows a representative image of intracellular ROS levels in striatal cells expressing wild-type Htt (STHdhQ7/Q7), mutant Htt (STHdhQ111/Q111), or STHdhQ111/Q111 cells treated with viniferin. Cells expressing mutant Htt had a greater increase in ROS levels in comparison

| Compound Code | Name | Structure | EC50 (estimated) |
|---------------|------|----------|-----------------|
| 25SH85A      | Trans-resveratrol | ![Structure](image) | ~300 nM |
| 25SH85B      | Trans-(c)-cinnamic acid | ![Structure](image) | ~30 nM |
| 25SH85D      | Amarensin | ![Structure](image) | >500 nM |
| 26TKE17C     | (E)-5-((benzoxo)xyloxy)-1,2-phenylene dibenzoate | ![Structure](image) | ~10 μM |
| 26TKE15B     | Dihydroresveratrol | ![Structure](image) | ~10 μM |
| 26TKE5C      | Resveratrol tricatate | ![Structure](image) | ~10 μM |
with cells expressing wild-type Htt (Fig. 2, A and B). Viniferin treatment significantly reduced intracellular ROS accumulation in cells expressing mutant Htt (Fig. 2, A–C).

**Viniferin Prevents Mitochondrial Dysfunction, Promotes Mitochondrial Biogenesis, and Attenuates PGC-1/\(^{\alpha}\) Depletion in Striatal Cells Expressing Mutant Htt**—Mutant Htt impaired mitochondrial function, as indicated by loss of mitochondrial membrane potential and lower NAD\(^+/\)NADH ratios in cells expressing mutant Htt when compared with those expressing wild-type Htt (Fig. 3, A–D). To determine whether viniferin prevents mitochondrial dysfunction, cells expressing wild-type or mutant Htt were treated with 1 \(\mu\)M viniferin for 48 h and then loaded with the mitochondrial dye TRMK, and changes in mitochondrial membrane potential were measured by FACS. Cells expressing mutant Htt exhibited lower mitochondrial membrane potential after serum withdrawal than did cells expressing wild-type Htt (Fig. 3, A and B). To gain insight into the mechanism by which viniferin regulates energy metabolism, we measured intracellular NAD\(^+/\)NADH levels. Consistent with our hypothesis, viniferin increased the NAD\(^+/\)NADH ratio (Fig. 3D).

We also evaluated the role of viniferin in mitochondrial biogenesis. To measure mitochondrial DNA directly, we isolated total DNA and determined the relative copy number of mitochondrial DNA by a quantitative PCR assay of the mitochondrial DNA-encoded COX II and 12S rRNA in striatal cells. Mutant Htt resulted in a significant reduction of mitochondrial DNA, indicating reduced mitochondrial biogenesis (Fig. 3, E and F). Viniferin treatment significantly increased the number of mitochondrial DNA copies, indicating increased mitochondrial biogenesis (Fig. 3, E and F).

**PGC-1/\(^{\alpha}\), a potent transcriptional coactivator, is the major regulator of mitochondrial biogenesis** (31). It has been reported that mutant Htt interrupts PGC-1/\(^{\alpha}\) transcription and results in decreased PGC-1/\(^{\alpha}\) levels (31–33); therefore, we next determined the effect of viniferin on PGC-1/\(^{\alpha}\) levels. As we predicted, PGC-1/\(^{\alpha}\) mRNA levels in STdh\(^{Q111/Q111}\) cells were significantly
lower than were levels in SThdh\textsuperscript{Q7/Q7} cells (Fig. 3G). Viniferin ameliorated the impairment of PGC-1α expression in HD cells (Fig. 3G).

Viniferin Ameliorates Mutant Htt-induced SIRT3 Decline—To further understand the molecular mechanisms underlying protection by viniferin, we focused on mitochondria. SIRT3 is a mitochondrial sirtuin that plays an important role in energy metabolism and mitochondrial function. Murine SIRT3 includes a long inactive isoform (about 37 kDa) and a short active isoform (about 28 kDa) (34–36). Both isoforms were significantly reduced in cells expressing mutant Htt (SThdh\textsuperscript{Q111/Q111} cells) (Fig. 4A), and viniferin significantly increased the levels of both isoforms of SIRT3 protein in SThdh\textsuperscript{Q111/Q111} cells (Fig. 4B). To determine whether SIRT3 decrease is the cause or consequence of mitochondria depolarization, we did a time course study. Our results indicated that mutant Htt-
expressing STHdhQ111/Q111 cells displayed mitochondrial depolarization before detectable SIRT3 decrease (Fig. 4, C and D), suggesting that the SIRT3 might represent a compensatory response to mitochondrial dysfunction. However, SIRT3 protein levels declined along with persistent mitochondrial dysfunction (Fig. 4C), viniferin preserved SIRT3 protein levels (particularly the 28-kDa active isoform), and this protection lasted longer than its analog resveratrol (Fig. 4, E and F).

We then determined whether viniferin also altered the SIRT3 deacetylase activity by measuring the acetylated Mn-SOD, a SIRT3 substrate. We incubated cells with viniferin for 4 h and...
FIGURE 4. Viniferin increases levels of SIRT3 protein and its deacetylase activity. A and B, striatal cells expressing mutant Htt (STHdh^{Q111/Q111} (Q111)) exhibited lower SIRT3 protein levels than cells expressing normal Htt (STHdh^{Q7/Q7} (Q7)) at 24 h following serum withdrawal. Panel A shows representative blots, and panel B shows quantification of densitometry of the 28-kDa band from three independent Western blots. *, p < 0.05 versus values in STHdh^{Q7/Q7} cells by standard Student’s t tests. W/O, untreated control. C and D, levels of SIRT3 protein (28 kDa) and mitochondrial membrane potential (MMP) in STHdh^{Q7/Q7} or STHdh^{Q111/Q111} cells following serum withdrawal at the indicated time points. Mean ± S.D., n = 3. *, p < 0.05 versus corresponding STHdh^{Q7/Q7} cells by standard Student’s t tests. MFI, mean fluorescence intensity. E and F, effects of viniferin and resveratrol on mutant Htt-induced decrease of SIRT3 protein levels in STHdh^{Q111/Q111} cells. Cells were treated with viniferin (1 μM) or resveratrol (10 μM) for the indicated times. SIRT3 protein levels were determined by Western blotting. *, p < 0.05 versus STHdh^{Q7/Q7} cells, & p < 0.05 versus DMSO-treated STHdh^{Q111/Q111} cells by Standard Student’s t tests. G, cells were treated with viniferin for 4 h. Cell extracts were immunoprecipitated (IP) with anti-Mn-SOD antibody, and the immunoprecipitation product was blotted with acetyl-lysine antibody to detect the levels of acetylated Mn-SOD. Note that there is an interaction between Mn-SOD and SIRT3, and viniferin treatment decreased acetylated Mn-SOD levels. The left panel shows representative blots, and the right panel shows quantification of densitometry from three independent Western blots (IB). *, p < 0.05 versus values in vehicle-treated cells by standard Student’s t tests.
found that viniferin reduced acetylated Mn-SOD levels, indicating that viniferin increases SIRT3 deacetylase activity (Fig. 4G).

**Viniferin Increases SIRT3-dependent AMPK Activation**—AMPK is a ubiquitous heterotrimeric serine/threonine protein kinase, which functions as a fuel sensor. Importantly, activated AMPK stimulates ATP-generating catabolic pathways, such as cellular glucose uptake and fatty acid oxidation. AMPK activation also represses ATP-consuming processes to restore intracellular energy balance (37, 38). To gain insight into the mechanism by which viniferin protects mitochondrial function, we treated striatal cells expressing mutant Htt with viniferin for 30 min and analyzed the levels of activated AMPK by Western blotting. Viniferin robustly increased the levels of activated AMPK (phosphorylated AMPK) in STHdhQ111/Q111 cells, but total AMPK protein levels remained unchanged (Fig. 5A). This increase in AMPK activation in response to viniferin is more sensitive in STHdhQ111/Q111 cells as we only detected increased AMPK phosphorylation after treatment with high concentrations of viniferin in STHdhQ7/Q7 cells (data not shown). To determine whether activation of AMPK is due to increased SIRT3 mediated by viniferin, we blocked the increase of SIRT3 with SIRT3 siRNA, which abolished the AMPK activation induced by viniferin (Fig. 5B), suggesting that SIRT3 increase is upstream and is required for AMPK activation mediated by viniferin.

**FIGURE 5. Viniferin increases AMPK activation, and SIRT3 is required for viniferin-mediated AMPK activation.** A, Western blotting of STHdhQ111/Q111 cells treated with viniferin (V) or vehicle DMSO. The upper panel shows representative blots, and the bottom panel shows quantification of densitometry of Western blots. STHdhQ111/Q111 cells were collected at 30 min after the treatment, and soluble cell extracts were analyzed by Western blotting with phosphorylated AMPK (p-AMPK) antibody or total AMPK antibody (t-AMPK). β-Actin (Actin) was used as a loading control. *, *p < 0.05 versus vehicle-treated group by standard Student’s t tests.

B, STHdhQ111/Q111 cells were treated with SIRT3 siRNA (10 nM) for 24 h and then with viniferin at the indicated concentrations or with DMSO for 30 min. Levels of phosphorylated AMPK and total AMPK antibody were determined by Western blot analysis. *, *p < 0.05 versus vehicle-treated group by standard Student’s t tests.
Viniferin Attenuates Mutant Htt-induced Hyperacetylation of LKB1—AMPK is allosterically activated by AMP and by phosphorylation at Thr-172 in the catalytic α-subunit, mainly by an upstream AMPK kinase, LKB1 (39, 40). Because LKB1 regulates AMPK activity (41), we first sought to determine whether LKB1 activity is altered by mutant Htt, and if so, whether viniferin modifies LKB1 activity, which is regulated by phosphorylation or acetylation. First, we measured the levels of phospho-LKB1 in cells expressing mutant Htt versus normal Htt and found that mutant Htt did not alter the levels of phosphorylated LKB1 (data not shown). Viniferin had no effect on LKB1 phosphorylation (data not shown). Remarkably, mutant Htt increased levels of acetylated LKB1, although total LKB1 levels were not altered in cells expressing mutant Htt (Fig. 6A). Viniferin significantly reduced acetylated LKB1 levels in striatal cells expressing mutant Htt (Fig. 6B). Because LKB1 is a substrate of SIRT3, to determine whether the increased acetylated LKB1 is due to the mutant Htt-induced decrease of SIRT3, we performed immunoprecipitation. SIRT3 interacts with LKB1, and viniferin did not alter this interaction (data not shown). Next, to determine whether SIRT3 mediated the effect of viniferin on LKB1 acetylation, we treated cells with SIRT3 siRNA. Inhibition of SIRT3 increased LKB1 acetylation and also abolished the effect of viniferin on the acetylation of LKB1 (Fig. 6C).

**FIGURE 6.** Viniferin decreases mutant Htt-induced hyperacetylation of LKB1, and this action is SIRT3-dependent. A, cells expressing mutant Htt exhibit increased levels of acetylated LKB1. The left panel shows representative blots, and the right panel shows quantification of blots, n = 3, *, p < 0.05 versus STHdhQ7/Q7 (Q7) cells. Q11, STHdhQ111/Q111 cells. IP, immunoprecipitation; IB, Western blot. B, viniferin decreases levels of acetylated LKB1 in STHdhQ111/Q111 cells. The left panel shows the representative blots, and the right panel shows quantification of acetylated LKB1 levels. *, p < 0.05 versus DMSO-treated STHdhQ111/Q111 cells. C, SIRT3 is required for effects of viniferin on LKB1 deacetylation. Cells were treated with SIRT3 siRNA (10 nM) or scrambled RNA for 24 h before treatment with viniferin at the indicated concentrations or DMSO as vehicle for 2 h, and acetylated LKB1 levels were determined by immunoprecipitation with LKB1 antibody and then blotting with anti-lysine (AcK) antibody. The left panel shows the representative blots, and the right panel shows the quantification of blots. n = 3, mean ± S.D. *, p < 0.05 when compared with the DMSO-treated culture in scrambled RNA-pretreated group by standard Student’s t tests.
SIRT3 Is Required for Viniferin-mediated Neuroprotection against Mutant Htt—Viniferin induced SIRT3 expression and protected cells against mutant Htt-induced neurotoxicity. We then asked whether SIRT3 is a critical mediator of neuroprotection by viniferin. Striatal cells were treated with SIRT3 siRNA before viniferin treatment, and we found that SIRT3 siRNAs completely abolished the protective effects of viniferin on mutant Htt (Fig. 7, A and B), suggesting that SIRT3 is indeed the critical mediator of the neuroprotection of viniferin against mutant Htt.

DISCUSSION

Our data support a working model for viniferin shown in Fig. 8. Viniferin attenuates mutant Htt-induced depletion of mitochondrial SIRT3, a soluble protein that controls global mitochondrial protein acetylation levels. For example, SIRT3 deacetylates its substrate Mn-SOD and enhances its antioxidant activity; SIRT3 also deacetylates LKB1, resulting in activation of AMPK, and activated AMPK promotes mitochondrial biogenesis and homeostasis of energy metabolism, thereby protecting cells from mutant Htt-mediated mitochondrial dysfunction. Activation of AMPK leads to increased levels of cellular NAD⁺, which in turn activates SIRT3 and subsequently affects multiple pathways involved in energy metabolism. Our results demonstrate that the effect of viniferin on enhancing mitochondrial function is mediated through SIRT3.

SIRT3 has been shown to play important roles in various mitochondrial functions, such as maintaining basal ATP levels and regulating apoptosis and energy homeostasis (42). Human SIRT3 exists in two isoforms, a full-length protein (~44 kDa) and a shorter, active form (~28 kDa) lacking the N-terminal 142 amino acids (43). The molecular mass of mouse SIRT3 is controversial, however. Our data confirmed the presence of two forms of endogenous mouse SIRT3 (37 and 28 kDa).

Recent studies have indicated that neuronal SIRT3 protects cells against excitotoxicity (44). Notably, we found that mutant Htt depleted SIRT3 protein levels. Although the molecular mechanisms underlying this regulation remain to be investigated, our findings open a new avenue for therapeutic intervention for HD. In this study, we demonstrate that viniferin, a stilbene resveratrol dimer with a five-membered oxygen heterocyclic ring, increases SIRT3 protein levels and plays a neuroprotective role in HD at a range of 100 nM–1 μM. The preservation of SIRT3 protein levels by viniferin is not at the transcriptional level; it might result from enhancing protein translation or slowing down protein degradation. The role of SIRT3 in the maintenance of ATP levels and in regulating mitochondrial electron transport was shown with SIRT3 knock-out mice (45). Constitutive expression of SIRT3 promotes the expression of mitochondrial genes, leading to enhanced mitochondrial electron transport activity (46). Viniferin increased levels of SIRT3, thereby promoting mitochondrial biogenesis and maintaining mitochondrial function.

The role of SIRT3 in regulating ROS production has been demonstrated in that overexpression of SIRT3 reduces ROS in adipocytes (46–48), whereas embryonic fibroblasts from SIRT3 knock-out mice exhibited increased levels of superoxide (49). ROS accumulation is associated with the pathogenesis of HD. The increased Mn-SOD activity by viniferin enhanced the antioxidant state in mitochondria accompanied by a significant decrease in ROS levels. ROS can react with DNA, proteins, and lipids and play important roles in many physiological and pathophysiological conditions, including neurodegenerative diseases and aging (50). Although ROS are produced in multiple cell compartments, the majority of cellular ROS (about 90%) contribute to mitochondrial energy metabolism. The mitochondrial electron transport chain complex I and complex III are presumed to be major sites of ROS generation (50), where electrons escape the electron transport chain and react with molecular oxygen, leading to the generation of superoxide. There are two main ways in which ROS production is limited in vivo. The first is through the action of detoxifying enzymes, including glutathione peroxidase-1 (GPx1) and superoxide dismutases (SODs); the second is through the uncoupling proteins (51). Our data suggest that viniferin...
promotes deacetylation of Mn-SOD and enhances its antioxidant activity, thereby reducing ROS levels.

The mechanism(s) by which different stimuli modulate SIRT3 and activate AMPK in neurons remains to be fully elucidated. For example, activation of acetyl-CoA synthetase 2 (AceCS2) by SIRT3 may elevate the AMP/ATP ratio and consequently activate AMPK. Interestingly, it has also been shown that activation of AMPK causes an increase in the cellular NAD[^+]/NADH ratio, consistent with a positive feedback loop needed for prolonged activation of SIRT3. It is known that activated AMPK directly phosphorylates PGC-1α (52). SIRT3 promotes mitochondrial biogenesis via PGC-1α (53). We have found that viniferin also attenuates mutant Htt-mediated decline of PGC-1α. Although PGC-1α stimulates mitochondrial biogenesis and electron transport activity, it suppresses ROS production and protects neural cells from oxidative stress-induced death through the induction of several key ROS-detoxifying enzymes (54, 55).

Continuous supply of energy is crucial for neurons because their survival requires large amounts of energy coupled with their inability to store energy. Therefore, neurons are extremely susceptible to insults that lead to energy depletion. NAD is an essential molecule that has a pivotal role in energy metabolism, cellular redox reactions, and mitochondrial function. Recent studies have revealed that maintaining intracellular NAD is important in promoting cell survival in various types of disease. Loss of NAD decreases the ability of NAD-dependent cell survival factors to carry out energy-dependent processes, leading to cell death. Recent evidence has shown that SIRT3 is involved in mitochondrial energy metabolism and biogenesis (56) and preservation of ATP biosynthetic capacity in the heart (45). SIRT3 knock-out resulted in a marked decrease in basal ATP in vivo (57) and eliminated the role of SIRT3 in protection of cells from oxidative stress (58). Our study suggests that viniferin increases SIRT3, stimulates AMPK activities, and preserves NAD levels, and therefore, could have a pivotal role in protecting neurons from mutant Htt, which promotes bioenergetic failure.

Substantial levels of SIRT3 are expressed in the brain (46). Nonetheless, its pathophysiological role remains unclear. In this study, we show, to our knowledge for the first time, that mutant Htt down-regulates SIRT3 protein levels and provide evidence that the neuroprotective role of viniferin is mediated by SIRT3 and that viniferin further enhances the activity of the antioxidant enzyme Mn-SOD and activates AMPK. Taken together, our results indicate that viniferin enhances mitochon-

![Diagram of the protective mechanism of viniferin.](image-url)

**Figure 8. Diagram of the protective mechanism of viniferin.** Viniferin counteracts mutant Htt-induced depletion of SIRT3 and facilitates its deacetylase activity. SIRT3 deacetylates its substrates Mn-SOD and then enhances its antioxidant activity. SIRT3 also deacetylates LKB1, resulting in activation of AMPK, and activated AMPK promotes mitochondrial biogenesis and homeostasis of energy metabolism, thereby protecting cells from mutant Htt-mediated mitochondrial dysfunction and toxicity. P indicates phosphorylation.
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