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Original Contribution

Mitochondrial UCP5 is neuroprotective by preserving mitochondrial membrane potential, ATP levels, and reducing oxidative stress in MPP^+ and dopamine toxicity

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ABSTRACT

We explored the protective mechanisms of human neuronal mitochondrial uncoupling protein-5 (UCP5) in MPP^+- and dopamine-induced toxicity after its stable overexpression in SH-SY5Y cells. We raised specific polyclonal antibodies. Overexpressed UCP5 localized in mitochondria but not in cytosol. UCP5 overexpression increased proton leak, decreased mitochondrial membrane potential (MMP), reduced ATP production, and increased overall oxygen consumption (demonstrating uncoupling activity). UCP5 overexpression did not affect other neuronal UCP expression (UCP2 and UCP4). Overexpressing UCP5 is protective against MPP^+- and dopamine-induced toxicity. MPP^+ and dopamine exposure for 6 h reduced MMP and increased superoxide levels. ATP levels in UCP5-overexpressing cells were preserved under MPP^+ and dopamine toxicity, comparable to levels in untreated vector controls. At 24 h, UCP5 overexpression preserved MMP, ATP levels, and cell survival; attenuated superoxide generation; and maintained oxidative phosphorylation as indicated by lower lactate levels. MPP^+ and dopamine exposure induced UCP5 mRNA transcription but did not decrease transcript degradation, as inhibition of transcription by actinomycin-D abolished induction by either toxin. Compared with our previous studies on UCP4, we observed functional differences between UCP4 and UCP5 in enhancing mitochondrial efficiency. These neuronal UCP homologues may work synergistically to maintain oxidative balance (through uncoupling activities) and ATP production (by modifying MMP).

Introduction

Mitochondrial oxidative phosphorylation generates cellular energy in the form of ATP. It involves electron flow along the electron transport chain in mitochondria from Complex I to Complex IV to molecular oxygen. Along with this electron flow, protons are pumped from the mitochondrial matrix to the intermembrane space, creating the proton-motive force, comprising the proton gradient and mitochondrial membrane potential (MMP) across the inner membrane [1]. Complex V (ATP synthase) uses this proton gradient by channeling the protons back to the matrix to drive ADP phosphorylation and generate ATP. Oxidative phosphorylation also produces superoxide free radicals when single electrons are transferred to molecular oxygen [2]. Only small amounts of superoxide free radicals are produced under normal physiological conditions, and they are rapidly sequestered by antioxidant enzyme systems within the mitochondria [3–5]. Mitochondrial dysfunction causes excessive production of these reactive oxygen species (ROS) leading to oxidative stress and cell death.

Uncoupling proteins (UCPs) belong to a family of mitochondrial solute carriers in the inner mitochondrial membrane. They uncouple the electron flow during biofuel oxidation from ATP synthesis by providing an alternate route for proton flow through the inner mitochondrial membrane to the matrix without ATP synthesis [6–8]. Five homologues of UCPs have been identified (UCP1 to UCP5) and they are differentially expressed in various tissues. UCP1 is localized mainly in brown adipose tissue [6]; UCP2 is ubiquitously expressed, at varying levels in different tissues including neurons [9]; UCP3 is mainly found in skeletal and heart muscles [10]; UCP4 and 5 are predominantly expressed in brain [11–13].

UCP1, UCP2, and UCP3 have been extensively studied [14–19]. The function and physiological significance of neuronal UCPs (UCP2, 4, and

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5) are unclear. Mild (or partial) uncoupling has been shown to be neuroprotective against various cellular insults [20]. The "mild uncoupling hypothesis" has been proposed to explain the protective functions of UCPs in alleviating oxidative stress through their uncoupling activities by decreasing the proton gradient and reducing ROS levels generated during oxidative phosphorylation [16–18]. Supportive evidence includes studies of antioxidative functions of UCP2 and UCP3 [21–25]. UCP2 knockout mice have reduced but not ablated uncoupling in the substantia nigra and increased in vivo ROS production and are more susceptible to MPTP toxicity [25]. These UCP2 knockout mice had reduced dopamine turnover in the striatum and reduced tyrosine hydroxylase immunoreactivity in the substantia nigra, striatum, and nucleus accumbens [24]. Conversely, overexpression of human UCP2 in mice showed increased uncoupling and decreased in vivo ROS production [25], indicating that UCP2 has a protective role in neuronal function [26–28].

UCP5 (also known as BMCP1, brain mitochondrial carrier protein-1) is predominantly expressed in brain [11,13]. It shares only 34 and 38% amino acid identity with UCP1 and UCP2, respectively [11]. There is little information on the physiological functions of UCP5. Because of its abundant expression in brain, modulation of UCP5 expression levels under various stresses [29,30] indicates that UCP5 may be important in adaptive responses to various neuronal disorders. Overexpression of UCP5 in murine GT1-1 cells regulated MMP and reduced oxidative stress [31]. We have previously shown that the mitochondrial toxin 1-methyl-4-phenylpyridinium iodide (MPP^+) induced mRNA expression of UCP5 in a time- and dose-dependent manner [32]. We also found that knockdown of UCP5 expression in neuronal culture increased ROS levels and susceptibility to neuronal cell death after MPP^+ exposure [33]. Recent studies also reported that greater oxidative and phosphorylative mitochondrial capacities observed in female aged rodents are associated with a comparatively higher expression of UCP5 in the female brain [34].

We investigated the neuroprotective properties of human neuronal UCP5 by comparing the levels of ATP production, the extent of glycolysis, MMP, superoxide levels, and cell viability in response to two toxins, MPP^+ and dopamine, in SH-SY5Y neuroblastoma cells stably overexpressing human UCP5.

Materials and methods

Materials

Human SH-SY5Y neuroblastoma cells were obtained from American Tissue Culture Collection (Manassas, VA, USA; CRL-2266). Dulbecco's modified Eagle medium + GlutaMAX-1 Nutrient Mixture F-12 (DMEM-F12), fetal bovine serum (FBS), penicillin–streptomycin, Opti-MEM, Lipofectamine 2000, pcDNA3.1(+)expression vector, and Trizol were from Invitrogen Life Technologies (San Diego, CA, USA); Marathon-Ready cDNA kit was from Clontech Laboratories (Palo Alto, CA, USA); LDH release assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and Titan One Tube RT-PCR Kit were from Roche Applied Science (Mannheim, Germany); mitochondrial isolation kit was obtained from Pierce (Rockford, IL, USA); adenosine 5′-diphosphate potassium salt (ADP) was from Calbiochem (San Diego, CA, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and dihydroethidium (DHE) were from Molecular Probes (Eugene, OR, USA); TaqMan EZ RT-PCR reagent kit, probes, Primer Express software version 2.0, and StepOne Real-Time PCR System and StepOne software version 2.0 were from Applied Biosystems (Foster City, CA, USA); Clark-type oxygen electrode in a water-jacketed microcell (Model MT200; 782 2-channel oxygen system) was from Strathkelvin Instruments (Scotland); D_C protein assay kit and polyvinylidene difluoride (PVDF) membrane were from Bio-Rad (Hercules, CA, USA); polyclonal anti-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal anti-COXIV antibody, ADP/ATP bioluminescence kit, and lactate colorimetric assay kit were from Abcam (Cambridge, UK); horseradish peroxidase-conjugated secondary antibodies were from DAKO (Glostrup, Denmark); ECL-Plus Western blotting detection system, Geneticin (G418), and protein G–Sepharose were from GE Healthcare (Buckinghamshire, UK); monoclonal anti-FLAG antibody, dimethyl sulfoxide (DMSO), dopamine–HCl, MPP^+, actinomycin-D, phenylmethylsulfonyl fluoride (PMSF), Triton X-100, mannitol, potassium chloride (KCl), magnesium chloride ($MgCl_2$), dipotassium hydrogen phosphate (K_2HPO_4), defatted bovine serum albumin, and *trans*-retinoic acid were from Sigma (St. Louis, MO, USA).

Affinity purification of human UCP5 antibodies

Two antibodies (anti-UCP5NT and anti-UCPCT) were generated against specific linear epitopes of human UCP5 in sheep in collaboration with The Binding Site Ltd. (Birmingham, UK) by immunization with antigenic peptides corresponding to two epitopes selected on the basis of their high hydrophilicity and antigenicity. The antisera were incubated with the corresponding antigenic peptides on controlled pore glass beads (1 ml) for 1 h at room temperature under constant agitation. The beads and the sheep IgG were poured into a small column (5 ml). After all the fluid was drained from the beads, they were washed with PBS at pH 7.0 (100 ml) until the absorbance of the eluent at 280 nm was similar to that of the initial PBS. Finally the column was eluted with 10 × 1-ml aliquots of 0.1 M glycine at pH 2.5. The pH of the eluted fractions was brought to 7.0 by 1 M Tris–HCl immediately. The three fractions showing the highest protein concentrations were pooled together.

Cell culture and treatments

Human SH-SY5Y cells were cultured in DMEM-F12 supplemented with 10% (v/v) FBS and 2 mM glutamine and 100 μg/ml penicillin–streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Empty-vector controls or UCP5-overexpressing SH-SY5Y cells were exposed to a variety of treatments: (a) ADP (2 mM), (b) MPP^+ (1 mM), and (c) dopamine (200 μM) for 6 and 24 h. The following parameters assayed: MMP, superoxide levels, intracellular ATP concentration, and lactate levels. To study the effects of these toxins on the steady-state UCP5 mRNA expression, cells were exposed to either MPP^+ or dopamine for 1 h, after which the medium was changed to one containing 5 μg/ml actinomycin-D or not. Total RNA was extracted at time 0, 30, 60, and 120 min after addition of actinomycin-D. UCP5 mRNA levels were normalized against 18S rRNA levels and compared with and without the addition of actinomycin-D. The UCP5 mRNA levels of actinomycin-treated samples were expressed as the percentage of the UCP5 mRNA level in samples without actinomycin-D exposure.

Elucidation of 5′-untranslated region (5′ UTR) of UCP5

The 5′ UTR of UCP5 was determined by using the Marathon-Ready cDNA kit. The kit contains premade "libraries" of adaptor-ligated double-stranded (ds) human brain cDNAs that act as templates for cDNA amplification. An internal primer specific to UCP5 and a Marathon adaptor primer (AP1) were used to prime the adaptor-ligated dsUCP5 cDNAs, which were then amplified by PCR. The sequence of AP1 was 5′-CCATCCTAATACGACTCACTATAGGGC-3′ and the internal UCP5-specific primer was 5′-CCGTTTTCAATGTCACCCATCA-3′. The PCR conditions were denaturing at 94 °C for 30 s, 30 cycles of annealing and amplification at 94 °C for 5 s and 68 °C for 2 min, and cool down to 4 °C. The resultant PCR product was DNA sequenced to determine the full length of the 5′ UTR and the transcription start site.