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ORIGINAL ARTICLE

DT-Diaphorase Prevents Aminochrome-Induced Lysosome Dysfunction in SH-SY5Y Cells

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Abstract

Aminochrome has been reported to induce lysosomal dysfunction by inhibiting the vacuolar H-type ATPase localized in lysosome membrane. DT-diaphorase has been proposed to prevent aminochrome neurotoxicity but it is unknown whether this enzyme prevents aminochrome-induced lysosomal dysfunction. In the present study, we tested the protective role of DT-diaphorase in lysosomal dysfunction by generating a cell line (SH-SY5YsiNQ7) with a stable expression of a siRNA against DT-diaphorase with only 10% expression of mRNA enzyme. The cells differentiated with retinoic acid and 12-*o*-tetradecanoylphorbol-13-acetate show a significant increase in the expression of tyrosine hydroxylase, vesicular monoamine transporter-2, and dopamine transporter. The incubation of SH-SY5YsiNQ7 cells with 10 μM aminochrome resulted in a significant decrease of lysosome pH determined by using acridine orange, while aminochrome has no effect on SH-SY5Y cells. These results support the proposed protective role of DT-diaphorase against aminochrome-induced lysosomal dysfunction.

Keywords DT-Diaphorase · Lysosomal dysfunction · Aminochrome · Dopamine · Neurotoxicity · Neuroprotection

Aminochrome forms adducts with lysosomal vacuolar-type H+-ATPase, resulting in a pH increase (Huenchuguala et al. 2014). Lysosomes play a key role in protein turnover and in the degradation of damaged proteins and organelles such as mitochondria. Lysosomal protein degradation depends on a low pH environment in the lysosomes in order to facilitate protease activity by inducing protein denaturation. The acidification is dependent on the vacuolar-type H+-ATPase, which pumps protons into the lumen (Ishida et al. 2013). In addition, aminochrome is also neurotoxic as it induces mitochondrial dysfunction, aggregation of alpha-synuclein to neurotoxic oligomers, proteasomal system dysfunction, neuroinflammation, and oxidative and endoplasmic reticulum stress (Aguirre et al. 2012; Arriagada et al. 2004; Huenchuguala et al. 2014; Muñoz et al. 2015; Xiong et al. 2014; Zafar et al. 2006;). Therefore, it has been proposed that aminochrome triggers the neurodegeneration of the nigrostriatal system in Parkinson's disease. Interestingly, neuromelanin formation and accumulation over time is a normal pathway in healthy individuals (Zecca et al. 2002) due to the existence of two enzymes, DT-diaphorase and glutathione transferase M2-2 that inhibit the activation of aminochrome in dopaminergic neurons and astrocytes (Lozano et al. 2010; Huenchuguala et al. 2014). Glutathione transferase M2-2 prevents aminochrome-induced lysosomal dysfunction but it is not known whether DT-diaphorase is able to prevent aminochrome-induced lysosomal dysfunction. Therefore, the aim of this paper is to demonstrate the protective role of DT-diaphorase by preventing lysosomal dysfunction in a differentiated cell line of dopaminergic features with the silenced expression of DT-diaphorase.

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Material and Methods

Chemicals

Dopamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Aminochrome was synthesized by oxidizing dopamine as described before (Paris et al. 2010) but 25 mM potassium phosphate buffer at pH 6.0 was used instead of MES buffer. The aminochrome concentration was calculated

using the molar extinction coefficient 3058 M^{-1} cm^{-1} at a wavelength of 480 nm (Segura-Aguilar and Lind 1989).

Cell Lines

To test the ability of DT-diaphorase to prevent lysosomal dysfunction, we generated a cell line with a silenced expression of the DT-diaphorase gene.

SH-SY5Y cells were transduced with retroviral particles containing a pSuper.retro.puro plasmid (pSR) coding a siRNA against DT-diaphorase (SH-Sy5YsiNQ7 cells) or with empty plasmid (SH-SY5YpSR cells), as described before (Lozano et al. 2010). The cells grow in a culture medium which included DMEM/F12 culture medium (Hyclone Sh300004.04) adjusted to pH 7.4 and supplemented with 7.5% bovine adult serum; 2.5% bovine fetal serum, 1X non-essential amino acids (MEM, Hyclone sh30238.01), 1X antibiotic/antifungal (Biological Industries 03-033-1B), at 37 °C, with 5% carbon dioxide. Cell differentiation was performed with 10 mM retinoic acid over 3 days and, later, in 80 nM 12-O-tetradecanoylphorbol-13-acetate for 3 days. The cells were incubated as described by Cuevas et al. (2015). Cell death was measured as described before (Paris et al. 2010) using calcein AM (L3224 Invitrogen, USA) and propidium iodide (Sigma-Aldrich, USA). The non-fluorescent calcein AM is a hydrophobic compound that penetrates intact live cells. Inside the cells calcein AM is hydrolized by intracellular esterases generating a hydrophilic compound with strong fluorescence, which is well-retained in the intact cell cytoplasm. Propidium iodide intercalates DNA of cells with damaged cell membrane.

Acridine Orange Staining

The fluorescent dye acridine orange (Invitrogen, no. A3568) was used to determine changes in lysosome pH. The cells treated with 10 μM aminochrome for 24 h were stained with 2 μg ml^{-1} acridine orange (Molecular Probes, Life Technologies, Invitrogen). After 30 min, the cells were washed three times with cold PBS, and observed immediately in a fluorescence microscope (Leica DMI6000B) with a × 40 magnification with excitation wavelength 488 nm, green emission wavelength 510–530 nm, and red 650 nm. The number of cells showing red acridine orange fluorescence was plotted (Huenchuguala et al. 2014).

Real-time PCR

Real-time PCR was performed as previously described (Huenchuguala et al. 2014). Total RNA from the cells was extracted with TRIZOL reagent (Invitrogen, 15596-026) according to the manufacturer's protocol, and was quantified using Nanodrop 3300 (Thermo). The cDNA was synthesized using oligo-dT (IDT) and epicenter RT reagents according to the manufacturer's instructions (Huenchuguala et al. 2014). Comparative quantitation real-time PCR for the DT-diaphorase gene (Fw 5′-GCCGCAGACCTTGTGATATT-3′; Rv 5′-CGGAAGGGTCCTTTGTCATA-3′) was performed in triplicate using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, 600886) in a Stratagene Mx3000p Detection System.

Immunofluorescence The treated cells were fixed by adding 4% paraformaldehyde (PFA) to each well and incubated for 30 min. After 30 min, the PFA was removed and the cells were washed three times with PBS for 5 min. The cells were incubated 1 h with blocking solution (1% BSA with 0.1% Triton X-100 in PBS) and then primary antibodies overnight at 4 °C in a solution containing 0.5% BSA with 0.1% Triton X-100 in PBS.

The primary and secondary antibodies for expression of VMAT-2 were sc-7721-01 (dilution 1:100, Santa Cruz Technology, USA) and Alexa Fluor 488 (Jackson 705-545-003, Jackson ImmunoResearch, USA); dopamine transporters were sc-7515 (dilution 1:100, Santa Cruz Technology, USA) and donkey anti-rabbit 488 (Jackson InmunoResearch 711-545-152); and tyrosine hydroxylase were sc-7847 (dilution 1:100, Santa Cruz Technology, USA) and Alexa Fluor anti-rabbit (Invitrogen, USA). The immunofluorescence was determined by using a Zeiss microscope (Gottingen, Germany, model Axio Observer-Z1).

Statistical Analysis All data were expressed as mean ± SEM values. The statistical significance was assessed using an analysis of variance (ANOVA) for multiple comparisons and Student's *t* test.

Results and Discussion

Lysosomal dysfunction can account for destruction of neuromelanin-containing dopaminergic neurons in Parkinson's disease (Herrera et al. 2017). Integral lysosomal integrity is essential to mitochondrial function and production of cellular energy (ATP). Lysosomal dysfunction prevents the recycling of damaged mitochondria, resulting in mitochondrial dysfunction (inhibition of ATP production and a decrease in the mitochondrial membrane potential) and cell death (Huenchuguala et al. 2017; Segura-Aguilar and Huenchuguala 2018). To demonstrate the protective role of DT-diaphorase in aminochrome-induced lysosome dysfunction, we generated a SH-5Y5Ycell line

with silenced expression of DT-diaphorase gene. The transduced cells (SH-SY5YsiNQ7) with the siRNA against DT-diaphorase have only 10% mRNA expression compared to SH-SY5Y cells or SH-SY5YpSR cells (Fig. 1a). SH-SY5Y cells were differentiated in order to increase the expression of dopaminergic markers such as VMAT-2, dopamine transporter (DAT), and tyrosine hydroxylase. Compared to the immunofluorescence of undifferentiated cells, SH-SY5Y cells showed a significant 80-fold increase in tyrosine hydroxylase ($P < 0.001$), a 19-fold increase in vesicular monoamine transporter-2 (VMAT-2; $P < 0.001$), and a nine-fold increase in dopamine transporter (DAT; $P < 0.001$; Fig. 1b). Compared to the immunofluorescence of undifferentiated cells, SH-SY5YsiNQ7 cells showed a significant 18-fold increase in tyrosine hydroxylase ($P < 0.001$), a 21-fold increase in vesicular monoamine transporter-2 (VMAT-2; $P < 0.001$), and a 12-fold increase in dopamine transporter (DAT; $P < 0.001$; Fig. 1b).

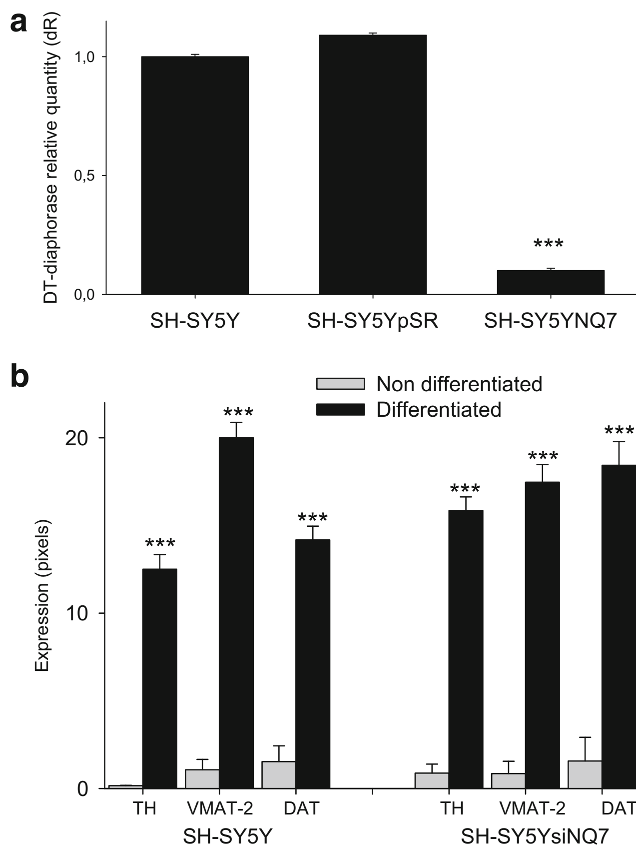

Fig. 1 DT-diaphorase and dopaminergic marker expression in SH-SY5Y cell lines. Differentiated SH-SY5Y and derived cell lines were used as cell models to test the protective role of DT-diaphorase against aminochrome-induced lysosomal dysfunction. SH-SY5Y cells and SH-SY5YpSR (expressing pSR empty plasmid) were used as control cells and SH-SY5YsiNQ7 cells expressing siRNA to silence the expression of DT-diaphorase. DT-diaphorase expression determined by real-time PCR in SH-SY5Y, SH-SY5YpSR (expressing pSR empty plasmid), and SH-SY5YsiNQ7 cells expressing siRNA is shown in **a**. The siRNA against DT-diaphorase silences 90% of this enzyme expression. The expression of the dopaminergic markers, VMAT-2, dopamine transporter (DAT), and tyrosine hydroxylase (TH), determined using immunofluorescence, is shown in **b**. The differentiation of SH-SY5Y, SH-SY5YpSR, and SH-SY5YsiNQ7 cells was performed with retinoic acid and TPA, as described in the section "Material and Methods." The values are the mean ± SEM, $n = 3$. The significance was measured with ANOVA for multiple comparisons and Student's *t* test (***$P < 0.001$)

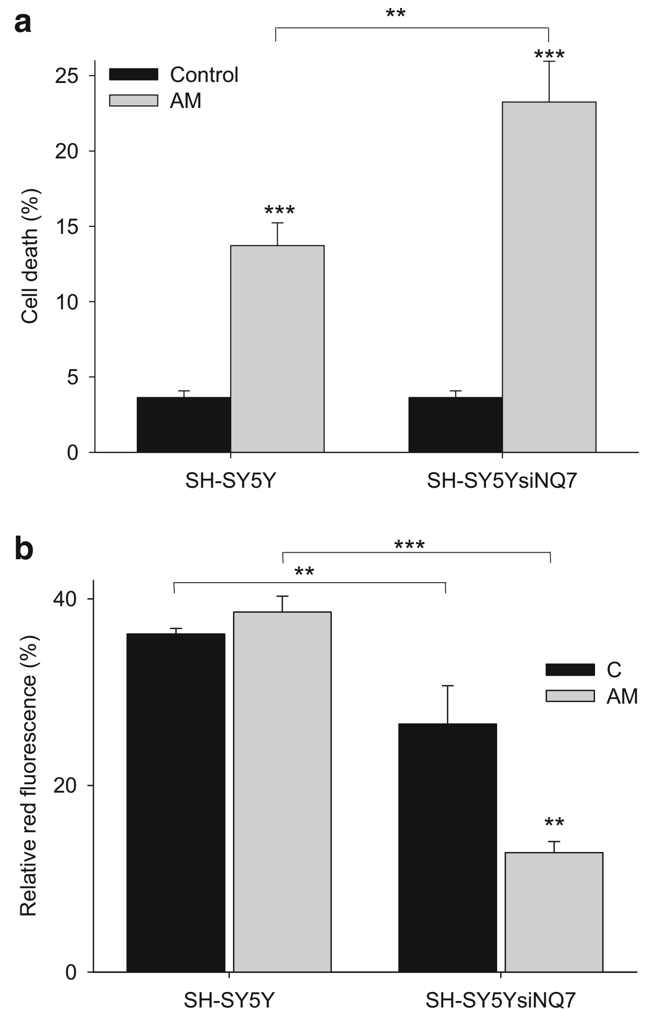

Fig. 2 The effect of DT-diaphorase on aminochrome-induced cell death and acridine orange staining. **a** The incubation of SH-SY5Y and SH-SY5YsiNQ7 cells with 10 μM aminochrome during 24-h induced significant cell death, in comparison to that with cells incubated with a culture medium. Compared with SH-SY5Y cells incubated with 10 μm aminochrome, a significant increase in cell death was observed in SH-SY5YsiNQ7 cells. **b** Aminochrome inhibits the vacuolar H-type ATPase localized in lysosome membrane that pumps protons into the lysosomes in order to maintain a low pH, which helps the denaturation of proteins that are going to be degraded by the action of proteolytic enzymes. The fluorescent dye acridine orange was used to determine changes in lysosome pH. Aminochrome induces a significant decrease in red acridine orange fluorescence in SH-SY5YsiNQ7 cells incubated with 10 μM aminochrome in comparison with SH-SY5Y cells or with SH-SY5YsiNQ7 cells incubated with a cell culture medium. The significance was measured with ANOVA for multiple comparisons and Student's *t* test (***$P < 0.001$; **$P < 0.01$)

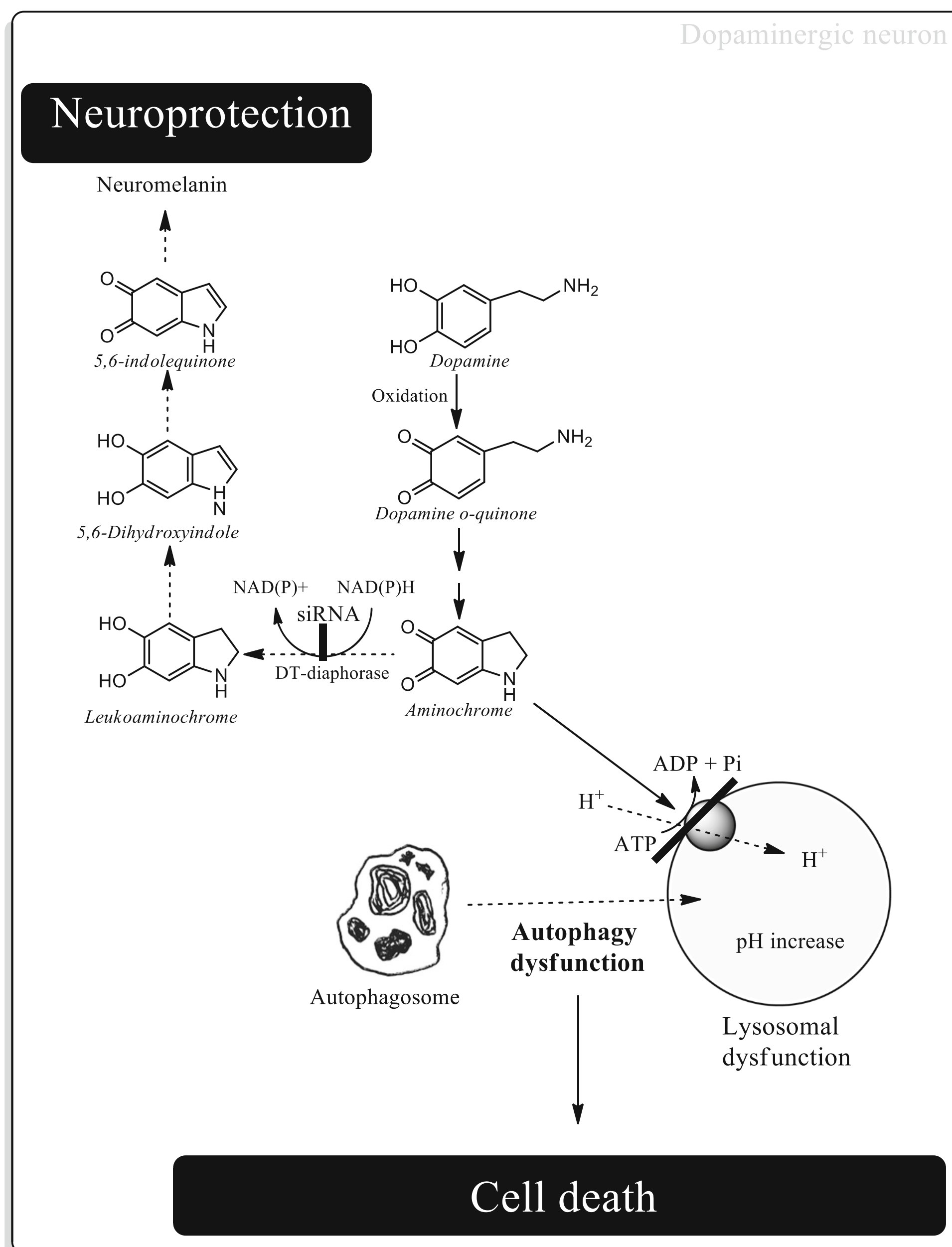

Fig. 3 DT-diaphorase prevents lysosome dysfunction-induced cell death. Dopamine oxidation to neuromelanin generates the formation of dopamine *o*-quinone that it is unstable at physiological pH and the amino group cyclizes to aminochrome. In the presence of DT-diaphorase, aminochrome is reduced to leukoaminochrome that finally forms neuromelanin after rearrangement to 5,6-dihydroxyindole and its oxidation to 5,6-indolequinone, which is the precursor of neuromelanin. Aminochrome forms adducts with the vacuolar-type H+-ATPase that pumps protons into the lysosomes, resulting in a lysosome dysfunction as a consequence of an increase in pH when DT-diaphorase is inhibited or silenced as in SH-SY5YsiNQ7 cells. The lysosomal protein degradation system plays a key role in cell viability by removing damaged mitochondria and proteins. An increase of lysosomal pH inhibits denaturation of proteins that is required for degradation of proteins catalyzed by proteolytic enzymes. However, our results show that DT-diaphorase prevents aminochrome-induced cell death and lysosome dysfunction

Aminochrome (10 μM, during 24 h) induces a significant increase in cell death in both SH-SY5Y and SH-SY5YsiNQ7 cells. However, a more significant increase in cell death was observed in SH-SY5YsiNQ7 cells compared with SH-SY5Y cells treated with 10 μM aminochrome ($P < 0.01$; Fig. 2a).

The incubation of SH-SY5Y cells with 10 μM aminochrome did not show a significant change in acridine orange staining in comparison with cells incubated with cell culture medium, demonstrating the protective role of DT-diaphorase.

The incubation of SH-SY5YsiNQ7 cells with 10 mM aminochrome induced a significant decrease in red acridine orange fluorescence compared to both with SH-SY5YsiNQ7 cells incubated with a culture medium (52%; $P < 0.01$) and SY-SY5Y cells incubated with 10 μM aminochrome (65%; $P < 0.001$), respectively. Interestingly, SH-SY5YsiNQ7 cells incubated with a cell culture medium in the absence of aminochrome exhibited a significant decrease in red acridine orange fluorescence compared with SH-SY5Y cell incubated with a cell culture medium alone ($P < 0.01$; Fig. 2b).

We conclude that DT-diaphorase prevents aminochrome-induced lysosomal dysfunction and, in Parkinson's disease, supports its protective role against aminochrome neurotoxicity (Fig. 3) (Lozano et al. 2010; Arriagada et al. 2004; Herrera et al. 2017; Huenchuguala et al. 2016; Muñoz and Segura-Aguilar 2017; Muñoz et al. 2012).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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