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ORIGINAL PAPER

Loss of SDHB Elevates Catecholamine Synthesis and Secretion Depending on ROS Production and HIF Stabilization

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Abstract Germline mutations in genes encoding succinate dehydrogenase subunits are associated with the development of familial pheochromocytomas and paragangliomas [hereditary paraganglioma/pheochromocytoma syndrome (HPPS)]. In particular, a mutation in succinate dehydrogenase subunit B (*SDHB*) is highly associated with abdominal paraganglioma and subsequent distant metastasis (malignant paraganglioma), indicating the importance of *SDHB* genetic testing. The discovery of HPPS suggests an association among genetic mitochondrial defects, tumor development, and catecholamine oversecretion. To investigate this association, we transfected pheochromocytoma cells (PC12) with *SDHB*-specific siRNA. *SDHB* silencing virtually abolished complex II activity, demonstrating the utility of this in vitro model for investigating the pseudo-hypoxic drive hypothesis. Lack of complex II activity resulting from RNA interference of *SDHB* increased tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine biosynthesis) activity and catecholamine secretion. Reduced apoptosis was observed accompanied by Bcl-2 accumulation in PC12 cells, consistent with the phenotypes of paragangliomas with *SDHB* mutations. In addition, *SDHB* silencing increased reactive oxygen species (ROS) production and nuclear HIF1α stabilization under normoxic conditions. Furthermore, phenotypes induced by complex II activity knockdown were abolished by pretreatment with *N*-acetyl cysteine (an ROS scavenger) and by prior *HIF1α* knockdown, indicating an ROS- and HIF1α-dependent mechanism. Our results indicate that increased ROS may act as signal transduction messengers that induce HIF1α stabilization and may be necessary for the pseudo-hypoxic states observed in our experimental model. To our knowledge, this is the first study demonstrating that pseudo-hypoxic states resulting from *SDHB* knockdown are associated with increased TH activity and catecholamine oversecretion.

Keywords Succinate dehydrogenase · Hereditary paraganglioma/pheochromocytoma syndrome · Catecholamine · Reactive oxygen species

Introduction

Succinate dehydrogenase (SDH; also known as succinate-ubiquinone oxidoreductase) is a highly conserved, heterotetrameric protein present in the mitochondrial matrix with two catalytic subunits; succinate dehydrogenase subunit A (SDHA) and succinate dehydrogenase subunit B (SDHB). SDH is anchored to the inner membrane by SDHC and SDHD that also provide the binding

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site for ubiquinone. All subunits are encoded by nuclear genes and are subsequently imported into the mitochondria.

Germline mutations of *SDHx,* genes encoding SDH subunits, are associated with the development of familial pheochromocytomas and paragangliomas [hereditary paraganglioma/pheochromocytoma syndrome (HPPS)] [1–10]. In particular, a mutation in *SDHB* is strongly related to abdominal paraganglioma and subsequent distant metastasis (malignant paraganglioma), thereby indicating the importance of *SDHB* genetic testing [6]. However, HPPS discovery provided the first link between a genetic mitochondrial defect and tumor development [11–17].

Although the precise mechanism by which the disruption of SDH in mitochondrial metabolism leads to tumorigenesis remains unknown, two prominent hypotheses have been proposed. The first hypothesis states that redox stress results from increased mitochondrial reactive oxygen species (ROS) production [13]. The second hypothesis states that succinate plays a role in metabolic signaling as an intracellular messenger between the mitochondria and cytosol [17]. Currently, the effect of *SDH* mutations on mitochondrial metabolism involves their capacity to induce pseudo-hypoxic responses such as the abnormal stabilization of hypoxia inducible factors (HIFs) under normoxic conditions (the pseudo-hypoxic hypothesis). SDH inactivation leads to HIF stabilization via the inhibition of HIF hydroxylation by prolyl-4-hydroxylases, which is necessary for their recognition by Von Hippel–Lindau disease tumor suppressor (pVHL) [18, 19]. ROS-mediated stabilization of HIFs may play an important role in tumorigenesis induced by SDH enzyme deficiency. Guzy et al. [13] demonstrated that *SDHB* inhibition, either pharmacologically or via RNA interference, increases hypoxia-inducible factor alpha (HIF1α) stabilization in an ROS-dependent manner under normoxic conditions. However, the specific targets of HIF1α involved in tumorigenesis remain unknown.

The precise mechanisms responsible for the elevated catecholamine secretion and synthesis observed in HPPS tumors remains to be elucidated. Tyrosine hydroxylase (TH) is the rate-limiting enzyme of catecholamine biosynthesis. TH activity is regulated by both short- and long-term mechanisms. Short-term regulation of TH activity occurs at the post-transcriptional level and is dependent on the activation of TH phosphorylation [20, 21]. TH is phosphorylated and activated by various protein kinases, although only three serine residues (Ser19, Ser31, and Ser40) are phosphorylated in vivo. The most likely physiological candidates for TH phosphorylation at Ser19, Ser31, and Ser40 are Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinase (ERK), and protein kinase A (PKA)/protein kinase C (PKC), respectively. However, only phosphorylation at Ser40 has a substantial effect on TH activity. In addition to the short-term regulation of TH activity, long-term control occurs at the translational level after *TH* gene transcription [22]. *TH* belongs to the group of hypoxia-inducible genes, with low oxygen levels shown to induce *TH* expression [23–26].

We aimed to determine the mechanisms underlying the disruption of mitochondrial metabolism leading to catecholamine oversecretion and to apoptosis resistance in HPPS. To investigate the abovementioned mechanisms, we initially introduced *SDHB*-specific siRNA into PC12 cells. *SDHB*-specific siRNA was shown to almost entirely abolish complex II activity, indicating that this approach is useful as an in vitro model for investigating the pseudo-hypoxic drive hypothesis. Using transfected PC12 cells, we found that SDH knockdown increased TH activity and catecholamine secretion. SDH knockdown reduced apoptosis and induced the accumulation of the anti-apoptotic protein B cell lymphoma 2 (Bcl-2). Observed responses were consistent with the phenotypes of paragangliomas with mutated *SDHB*, which are dependent on ROS accumulation and subsequent HIF1α stabilization.

Materials and Methods

Reagents

All reagents were purchased from Wako Seiyaku (Tokyo, Japan), unless otherwise stated.

Cell Culture and Treatment

The PC12 cell line (RCB009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). Cells were grown in 75-cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10 % inactivated horse serum (Gibco BRL) and 10 % fetal bovine serum (Gibco BRL) in a humidified atmosphere of 5 % CO_2 and 95 % O_2 at 37 °C. Culture media was changed three times per week. Cells were removed from flasks for subculture and for plating into assay dishes using a Ca^{2+}/Mg^{2+}-free dissociation solution comprising 172 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, and 5.6 mM glucose at pH 7.4. After approximately 2 min in dissociation solution, cells were detached by agitation. Cells (1×10^6) were plated into 35-mm polystyrene dishes and cultured with 2 ml DMEM for 2 days under similar conditions to those described above. All subsequent experiments were conducted under serum-starved conditions [27, 28]. ROS inhibition were performed by pre-incubated of NAC (10 μM) for 24 h.

Immunoblotting

Immunoblotting was performed as described previously [27, 28]. Anti-SDHB (Abcam), anti-HIF1α (Abcam), anti-Bcl-2 (Cell Signaling Technology), anti-pTH (Ser19; Santa Cruz), anti-pTH (Ser31; CALBIOCHEM), anti-pTH (Ser40; Santa Cruz), anti-αTubulin (SIGMA), anti-Bax, anti-Bim, anti-Cyclin D1, anti-Cyclin D3, and anti-β-actin (Cell Signaling Technology) antibodies were used. Band intensities were measured by densitometry.

Complex II Assay

Complex II activity was measured using a Complex II Enzyme Activity Microplate Assay Kit (abcam). PC12 cells were seeded into the culture medium with 10 % FBS for 24 h before siRNA transfection for 48 h. Cells were washed and collected in PBS. Detergent extraction with detergent buffer was then performed for 30 min on ice followed by centrifugation at 25,000×*g* for 20 min and removal of supernatant. Sample concentrations were then adjusted to recommended dilutions for loading plates using incubation buffer; subsequently, the samples were loaded onto plates and were incubated at room temperature for 2 h. Next, samples were washed twice with wash buffer before the addition of Lipid mix and succinate activity solution. Finally, complex II activity was measured at OD 600 at 1 min intervals for 1 h at room temperature using a spectrophotometer.

Proliferation Assay

Cells were plated onto 96-well culture plates at a density of 10,000 cells/well. At the indicated time points, culture medium was removed and 100 µl DMEM containing 0.5 mg/ml 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) was added. Cells were then incubated for 45 min at 37 °C in a CO_2 incubator. Finally, MTT-containing medium was removed and purple formazan crystals were dissolved by adding 100 µl of dimethyl sulfoxide to each well. Absorbance at 570 nm was measured in a microplate reader with a reference filter set at 655 nm.

Apoptosis Assay

TUNEL was performed using commercially available TMR-red kits (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. Apoptosis was assessed using Cell Death Detection ELISA PLUS kits (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions [28]. In brief, 5,000 cells per well were seeded into 96-well plates in culture medium containing 10 % FBS and cultured for 24 h before transfection with siRNA for 24 h. After treatment, apoptotic cells were detected by a photometric enzyme-immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono-and oligonucleosomes).

Determination of Catecholamine Secretion

Catecholamine concentrations were determined in the following manner. Cells were cultured in 35-mm polystyrene dishes before the replacement of culture media with 2 ml DMEM containing 0.1 % bovine serum albumin (BSA). Cells were then incubated at 37 °C for 2 h. Cells were washed twice by incubation in 2 ml HEPES-buffered Krebs buffer (128 mm NaCl, 4.6 mm KCl, 10 mm glucose, 25 mm HEPES-sodium, 1.2 mm MgSO4, 1.1 mm CaCl2; pH 7.4) containing 0.1 % BSA for 10 min. Experiments were initiated by replacing the medium with HEPES-buffered Krebs buffer containing the test substance. Cells were then incubated at 37 °C for 5 min. Aliquots of incubation media (0.5 ml) were removed and analyzed to determine catecholamine concentrations. Samples were acidified by adding 10 µl of 2 N HCl and diluting up to 200-fold prior to assessment using a catecholamine autoanalyser (TOSOH, H8030) with a built-in HPLC unit and spectrofluorometer.

TH Activity Measurements

TH activity was measured using a previously reported method [3]. Cells were incubated with either dimethyl sulfoxide (DMSO; control) or with sample at 37 °C for 12 h. Cells were then homogenized in 0.25 M sucrose (50 volumes) using a glass tissue grinder. The standard incubation medium comprised the following components in a total volume of 250 µl: 100 µl cell homogenate, 40 µl of 1 M sodium acetate buffer (pH 6.0), 40 µl of 1 mM L-tyrosine or D-tyrosine, 20 µl of 1 M 6-methyl-5,6,7,8-tetrahydropterine in 1 M 2-mercaptoethanol, 20 µl of 20 mg/ml catalase, and 30 µl water. Reactions were performed at 37 °C for 30 min before being stopped because of the addition of 1 M perchloric acid containing dihydroxy benzylamine as an internal standard and 0.2 M EDTA and placed on ice. Next, 1 M potassium carbonate and 0.2 M Tris HCl (pH 8.5) containing 2 % EDTA was added. 3,4-dihydroxyphenylalanine (DOPA) was extracted using the aluminum oxide method by mixing 240 µl of extracted medium with 0.1 N NaOH and Amberlite CG-50 (MP Biomedicals, Illkirch, France) prior to analysis by HPLC. The mobile phase comprised the following components: 50 mM sodium acetate, 20 mM citric acid, 20 mM sodium octyl sulfate, 1 mM di-*n*-butylamine, and 0.134 mM EDTA. All separations were isocratically performed at a

flow rate of 0.6 ml/min at 29 °C. Enzyme activity was calculated as the amount of DOPA formed from tyrosine per mg of protein in 30 min.

ROS Assay

Cells were incubated with 5 μM 3′-(p-aminophenyl) fluorescein (APF; Molecular Probes) for 30 min at 37 °C. Cells were than washed in PBS to remove the probe. Fluorescence excitation and emission maxima used for analysis were 490 and 515 nm, respectively.

HIF1α Immunohistochemistry

Immunocytochemical analyses were performed on PC12 cells [29]. Cells were fixed with 4 % paraformaldehyde and immunostained with anti-HIF1α IgG (abcam) and anti-mouse IgG Fab2 Alexa Fluor 555 (Cell Signaling). 4′,6-Diamidino-2-phenylindole (DAPI) (1 mg/ml) was used for nuclear DNA staining. Fluorescence images were captured using an inverted microscope (IX71) and an image acquisition system (DP2-BSW; Olympus, Japan).

Small Interfering RNA Transfection

RNA interference experiments were performed as previously described [27]. In brief, PC12 cells were seeded in 6-well plates for 24 h before transfection with Silencer Select small interfering RNAs (Applied Biosystems) targeting SDHB and/or HIF1α using RNAiMAX Reagent (Invitrogen), according to the manufacturer's protocol. Concurrently, cells cultured under identical conditions were transfected with non-targeting Silencer Select siRNA (Applied Biosystems) as a negative control.

Statistical Analysis

All data are expressed as mean ± SE. Statistically significant differences between groups were determined using Student's *t* test. *P* values of <0.05 were considered to be statistically significant.

Results

Inhibition of *SDHB* Using Specific siRNA Almost Entirely Abolishes Complex II Activity in PC12 Cells

To investigate the effects of SDH knockdown in PC12 cells, we targeted *SDHB* gene expression using siRNA transfection. *SDHB* knockdown using specific siRNA reduced *SDHB* mRNA and SDHB protein levels but did not affect the levels of tubulin protein, which was used as an internal marker (Supplemental Fig. 1a). Consistent with reduced SDHB protein levels, *SDHB* knockdown almost entirely abolished complex II activity in PC12 cells, reducing activity to <5 % of complex II activity in control cells (Supplemental Fig. 1b). Thus, complex II activity was almost completely absent in *SDHB*-knockdown PC12 cells; this was in agreement with the complete abolition of complex II activity observed in HPPS tumors as a result of heterozygous constitutive *SDHB* mutation and associated somatic loss of heterozygosity (LOH). These results indicate that PC12 cells with SDH deficiency may have utility as an in vitro model of HPPS [5, 7].

SDHB Knockdown Increases ROS and Stabilizes HIF1α Under Normoxic Conditions

SDHB-silenced PC12 cells were used to determine the effect of SDH activity on ROS levels in PC12 cells. As shown in Fig. 1a, cytoplasmic ROS levels increased in response to *SDHB* knockdown. Next, to examine the effect of *SDHB* knockdown on the translocation of HIF1α from the cytoplasm to the nucleus, HIF1α (red; Fig. 1b, left) and DAPI (a nuclear marker; blue; Fig. 1b, middle) were examined by immunohistochemistry. Merging of HIF1α (red) and DAPI (blue) immunohistochemical images demonstrated the translocation of HIF1α to the nucleus (purple; Fig. 1b, right). A significant increase in the number of cells with the nuclear co-localization of HIF1α and DAPI staining was observed following *SDHB* knockdown under normoxic conditions (Fig. 1b, lower panel; merged image and histogram). In contrast, little nuclear HIF1αHstaining was observed in cells treated with control siRNA (Fig. 1b, upper panel; merged image). These findings indicate that *SDHB* knockdown induces SDH deficiency in PC12 cells resulting in HIF1α stabilization under normoxic conditions (pseudo-hypoxic states).

SDHB Knockdown Increases Cell Survival (Resistance to Apoptosis) in PC12 Cells

To determine the precise mechanisms by which the disruption of SDH leads to tumorigenesis, we measured apoptosis in *SDHB*-knockdown PC12 cells. As shown in Fig. 2a, b, a significant decrease in TUNEL-positive cells (green) was observed following *SDHB* knockdown. This finding was confirmed by assessing oligonucleosomal DNA fragmentation. As shown in Fig. 2c, apoptosis was significantly reduced in *SDHB*-knockdown cells compared with control cells.

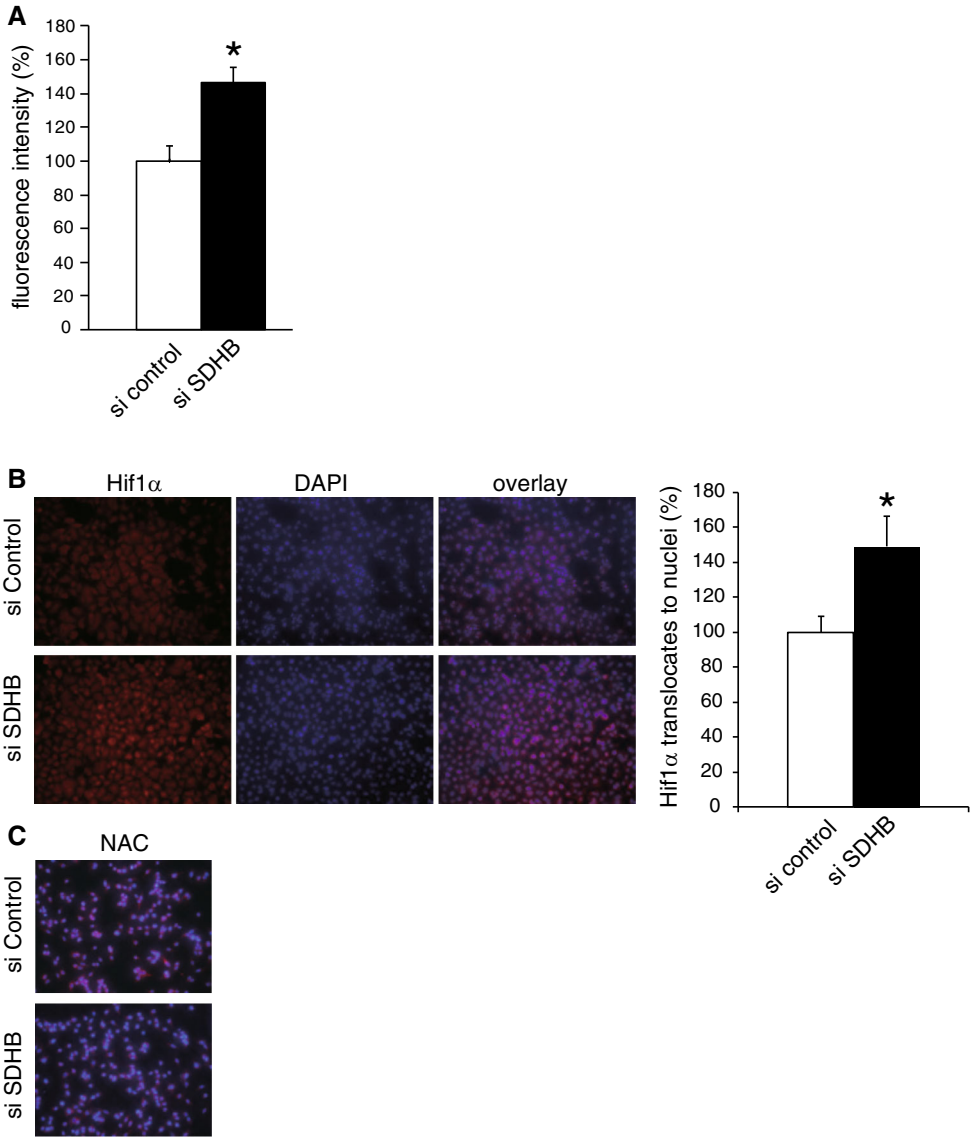

Fig. 1 Silencing of *SDHB* increases ROS and stabilizes HIF1α under normoxic conditions. **a** *SDHB* knockdown leads to increased levels of cytosolic ROS. Superoxide production was assessed by aminophenyl fluorescence. Values shown represent the mean ± SD (n = 4–6). *$P < 0.05$ versus control siRNA-treated values. **b** *SDHB* knockdown stabilizes HIF1α in PC12 cells. This study aimed to determine the effect of *SDHB* knockdown on HIF1α translocation from the cytoplasm to the nucleus (pseudo-hypoxic response). Nuclear HIF1α protein was detected by immunostaining with an anti-HIF1α antibody shown in *red* (*left*). DAPI was used for nuclear DNA staining shown in *blue* (*middle*). In the merged image shown on the *right*, nuclear translocation of HIF1α (merged *red* and *blue* images producing *purple* image) indicates the stabilization of HIF1α. Representative data are shown. Histogram shows the average number of *purple* cells per total cells. Details are described in the "Materials and Methods" section. *$P < 0.05$ versus control siRNA-treated values. **c** *SDHB* knockdown stabilizes HIF1α in an ROS-dependent manner. Pretreatment (24 h) with 10 μM NAC (an antioxidant and ROS scavenger) reversed the *SDHB* knockdown-induced stabilization of HIF1α to control levels (control siRNA-treated +NAC)

SDHB Knockdown Increases Bcl-2 Expression in PC12 Cells

Consistent with the observed anti-apoptotic effect of *SDHB* knockdown, increased levels of the anti-apoptotic protein Bcl-2 was observed following *SDHB* knockdown (Fig. 3a). *SDHB* knockdown had no effect on the levels of other apoptosis-related proteins, including P53, B-cell lymphoma-extra large (Bcl-xL), BCL2-like 11 (Bim), P-ERK, P-AKT, P-JNK, and p-38 (data not shown). Decreased levels of the pro-apoptosis protein Bax were observed following *SDHB*/HIF1α knockdown; however, Bim levels were unaffected indicating a lack of Bim-dependent activation of the Akt pathway (Supplemental Fig. 2a and b). In addition, knockout of SDHB or HIF-1α had no effect on cell proliferation (Supplemental Fig. 2c and d).

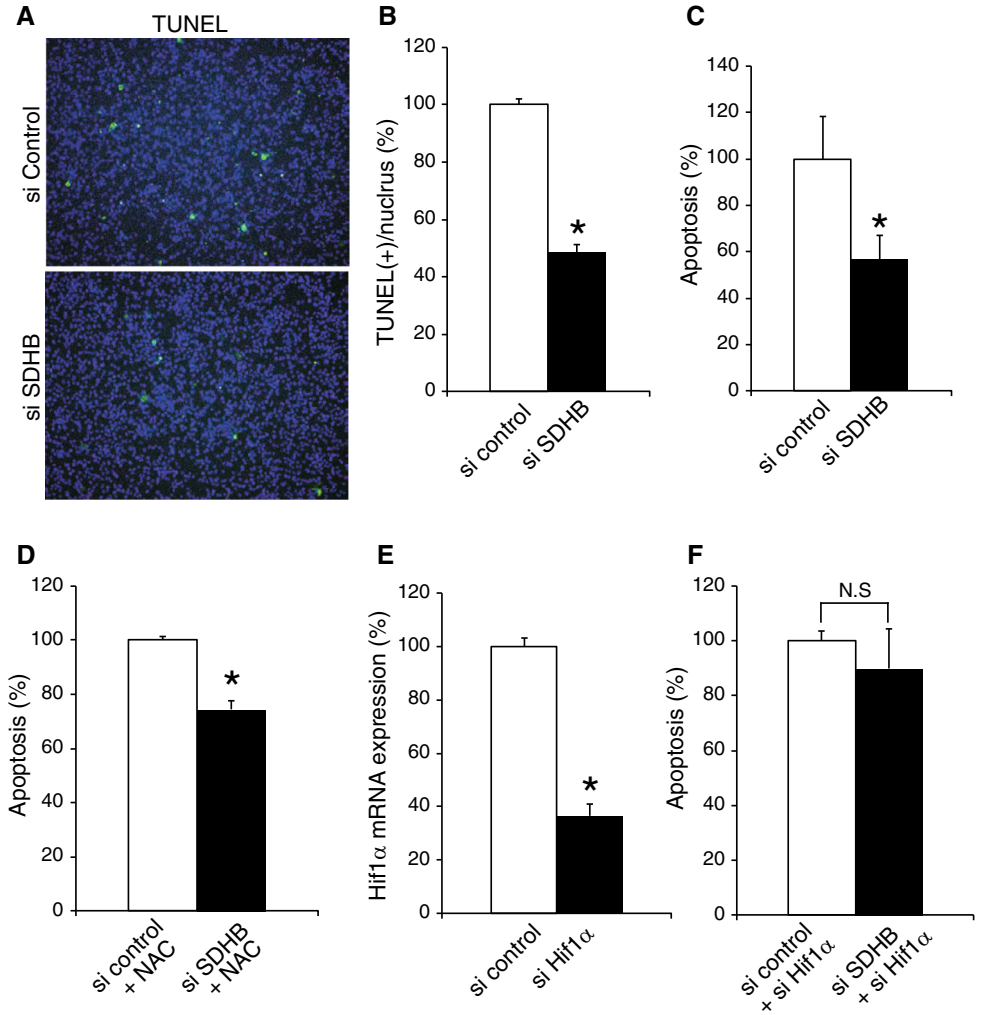

Fig. 2 *SDHB* knockdown results in increased survival of PC12 cells (reduction of apoptosis) in a ROS- and HIF1α-dependent manner. **a** Representative images of TUNEL staining (*green*) are shown. Data were reproducible in three independent experiments. **b** Histogram showing the average number of TUNEL-positive cells (*green*) per total cells. Significantly decreased number of TUNEL-positive cells (*green*) were observed in PC12 cells following *SDHB* knockdown (*$P < 0.05$). **c–f** Cytoplasmic histone-associated DNA fragments. **c** *SDHB* knockdown resulted in increased cell survival (reduction of apoptosis). **d** *SDHB*-knockdown PC12 cells pretreated (24 h) with or without 10 μM NAC. **e** Confirmation of decreased HIF1α mRNA levels in response to siRNA specifically directed against HIF1α. **f** HIF1α knockdown prior to treatment with either si*SDHB* or control siRNA. Cytoplasmic histone-associated DNA fragments, used as markers of apoptosis, were determined by ELISA. Details are described in the "Materials and Methods" section. Data represent the mean ± SE of two independent experiments (one experiment was performed with three samples). *$P < 0.05$ versus control siRNA-treated values

SDHB Knockdown Increases TH Activity in PC12 Cells

As TH is the initial and rate-limiting enzyme in catecholamine biosynthesis, we measured TH activity in SDH-deficient PC12 cells. As shown in Fig. 4a, *SDHB* knockdown significantly increased TH activity by approximately 1.3-fold compared with the control value. To confirm this finding, we examined the effect of *SDHB* knockdown on the phosphorylation of TH at Ser19, Ser31, and Ser40. Similar increases in TH phosphorylation were observed at Ser19, Ser31, and Ser40 (Fig. 4d). In addition, we examined the effect of reduced SDH activity on the long-term regulation of TH activity. As shown in Fig. 4d, *SDHB* knockdown significantly increased the level of TH protein by approximately threefold compared with the control value.

SDHB Knockdown Results in Elevated TH Catecholamine Secretion by PC12 Cells

We next examined the effect of *SDHB* knockdown on catecholamine secretion. As shown in Fig. 5a, *SDHB* knockdown significantly increased catecholamine secretion

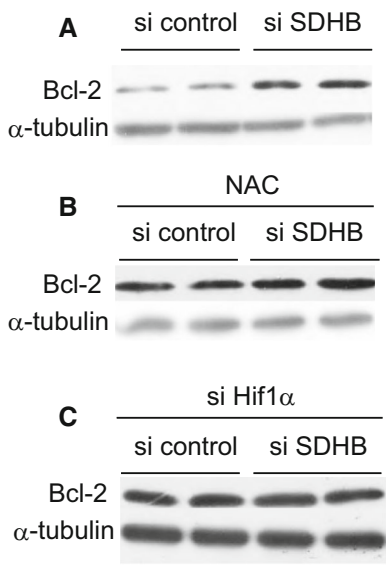

Fig. 3 *SDHB* knockdown increases Bcl-2 expression in PC12 cells in a ROS- and HIF1α-dependent manner. **a** *SDHB* knockdown increases Bcl-2 expression. **b** *SDHB* knockdown (si*SDHB*) or control siRNA-treated cells pretreated (24 h) with 10 μM NAC. **c** *HIF1α* knockdown prior to treatment with either si*SDHB* or control siRNA. After treatment, cells were cultured for 48 h and cell lysates were subjected to SDS-PAGE and immunoblotted with either anti-Bcl-2 or anti-tubulin antibodies. Details are described in the "Materials and Methods" section. Representative data are shown. Data were reproducible in three independent experiments

by approximately 2.5-fold compared with the control value.

Role of Redox Stress in HIF1α Stabilization, Apoptosis Reduction, and Catecholamine Oversecretion in *SDHB*-Silenced PC12 Cells

To determine the effect of ROS accumulation on HIF1α stabilization, apoptosis reduction, and catecholamine oversecretion, we assessed the ability of *N*-acetylcysteine (NAC) to alleviate glutathione redox stress. NAC reversed HIF1α stabilization, apoptosis reduction, and catecholamine oversecretion induced by *SDHB* knockdown to levels observed in siRNA-treated control cells. Pretreatment with NAC ameliorated nuclear HIF1α accumulation (Fig. 1c), reduced apoptosis levels (Fig. 2d), and increased Bcl-2 levels (Fig. 3b) observed in *SDHB*-silenced PC12 cells to levels comparable with those of siRNA-treated control cells. Furthermore, NAC pretreatment attenuated the increases in TH activity and the resultant phosphorylation at Ser40 of SDHB (Fig. 4b, e) and catecholamine secretion (Fig. 5b) observed in response to *SDHB* knockdown. Our results indicate that increased ROS may act as signal transduction messengers that induce HIF1α stabilization and may be necessary for the pseudo-hypoxic states observed in our experimental model.

Proposal of a HIF1α-Dependent Mechanism for Apoptosis Reduction (Apoptosis Resistance) and Catecholamine Oversecretion in SDH-Deficient PC12 Cells

To determine whether HIF1α is involved in apoptosis reduction and catecholamine oversecretion in response to *SDHB* knockdown, *HIF1α* knockdown was performed prior to *SDHB* knockdown. *HIF1α* knockdown using specific siRNA reduced *HIF1α* mRNA (Fig. 2e). Pre-knockdown (pre-KD) of HIF1α attenuated apoptosis reduction (Fig. 2f). Consistent with this finding, pre-KD of HIF1α attenuated the induction of Bcl-2 (Fig. 3b), increases in TH activity and the resultant phosphorylation at Ser40 of SDHB (Fig. 4c, f), and increased catecholamine secretion (Fig. 5c) observed in *SDHB*-silenced PC12 cells. These results indicate that increased HIF1α stabilization may be necessary for the reduction in apoptosis and elevation of catecholamine synthesis and secretion observed in SDH-deficient PC12 cells.

Underlying Signaling Mechanisms Responsible for Elevated TH Activity in SDH-Deficient PC12 Cells

As mentioned above, physiological candidates for TH phosphorylation at Ser19, Ser31, and Ser40 include CaMKII, ERK, and PKA/PKC, respectively. As shown in Fig. 4d, *SDHB* knockdown significantly increased TH phosphorylation at Ser19, Ser31, and Ser40. Thus, we examined whether ERK, PKA, and PKC may be responsible for the increased TH serine phosphorylation in response to *SDHB* knockdown observed at these sites. No increases in the levels of ERK, PKA, or PKC were observed following *SDHB* knockdown (data not shown). Possible reasons for this discrepancy are discussed below and summarized in Fig. 6.

Discussion

Our results provide insights into the potential mechanisms underlying the association between mitochondrial dysfunction and phenotypes observed in HPPS with *SDHB* mutation [6, 7]. Previously, *SDHB* mutations in patients with paraganglioma were considered to be associated with complete and selective loss of complex II electron transfer activity, leading to complete inhibition of the succinate dehydrogenase-mediated step of the Krebs cycle [7]. The complete abolition of complex II activity have been reported to be the result of heterozygous constitutive mutation of *SDHB* associated with a somatic LOH [7]. Although previous data have demonstrated incomplete

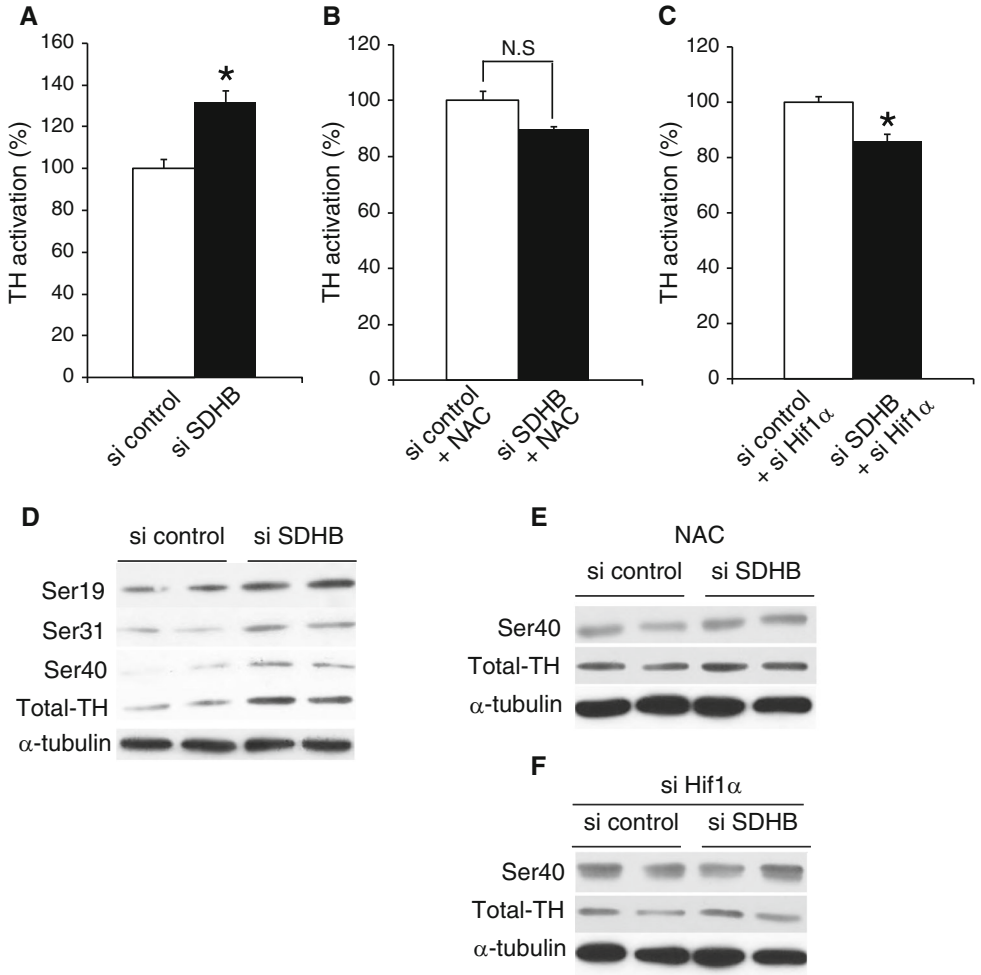

Fig. 4 Silencing *SDHB* increases TH activity in a ROS- and HIF1α-dependent manner in PC12 cells. **a** *SDHB* knockdown increases TH activity in PC12 cells. **b** *SDHB* knockdown or control siRNA-treated cells pretreated (24 h) with 10 μM NAC. **c** HIF1α knockdown prior to treatment with either si*SDHB* or control siRNA. **d** *SDHB* knockdown increases TH phosphorylation and protein level in PC12 cells. **e** *SDHB* knockdown or control siRNA-treated cells were pretreated (24 h) with 10 μM NAC. **f** HIF1α knockdown was performed prior to treatment with either si*SDHB* or control siRNA. Cell lysates were subjected to SDS-PAGE and immunoblotted with an anti-phospho-TH antibody specific for phosphorylation at Ser19, Ser31, or Ser40. Details are described in the "Materials and Methods" section. Representative data are shown. Data were reproducible in three independent experiments. Cells were cultured for 48 h and TH protein levels were measured by immunoblotting as described in the "Materials and Methods" section. Values in histograms represent the mean ± SD of densitometric measurements of each indicated parameter. A value of 100 % represents basal conditions. $*P < 0.05$ versus control siRNA-treated values

inhibition of SDH activity (approximately 50–70 %) following either *SDHB* or *SDHD* knockdown [11, 13, 17], *SDHB* knockdown in PC12 cells in the present study almost entirely abolished complex II activity (Supplemental Fig. 1b), corroborating previous studies that have demonstrated a lack of complex II activity in tumor cells [5, 7]. Thus, PC12 cells with *SDHB* knockdown may have utility as an in vitro model for the study of SDH-deficient HPPS.

Although the precise mechanism by which disruption of SDH in mitochondrial metabolism leads to tumorigenesis remains unknown, increases in ROS production at complex II caused by defects in SDHB could activate HIF1α by mimicking the hypoxia signaling pathway (pseudo-hypoxic drive; the aberrant activation of the cellular hypoxic response pathways, despite normal oxygen levels), thereby promoting cell proliferation, angiogenesis, survival, and tumor phenotypes observed clinically [13, 30]. Consistent with this hypothesis, high levels of HIF1α have been observed in HPPS tumor tissue [4, 10, 12, 16].

Consistent with the pseudo-hypoxic drive hypothesis, we demonstrated cytosolic ROS generation and subsequent nuclear HIF1α stabilization in SDH-deficient PC12 cells (Fig. 1a, b). In addition, pretreatment with NAC and silencing of HIF1α expression attenuated the reduced levels of apoptosis following *SDHB* knockdown in PC12

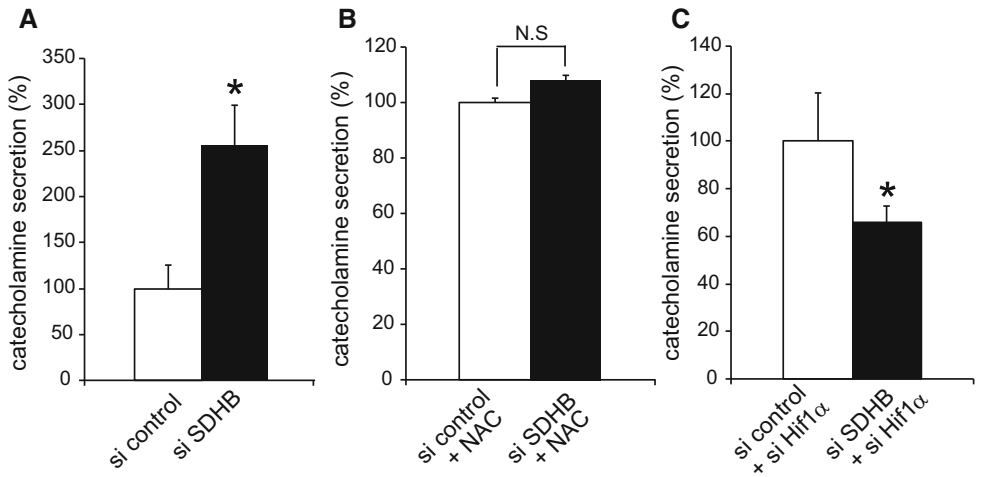

Fig. 5 Silencing *SDHB* increases catecholamine secretion in a ROS- and HIF1α-dependent manner in PC12 cells. **a** *SDHB* knockdown increases catecholamine secretion in PC12 cells. **b** *SDHB* knockdown or control siRNA-treated cells pretreated (24 h) with 10 μM NAC. **c** HIF1α knockdown prior to treatment with either si*SDHB* or control siRNA. After treatment, cells were cultured for 48 h before the culture medium was examined by HPLC according to the method described in the "Materials and Methods" section. Values in histograms represent the mean ± SD (n = 4–6). $*P < 0.05$ versus control siRNA-treated values

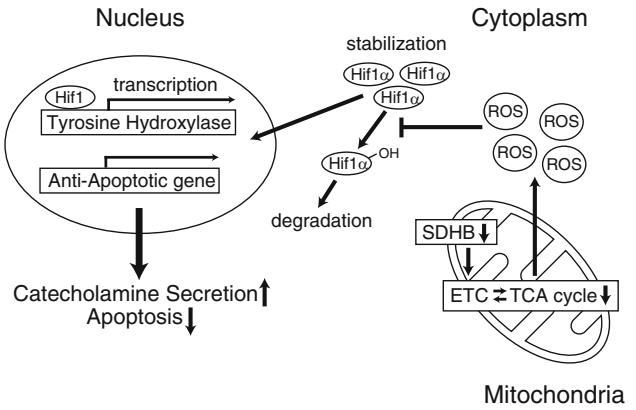

Fig. 6 Summary of a proposed hypothesis explaining the link between SDH mutations, elevated TH activity, and apoptosis resistance in HPPS. Data from the present study and previous reports indicate that SDH inactivation is involved in HPPS tumorigenesis. Loss of SDH function may result in the increased production of ROS. ROS may induce the hypoxic response under normoxic conditions (pseudo-hypoxia). The stabilization of nuclear HIF1α may, in turn, lead to the transcriptional activation of TH and Bcl-2. The resultant concomitant increase in TH activity, catecholamine secretion, and apoptosis resistance is consistent with the phenotypes of SDHB-mutated paragangliomas

cells (Fig. 2d, f). ROS directly inhibits the catalytic activity of proline hydroxylase domain (PHD), with an inverse relationship between prolyl-hydroxylated HIF1α and the previously shown intracellular ROS levels [18, 31] (Fig. 1c). In support of this hypothesis, *SDHB* inhibition has been shown to result in normoxic HIF1α stabilization through an ROS-dependent mechanism [13]. When considering our findings in light of these previous studies, ROS generation and subsequent HIF1 stabilization may be necessary for apoptosis resistance in SDH-deficient PC12 cells.

HIF1α plays an important role in promoting anti-apoptosis. However, the underlying mechanisms remain to be elucidated. Recently, the anti-apoptotic genes Bcl-xL [32], Mcl-1 [33], and survivin [34] were identified as HIF1α targets. Bcl-2 is a key molecule involved in hypoxia-induced resistance to cell death, and Bcl-2 overexpression has been associated with the prognosis of pheochromocytomas [35, 36]. However, the mechanism by which hypoxia induces Bcl-2 upregulation as well as the relationship between HIF1α and Bcl-2 remain unknown. In the present study, we identified Bcl-2 as an HIF1α target. Silencing of HIF1α attenuated apoptosis reduction (Fig. 2f) and Bcl-2 induction (Fig. 3c) in SDH-deficient PC12 cells, indicating HIF1α directly regulates Bcl-2 expression at the transcriptional level. These findings suggest that HIF1α-dependent Bcl-2 overexpression is an important mechanism by which HIF1α protects pheochromocytoma cells from apoptosis contributing to treatment failure.

An important unanswered question in the present study is the extent to which HIF-independent functions are involved in apoptosis reduction. In addition to pseudo-hypoxia, succinate may inhibit PHD3 (EglN3)-mediated developmental apoptosis of PC12 cells [37]. Moreover, HIF-independent pVHL functions have been implicated in the pathogenesis of renal cell carcinoma (RCC) [38].

A majority of HPPS cases with *SDHx* mutation are associated with an increase in catecholamine production. Consistent with this notion, *SDHB* knockdown increased TH activity in the present study. Moreover, increased TH

enzyme activity was associated with an increase in TH phosphorylation at Ser19, Ser31, and Ser40 as well as total protein level (Fig. 4a, d). In addition, *SDHB* knockdown significantly increased catecholamine secretion (Fig. 6). Scavenging of ROS by antioxidants (NAC) or the knockdown of *HIF1α* ameliorated elevated TH activity (Fig. 4b, c, e, f) and catecholamine oversecretion (Fig. 5b, c) in response to *SDHB* knockdown. Thus, as with the reduction in apoptosis levels, the accumulation of ROS and subsequent HIF1αcstabilization may be necessary for catecholamine oversecretion in SDH-deficient PC12 cells.

At present, the precise mechanism by which pseudo-hypoxic drive stimulates TH activity remains unknown. We detected increased TH phosphorylation at Ser40, Ser31, and Ser19 without changes in the levels of other protein kinases, such as PKA/C and ERK, in SDH-deficient cells. Although we cannot exclude the possibility that kinases other than PKA/C, ERK, and CaM may be involved in the phosphorylation of TH observed in the present study, increased TH phosphorylation may in part be a consequence of TH protein upregulation for the following reasons (summarized in Fig. 6):

1. Previously, increased TH activity in chronic sustained hypoxia was primarily attributed to the upregulation of TH protein [25, 26].
2. As the knockdown of *SDHB* increases TH protein level and phosphorylation to the same degree, it appears that upregulation of TH protein is the primary mechanism underlying increased levels of phosphorylated TH (Fig. 4d).
3. Transcription of the *TH* gene is activated by HIFs that interact with a specific hypoxia response element (HRE) in the proximal region of the TH promoter in PC12 cells [39–42]. Thus, HIF1α-dependent accumulation of TH protein underlies increased phosphorylated TH levels, consistent with the findings of the present study (Fig. 4f).

Hypoxia regulates TH promoter activity via three different groups of transcription factors that bind to the promoter sites HRE1, AP1, and CRE. It is unclear whether the transcriptional activation of TH by hypoxia requires the cooperative activity of these three groups of factors or whether the activation of multiple transcription factors represents a redundancy of mechanisms, which could assure the induction of TH transcription during hypoxia [23, 40, 41]. Further studies involving the use of TH promoter assays and mutagenesis of consensus sites are required to clarify the mechanisms underlying increased TH activity.

From a clinical point of view, our present findings raise the possibility that silencing of HIF1α may sensitize pheochromocytoma cells to therapeutic agents and ameliorate catecholamine oversecretion. Thus, HIF1α and related molecules may represent potential therapeutic targets in pseudo-hypoxic–driven pheochromocytomas [43].

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest, financial or otherwise.

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