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Altered dopamine homeostasis differentially affects mitochondrial voltage-dependent anion channels turnover

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ABSTRACT

Altered dopamine homeostasis plays a key role in the pathogenesis of Parkinson's disease. The generation of reactive oxygen species by spontaneous dopamine oxidation impairs mitochondrial function, causing in turn an enhancement of oxidative stress. Recent findings have highlighted the role of mitochondrial outer membrane proteins in the regulation of the correct disposal of damaged mitochondria. Here, we report the effect of altered dopamine homeostasis on the mitochondrial functionality in human neuroblastoma SH-SY5Y cells, a cellular model widely used to reproduce impaired dopamine homeostasis. We observed that dopamine significantly and relevantly reduces VDAC1 and VDAC2 levels without any change in the mRNA levels. Although mitochondria are depolarized by dopamine and mitochondrial calcium influx is reduced, dysfunctional mitochondria are not removed by mitophagy as it would be expected. Thus, alteration of dopamine homeostasis induces a mitochondrial depolarization not counteracted by the mitophagy quality control. As a consequence, the elimination of VDACs may contribute to the altered mitochondrial disposal in PD pathogenesis, thus enhancing the role of oxidative stress.

1. Introduction

Voltage-dependent anion channels (VDACs, also known as mitochondrial porins) are the most abundant proteins of the outer mitochondrial membrane. In mammals, the VDAC family consists of three proteins VDAC1, VDAC2 and VDAC3 [1]. They mediate the flow of ions and metabolites between the cytoplasm and the mitochondrial network and regulate Ca^{2+} signaling [2,3]. The recent studies about their role in regulating apoptosis, autophagy and energy production pointed out that they are much more than simple channels that passively allow the transit from and to mitochondria and that their activity is isoform-dependent [4–6].

Altered dopamine homeostasis and oxidative stress play a key role in the pathogenesis of Parkinson's disease (PD) [5–7]. Free cytosolic dopamine spontaneously oxidizes and generates reactive oxygen species (ROS) that impair mitochondrial functionality [8]. VDAC levels were observed to decrease in human neuroblastoma NMB cells after dopamine treatment, and this down-regulation was linked to dopamine-induced apoptosis [9]. Recently, the complete disappearance of one spot corresponding to VDAC2 was reported in the two-dimensional electrophoresis (2-DE) pattern of dopamine-treated human neuroblastoma SH-SY5Y cells [10]. Besides a strong evidence for a role of VDACs in regulating the apoptotic process, the biochemical mechanisms involved are not well characterized [4]. Ca^{2+} flux to mitochondria is considered an important signal for apoptosis induction [11]. In this view, the role of VDAC1 in the transmission of the low-amplitude apoptotic Ca^{2+} signal to mitochondria may justify its apoptotic feature [12].

A role of VDACs has been recently proposed also in guiding the mitophagic process [13]. It is still a matter of debate whether the three VDACs have an isoform-dependent role and whether they are dispensable in directing altered mitochondria to the autophagosome [13–15]. According to several reports, impaired mitochondria are labeled for mitophagy by parkin, a PD-related E3 ubiquitin ligase, after activation by PINK1, a PD-related protein kinase [14,16,17]. Strikingly, VDAC1 was identified as a target for parkin-mediated Lys27 poly-ubiquitinylation and mitophagy [14]. Thus, these data provide functional links between

Abbreviations: 2-DE, two-dimensional electrophoresis; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CMXRos, chloromethyl-X-rosamine; COX, cytochrome-c-oxidase; DAPI, 4′,6-diamidino-2-phenylindole; $\Delta\Psi_m$, mitochondrial membrane potential; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; KRB, Krebs–Ringer bicarbonate; MPP^+, 1-methyl-4-phenylpyridinium; mTOR, mammalian target-of-rapamycin; PD, Parkinson's disease; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel

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PINK1, parkin and the selective autophagy of mitochondria, which is implicated in the pathogenesis of PD [14].

In this view, the effect of impaired dopamine homeostasis on VDACs and on the related mitophagy pathway appears of great interest. To this purpose, we have investigated VDAC levels and the disposal of damaged mitochondria in human neuroblastoma SH-SY5Y cells, a cellular model widely used to reproduce impaired dopamine homeostasis [5].

2. Material and methods

2.1. Cells

Human neuroblastoma SH-SY5Y cells were obtained from the European Collection of Cell Cultures (Cat No. 94030304; Lot No. 11C016) and were cultured in 5% CO_2 humidified atmosphere at 37 °C in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All cell culture media and reagents were from Euroclone. Cells were treated for different times with 0.25 mM dopamine (Sigma-Aldrich) in the presence of 700 U/ml catalase (Sigma-Aldrich) to eliminate aspecific effects due to H_2O_2 arising from dopamine auto-oxidation [9].

For the time-course analysis, cells were treated or not for 3, 7 and 24 h with dopamine. To verify autophagy induction, 50 μM chloroquine was added to cells under 24 h dopamine treatment for the last 2 h. To induce autophagy, cells were treated overnight with 1 μM rapamycin. To inhibit proteasome, 1 μM MG132 (Sigma-Aldrich) was added to cells already treated with dopamine for 8 h, for other 16 h.

2.2. Quantitative Western blotting analysis

Cells were collected by centrifugation (300 ×*g*, 5 min, 25 °C) and resuspended in 100 μl RIPA buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% sodium deoxycholate) in the presence of protease (Sigma-Aldrich) and phosphatase (Roche) inhibitors (30 min, 4 °C under shaking). Extracts were cleared by centrifugation (10,000 ×*g*, 30 min, 4 °C) and protein concentration in the supernatants was determined spectrophotometrically with the bicinchoninic acid assay (Pierce).

Cell lysates (40 μg) were denatured in Laemmli sample buffer for 5 min at 98 °C and electrophoresed on 13% SDS-PAGE gel. Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) at 1.0 mA/cm^2, 1.5 h (TE77pwr, Hoefer). Membranes were saturated in 5% non-fat milk or 5% BSA in TBS-T (10 mM Tris–HCl pH 7.4, 150 mM NaCl and 0.05% Tween-20) and incubated in the same buffer at 4 °C overnight with rabbit anti-VDAC1 polyclonal antibody (Abcam), 1:600 dilution, with rabbit anti-VDAC2 polyclonal antibody (Sigma-Aldrich), 1:250 dilution, with rabbit anti-LC3 polyclonal antibody (Cell Signaling), 1:1000 dilution, with mouse anti-β-actin monoclonal antibody (GeneTex), 1:3000 dilution, or with rabbit anti-cytochrome-c-oxidase subunit 5B (COX5B) (Sigma-Aldrich), 1:1000 dilution. Membranes were then washed with TBS-T and incubated with peroxidase-conjugated anti-rabbit-IgG antibody (Thermo Scientific), 1:1000 in 5% milk-TBS-T, or anti-mouse-IgG antibody (Millipore), 1:3000 in 5% milk-TBS-T, respectively, for chemiluminescence detection (Millipore). Images (16 bit grayscale) were acquired with G:BOX Chemi XT4 (Syngene) system and analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Signal intensities were corrected for protein loading by normalization to β-actin intensity. Statistical significance was verified by Student's *t* test with Welch correction for heteroscedasticity.

2.3. RNA extraction, retrotranscription and quantitative PCR

RNA was extracted using SV total RNA isolation system (Promega) following the manufacturer's instructions and treated with rDNase in order to remove genomic DNA. The integrity of RNA was verified loading total RNA onto an agarose gel and visualizing the two bands of rRNA 28S (5 kb) and 18S (2 kb). One microgram of RNA was reverse transcribed into first-strand cDNA in a 20 μl final volume using the GoTaq® 2-Step RT-qPCR System (Promega). PCR was performed by Real-Time PCR (DNA Engine Opticon 2; MJ Research, USA) using a SYBR Green PCR mix (Promega). The expression of VDAC1 and VDAC2 was evaluated using primers reported in Table 1 after 2, 6 and 24 h dopamine treatment. Results were confirmed by three independent replicates. The specificity of the amplified products was checked by the melting temperature curve analysis and the expected size of the fragments was further visualized in a 2% agarose gel stained with ethidium bromide. Statistical significance was verified by Student's *t* test with Welch correction for heteroscedasticity.

2.4. Measurement of Ca^{2+} flux to mitochondria using Aequorin

Ca^{2+} measurements were performed by means of a luminescence system based on aequorin, a Ca^{2+}-sensitive globular protein containing a hydrophobic core cavity that accommodates the prosthetic group coelenterazine. Upon Ca^{2+} binding to aequorin EF-hand domains, coelenterazine is released (as coelenteramide) and a photon is emitted. Due to the huge dynamic range of aequorin-based probes, the technique is highly quantitative [18]. Activation of a membrane receptor coupled to a G_q protein leads to activation of PLCβ production of IP_3 and thus opening of ER Ca^{2+} releasing channel (IP_3R), with a consequent increase of [Ca^{2+}] both in the cytosol and in the mitochondrial matrix. SH-SY5Y cells express bradykinin receptors (type B2) that trigger the release of Ca^{2+} from the intracellular stores and thus activation of Ca^{2+} signaling [19].

In detail, 24 h before the transfection step, SH-SY5Y cells were plated on a 13 mm round coverslip at 30–50% confluence. Just before the transfection procedure, cells were washed with 1 ml of fresh medium. For each coverslip, 5 μl of 2.5 M $CaCl_2$ solution was added to 4 μg of MTS (mitochondrial target sequence)-aequorin (mtAEQ) DNA dissolved in 45 μl of Tris-EDTA (10 mM Trizma-base, 1 mM EDTA, pH 8). The solution was mixed and added to 50 μl of HEPES Buffered Saline Solution (HBS 2×, 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.12) while vortexing, and incubated for 30 min at RT. After 16 h, cells were washed three times with PBS and then treated or not with 0.25 mM dopamine for 24 h. One hour before measurements, cells were placed in KRB saline solution (in the presence of 1 mM $CaCl_2$ and 1.0 g/l glucose) supplemented with synthetic coelenterazine (5 μM) for aequorin reconstitution. The coverslip with transfected cells was transferred to the luminometer chamber and perfused with Krebs–Ringer bicarbonate (KRB) saline solution in the presence of 1 mM $CaCl_2$ and 1.0 g/l glucose to remove the excess of coelenterazine. Bradykinin (100 nM) was added to the perfusing medium. Each experiment was terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The light signal was collected by means of a low-noise photomultiplier placed in close proximity (2–3 mm) to aequorin expressing cells, and calibrated into [Ca^{2+}] values by an algorithm based on the Ca^{2+} response curve of aequorin at physiological conditions of pH, [Mg^{2+}] and ionic strength, as previously described [20]. Data are presented as representative traces and bar graphs showing the mean of the [Ca^{2+}] peak value reached during cellular stimulation.

Table 1
Sequences of specific primers: F = forward primer; R = reverse primer.

Gene	Primer sequence	Amplicon size (bp)
VDAC1 F	5′-GGGGCCCGGAAGGCAGAAGA-3′	82
VDAC1 R	5′-GCCCTTGGTGAAGACATCCTTGG-3′	
VDAC2 F	5′-CGGGGTCCTGTCTGGGAGAG-3′	70
VDAC2 R	5′-GCTGGAGGGCGAAGTGAAGG -3′	
GAPDH F	5′-GAGTCAACGGATTTGGTCGT-3′	238
GAPDH R	5′-TTGATTTTGGAGGGATCTCG-3′	