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Overexpression of VMAT-2 and DT-diaphorase protects substantia nigra-derived cells against aminochrome neurotoxicity

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ABSTRACT

We tested the hypothesis that both VMAT-2 and DT-diaphorase are an important cellular defense against aminochrome-dependent neurotoxicity during dopamine oxidation. A cell line with VMAT-2 and DT-diaphorase over-expressed was created. The transfection of RCSN-3 cells with a bicistronic plasmid coding for VMAT-2 fused with GFP-IRES-DT-diaphorase cDNA induced a significant increase in protein expression of VMAT-2 (7-fold; $P<0.001$) and DT-diaphorase (9-fold; $P<0.001$), accompanied by a 4- and 5.5-fold significant increase in transport and enzyme activity, respectively. Studies with synaptic vesicles from rat substantia nigra revealed that VMAT-2 uptake of ^{3}H-aminochrome 6.3 ± 0.4 nmol/min/mg was similar to dopamine uptake 6.2 ± 0.3 nmol/min/mg that which were dependent on ATP. Interestingly, aminochrome uptake was inhibited by 2 μM lobeline but not reserpine (1 and 10 μM). Incubation of cells overexpressing VMAT-2 and DT-diaphorase with 20 μM aminochrome resulted in (i) a significant decrease in cell death (6-fold, $P<0.001$); (ii) normal ultra structure determined by transmission electron microscopy contrasting with a significant increase of autophagosome and a dramatic remodeling of the mitochondrial inner membrane in wild type cells; (iii) normal level of ATP (256 ± 11 μM) contrasting with a significant decrease in wild type cells (121 ± 11 μM, $P<0.001$); and (iv) a significant decrease in DNA laddering (21 ± 8 pixels, $P<0.001$) cells in comparison with wild type cells treated with 20 μM aminochrome (269 ± 9). These results support our hypothesis that VMAT-2 and DT-diaphorase are an important defense system against aminochrome formed during dopamine oxidation.

1. Introduction

Vesicular monoamine transporter-2 (VMAT-2) is able to take up dopamine into monoaminergic vesicles by using a vesicular proton pump associated to ATPase that under hydrolysis of ATP to ADP and Pi one proton (H^+) is translocated into the vesicle, generating an electrochemical gradient of protons. VMAT-2 uses this electrochemical gradient to take up one molecule of dopamine by releasing 2 protons [1,2]. This electrochemical gradient creates a low pH environment inside the monoaminergic vesicles estimated to be 2 to 2.4 pH units lower than in the cytosol [3], where the protons of dopamine hydroxyl groups are very hard bound to the oxygen, preventing dopamine oxidation. However, in the cytosol the protons of dopamine hydroxyl groups can dissociate and in the presence of oxygen dopamine oxidizes spontaneously without the necessity of metal-ion catalysis [4]. Dopamine oxidation to aminochrome catalyzed by oxygen is accompanied with the formation of superoxide radicals that can enzymatically or spontaneously generate hydrogen peroxide, the precursor of hydroxyl radicals. This oxidative mechanism of dopamine can be potentiated by drugs such as methamphetamine. VMAT-2 is able to take up methamphetamine into monoaminergic vesicles, inducing the release of dopamine to the cytosol that is an important event for methamphetamine neurotoxicity. The role of cytosolic dopamine in methamphetamine neurotoxicity has been supported by the fact that the inhibition of dopamine synthesis protects against methamphetamine neurotoxicity while the inhibition of VMAT-2 and monoamine oxidase exacerbate methamphetamine neurotoxicity [for review see 5].

Dopamine oxidation to aminochrome and its polymerization to neuromelanin seems to be a natural occurring process which accumulates with age [6,7]. Motor symptoms in Parkinson's disease are primarily the result from a selective loss of the neuromelanin-containing dopaminergic neurons of the substantia nigra while the unpigmented dopaminergic neurons are spared [8,9]. However, the question is why melanin containing dopaminergic neurons degenerate during Parkinson's disease when the neuromelanin formation itself is not neurotoxic. One possible explanation is that aminochrome

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is able to participate in neurotoxic reactions such as (i) the formation of adducts with alpha synuclein to produce and stabilize the formation of neurotoxic protofibers [10,11]; (ii) inducing mitochondrial dysfunction [12]; (iii) inactivation of proteosomal system [13,14]; (iv) disruption of cytoskeleton architecture [15]; and (v) formation of reactive oxygen species [16].

A complete knockout of VMAT2 (−/−) induces that mice move little, feed poorly, and die within a few days after birth while amphetamine increases movement, promotes feeding, and prolongs their survival [17]. VMAT2-deficient animals have decreased motor function, progressive deficits in olfactory discrimination, shorter latency to behavioral signs of sleep, delayed gastric emptying, anxiety-like behaviors at younger ages, and a progressive depressive-like phenotype [18]. Overexpression of VMAT-2 decreases cytosolic dopamine and inhibit neuromelanin synthesis, preventing dopamine oxidation to *o*-quinones [19]. Experiments done with human brain tissues support this idea since they found an inverse relationship between the level of expression of VMAT-2 and the amount of neuromelanin and the vulnerability to neurodegeneration in Parkinson's disease [20]. Therefore, VMAT-2 should play a protective role in dopaminergic neuron by preventing dopamine oxidation in the cytosol to aminochrome.

DT-Diaphorase (EC.1.6.99.2) is the unique flavoenzymes that catalyzes the two-electron reduction of aminochrome to leukoaminochrome and that can use both NADH and NADPH as electron donators [21]. This reaction is neuroprotective since it prevents one-electron reduction to leukoaminochrome *o*-semiquinone radical that it's extremely reactive with oxygen and neurotoxic [15,16,22–24]. DT-Diaphorase has been reported to prevent the cytoskeleton disruption [15], mitochondria damage [16,24], the formation of alpha synuclein protofibrils [25,26] and protects against aminochrome-induced proteasome inhibition [13]. Therefore, the aim of this work was to investigate the possible protective role of both of VMAT-2 and DT-diaphorase against aminochrome neurotoxicity by creating a cell line with high expression of VMAT-2 and DT-diaphorase.

2. Materials and methods

2.1. Chemicals

Dopamine, reserpine, dicoumarol, DME/HAM-F12 nutrient mixture (1:1) and Dulbecco's phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. (St Louis. MO. USA). Lobeline was obtained from Fluka. Calcein AM and ethidium homodimer-1 were obtained from Molecular Probe (Eugene. OR. USA). Thermoscript RT-PCR system and Taq DNA polymerase were obtained from Life Technologies (California. USA). Trizol reagent was obtained from Invitrogen (California. USA). The primers were obtained from T-A-G-Copenhagen A/S (Copenhagen. Denmark).

2.2. Preparation of synaptic vesicles and uptake

The purification of presynaptic vesicles was performed by using a Synaptic Vesicles Isolation KIT (Sigma-Aldrich Co., St. Louis. USA). After the gradient centrifugation, the protein concentration of the fraction containing synaptic vesicles was determined by Bradford method. Synaptic vesicles (2.5 μg protein) were incubated at 37 °C during 0, 60, 90 and 120 min in a buffer containing 25 mM HEPES, 100 mM potassium tartrate, 100 mM sodium tartrate, 1.7 mM ascorbic acid, 0.05 mM EGTA, 0.1 mM EDTA, and 2 mM ATP-Mg^{+2} at pH 7.5 in the presence of 50 nM ^{3}H-dopamine or ^{3}H-aminochrome. The samples were filtrated with a Sephadex G-25 to remove extracellular ^{3}H-dopamine or ^{3}H-aminochrome. The vesicles were disrupted by adding RIPA buffer and the isotopes were measured the counts per minute by using scintillate instrument.

2.3. Cell culture

The RCSN-3 cell line was derived from the substantia nigra of a 4-month-old normal Fisher 344 rat. The RCSN-3 cell line grows in monolayer with a doubling time of 52 h at a plating efficiency of 21% and a saturation density of 56,000 cells/cm^2 when kept in normal growth media composed of: DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, and 40 mg/l gentamicine sulfate [27,28]. Cultures were kept in an incubator at 37 °C with 100% humidity and an atmosphere of 10% CO_2.

2.4. Cell transfection

The OmicsLink™ bicistronic plasmid pVMAT2GFPDTwith internal ribosome entry site (IRES) was used as expression vector systems of human synaptic vesicle monoamine transporter (accession no.: L09118), 1545 bp length fused in the C-terminal with GFP and *Homo sapiens* DT-diaphorase (NAD(P)H dehydrogenase quinone; accession no: NM_000903), and 825 bp length (GeneCopoeia). The transfection solutions was prepared by mixing 50 mM HEPES buffer, 30 mM NaCl, 1.5 mM Na_2HPO_4 pH 6.9, DNA plasmid and 2.5 M $CaCl_2$ and incubated at room temperature during 20 min. RCSN-3 cells in 60% confluence were transfected with this solution added slowly and mixing gently. The cells were incubated during 48 to 72 h at 37 °C.

2.5. Dot blot

Dot blots were performed by using a Bio-Rad Bio-Dot dot-blot apparatus assembled with a nitrocellulose membrane that previously was immersed in 20 mM Tris pH 7.6 containing 136 mM NaCl was added to each well before the addition of 50–200 μl samples containing 50 μg protein. The vacuum connected to the dot blot equipment is allowed to continue until the membrane is dry. The nitrocellulose membrane was blocked by incubating them in 20 mM Tris pH 7.6 containing 136 mM NaCl, 0.1% Tween 20, low fat milk 5% during 3 h at room temperature with gently shaking. Wash the membrane 3 times during 5 min by using a solution of 20 mM Tris pH 7.6 containing 136 mM NaCl, 0.1% Tween 20. Incubate the membrane in a solution of 20 mM Tris pH 7.6 containing 136 mM NaCl, 0.1% Tween 20, 5% BSA and polyclonal antibodies against DT-diaphorase diluted 1:1000 (SC-7012, Santa Cruz Biotechnology Inc). VMAT-2 diluted 1:1000 (AB1767, Millipore Chemicon) and actin diluted 1:1000 (SC-1615, Santa Cruz Biotechnology Inc). The membrane were washed 3 times 5 min and incubated in 20 mM Tris pH 7.6 containing 136 mM NaCl, 0.1% Tween 20, 5% BSA and secondary antibody conjugated with HRP (horseradish peroxidase) diluted 1:10,000. The quantification of dot blot bands was performed by scanning the nitrocellulose membranes with scion image program (NIH) and they were expressed as pixels.

2.6. Determination of GFP fluorescence with confocal microscopy

Cover slips were mounted on to slides with fluorescent mounting medium (Dako, Carpinteria, CA. USA) and kept in the dark at 4 °C. Confocal microscopy (Zeiss, Göttingen. Germany; model LSM-410 Axiovert-100) was used to study the cells. Sample illumination was carried out via a He–Ne laser with 543-nm excitation filter and emission filter over 560 nm. The nuclei were marked with DAPI staining.

2.7. VMAT-2 activity determination

VMAT-2 activity was determined by measuring ^{3}H-dopamine transport in RCSN-3 and RCSN3VMATGFPDT cells with stable overexpression of VMAT2. The cells were harvested and collected by centrifugation (2000 rpm for 5 min) in PBS, resuspended at 1.25×10^6 cells/ml in KT-HEPES buffer (25 mM HEPES; 100 mM potassium tartrate; 0.1 mM