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Brief communication

DT-diaphorase protects astrocytes from aminochrome-induced toxicity

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ABSTRACT

Astrocytes are exposed to aminochrome via the oxidation of dopamine that is taken up from the synaptic cleft after its release from dopaminergic neurons. Glutathione transferase M2-2 (GSTM2) has been shown to protect astrocytes from aminochrome-induced toxicity, but astrocytes also express DT-diaphorase, which has been shown to prevent aminochrome-induced neurotoxicity in dopaminergic neurons. Therefore, the question is whether DT-diaphorase also protects astrocytes from aminochrome-induced toxicity. DT-diaphorase is constitutively expressed in U373MG cells, and its inhibition by dicoumarol induced a significant increase of aminochrome-induced cell death. However, the inhibition of DT-diaphorase in U373MGsiGST6 cells, which have 74% of GSTM2 gene expression silenced, resulted in a more than 2-fold increase in cell death, suggesting that DT-diaphorase plays an important role in preventing aminochrome-induced toxicity in astrocytes.

1. Introduction

Oxidation of dopamine to neuromelanin seems to be a normal process that occurs in dopaminergic neurons that contain neuromelanin because it takes place in these neurons in healthy individuals. In Parkinson's disease, the loss of more than 60–70% of dopaminergic neurons that contain neuromelanin occurs before motor symptoms appear. The question is why these dopaminergic neurons that contain neuromelanin die. Dopamine oxidation generates several *o*-quinones in a sequential manner (dopamine → dopamine *o*-quinone → aminochrome → 5,6-indolequinone → neuromelanin). Aminochrome, the most stable *o*-quinone, can be neurotoxic when it forms adducts with proteins or when it is reduced by one electron by flavoenzymes, which catalyze one-electron transfers (Segura-Aguilar et al., 2014). Aminochrome induces (i) the formation of alpha synuclein neurotoxic oligomers (Muñoz et al., 2015), (ii) dysfunction of protein degradation in both the proteasomal and lysosomal systems (Zafar et al., 2006; Huenchuguala et al., 2014; Muñoz et al., 2012a), (iii) mitochondrial dysfunction (Aguirre et al., 2012; Muñoz et al., 2012b; Arriagada et al., 2004), (iv) oxidative stress (Arriagada et al., 2004; Segura-Aguilar et al., 1998), and (v) endoplasmic reticulum stress (Xiong et al., 2014).

Dopamine that is released during neurotransmission is removed from the synaptic cleft by dopamine transporters in dopaminergic neurons, but the synaptic terminals of dopaminergic neurons are surrounded by other neurons and astrocytes, which are also able to take up dopamine. Therefore, the oxidation of dopamine to aminochrome is feasible in astrocytes and astrocytes have constitutive expression of GSTM2 to catalyze the conjugation of aminochrome with glutathione. DT-diaphorase is expressed in both dopaminergic neurons and astrocytes, and the question being addressed here is whether this enzyme plays a role in the protection of astrocytes against aminochrome-induced toxicity.

2. Materials and methods

2.1. Chemical

A LIVE/DEAD Viability/Cytotoxicity kit was purchased from Invitrogen (Cat. # L3224). Aminochrome was prepared by using tyrosinase to oxidize dopamine (Sigma Aldrich, Cat. # T3824-50KU and H8502-10G, respectively) and purified according to methods we have described previously (Paris et al., 2010). We used an antibody against DT-diaphorase (NQO1) from Santa Cruz Biotechnology.

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2.2. Cell lines

In this study we used the U373MG cell line, which is a permanent human astrocytoma cell line with constitutive expression of GSTM2. We used U373MGsiGT6 cells in which GSTM2 expression is 74% silenced by siRNA and U373pSR cells, which are transduced with the plasmid vector pSuper.retro.puro (pSR) alone. Cells were cultured as described previously (Huenchuguala et al., 2014).

2.3. Western blot

Homogenates of U373MG cells (100 μg) were separated on SDS-PAGE (10% w/v) gels and transferred to 0.2 μn nitrocellulose membranes. The membranes were incubated with an antibody against DT-diaphorase, obtained from Santa Cruz Biotechnology or an antibody against GSTM2 obtained from Prof. Bengt Mannervik, Stockholm Universitet, Sweden, in TBS buffer containing 5% skim milk and 0.1% Tween 20. The antibody against actin was from Sigma Aldrich (A2668-2ML; polyclonal rabbit 1:1000). The membranes were stained with BCIP/NBT (Invitrogen, N6547). Protein ladder for western blot experiments were from New Englands Biolabs Inc. (Broad Range) for DT-diaphorase and Santa Cruz Biotechnology INC (Broad Range Markers: sc-2361) for GSTM2.

2.4. Cell death

Cell death was measured by staining with 0.5 μM calcein AM and 5 μM ethidium homodimer-1 for 45 min at room temperature in the dark (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes) and then counting live and dead cells. Calcein AM is a marker for live cells, and ethidium homodimer-1 intercalates into the DNA of dead cells. Cells were counted with a phase contrast fluorescent microscope using the following filters: calcein AM, 450–490 nm (excitation) and 515–565 nm (emission); and ethidium homodimer-1, 510–560 nm (excitation) and LP–590 nm (emission).

2.5. Statistical analysis

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

3. Results

To study the possible role of DT-diaphorase in astrocytes, we used U373MG cells derived from a human astrocytoma as a model cell line. We used western blotting to determine the constitutive expression of DT-diaphorase in U373MG cells and found that DT-diaphorase was in a dimeric form and had a molecular weight of approximately 62 kDa (Fig. 1A). GSTM2 was constitutively expressed in U373MG and U373MGpSR cells and silenced in U373MGsiGST6 cells expressing a siRNA against GSTM2 (Fig. 1B).

The incubation of U373MG cells with 30 μM aminochrome or 100 μM dicoumarol, an inhibitor of DT-diaphorase, separately, did not induce significant cell death. However, the incubation of U373MG cells with 30 μM aminochrome and 100 μM dicoumarol together induced significant cell death (24 ± 3%; $P < 0.001$). Recently, it has been reported that GSTM2 protects U373MG cells against aminochrome-induced toxicity by catalyzing aminochrome conjugation with GSH (Huenchuguala et al., 2014). Therefore, we used a U373MG cell line expressing a siRNA against GSTM2 (U373MGsiGST6 cells) to silence GSTM2 activity so that we could determine the protective role of DT-diaphorase against

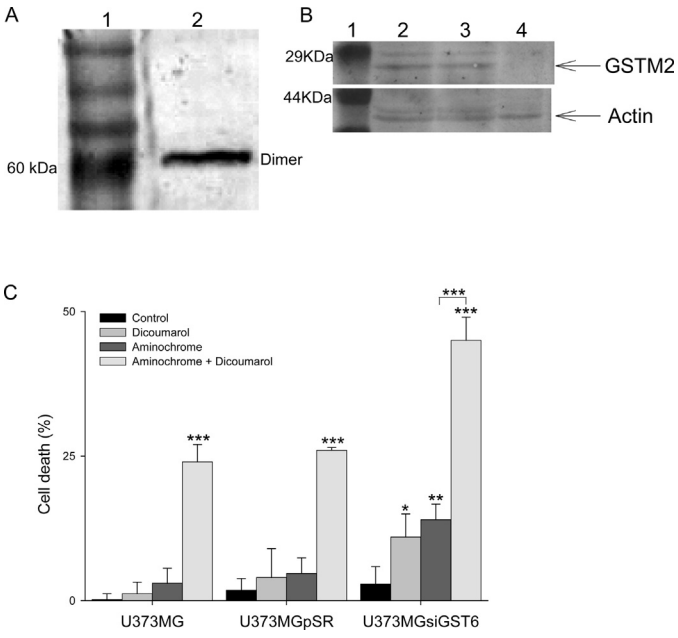

Fig. 1. The inhibition of DT-diaphorase with dicoumarol increases aminochrome-induced cell death in U373MGsiGST6 cells. (A) DT-diaphorase expression in U373MG cells was determined by western blot (100 μg protein; lane 2) and compared to a protein ladder (lane 1). (B) GSTM2 expression was determined by western blot (100 μg protein) in U373MG cells (ladder 2), U373MGpSR (ladder 3) and U373MGsiGST6 cells (ladder 4) and compared to a protein ladder (ladder 1). (C) Aminochrome (30 μM) induces significant cell death only in U373MGsiGST6 cells ($P < 0.01$) at 24 h, but inhibition of DT-diaphorase with 100 μM dicoumarol significantly increases aminochrome-induced cell death in U373MGsiGST6 cells that have 74% of the expression of GSTM2 silenced by a siRNA ($P < 0.001$). Aminochrome is only neurotoxic when DT-diaphorase is inhibited by dicoumarol both in wild type cells U373MG cells and in U373MG cells that have been transduced with an empty retrovirus plasmid. Interestingly, 100 μM dicoumarol induces cell death only in U373MGsiGST6 cells. We used a high concentration of dicoumarol (100 μM) to inhibit DT-diaphorase because dicoumarol form adducts with a number of proteins, which decreases its concentration in the cytosol. Dicoumarol at a high concentration only induces a rate of cell death of 1.2% in U373MG cells. The rate of cell death was measured by using a LIVE/DEAD Viability/Cytotoxicity kit from Molecular Probes, which uses calcein AM and ethidium homodimer-1 as markers for live and dead cells, respectively. The values given are the mean ± SD (n = 3) and statistical significance was assessed by using an analysis of variance (ANOVA) for multiple comparisons.

aminochrome. The incubation of U373MGsiGST6 cells with 30 μM aminochrome induced a rate of cell death of 14 ± 3% ($P < 0.01$). However, the incubation of U373MGsiGST6 cells with 30 μM aminochrome and 100 μM dicoumarol together induced a rate of cell death of 45 ± 4% ($P < 0.001$), which was significantly higher than the rate of cell death induced by 30 μM aminochrome ($P < 0.001$) or 100 μM dicoumarol ($P < 0.001$) alone. (Fig. 1C).

4. Discussion

Both GSTM2 and DT-diaphorase seem to play important roles in the prevention of aminochrome-induced toxicity in astrocytes given that the inhibition of DT-diaphorase resulted in a significant increase in cell death in U373MGsiGST6 cells, which have stable expression of a siRNA against GSTM2 (Fig. 1). It has been reported that aminochrome uptake increases in U373MG cells for 40 min (Cuevas et al., 2015). There is increasing evidence that the *o*-quinones, especially aminochrome, generated during the oxidation of dopamine, directly participate in the loss of neuromelanin containing dopaminergic neurons in the nigrostriatal system through their involvement in mitochondria dysfunction, aggregation of alpha synuclein to neurotoxic oligomers, dysfunction of protein degradation systems (both proteasomal and autophagy/

lysosomal), oxidative stress and endoplasmic reticulum stress (for review see Segura-Aguilar et al., 2014). The oxidation of dopamine to *o*-quinones (dopamine *o*-quinone → aminochrome → 5,6-indolequinone) eventually results in the formation of neuromelanin, which seems to play a protective role in dopaminergic neurons in the nigrostriatal system because these neurons are intact in healthy individuals. However, aminochrome, the most stable *o*-quinone formed during the oxidation of dopamine, can be toxic if it forms adducts with proteins or when it is reduced by one electron by flavoenzymes. Astrocytes seem to have greater protection against aminochrome-induced toxicity than dopaminergic neurons because they express both GSTM2 and DT-diaphorase, both of which prevent aminochrome-induced toxicity.

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