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The preservation of in vivo phosphorylated and activated uncoupling protein 3 (UCP3) in isolated skeletal muscle mitochondria following administration of 3,4-methylenedioxymethamphetamine (MDMA aka ecstasy) to rats/mice

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ABSTRACT

Previous researchers have demonstrated that 3,4-methylenedioxymethamphetamine (MDMA) induced hyperthermia, in skeletal muscle of animals, is uncoupling protein 3 (UCP3) dependent. In light of our investigations that in vivo phosphorylation of UCP1 is augmented under conditions of cold-acclimation, we set out to investigate whether (a) UCP3 was phosphorylated in vivo and (b) whether in vivo phosphorylation of UCP3 resulted in increased proton leak following MDMA administration to animals. Our data demonstrate that MDMA treatment (but not PBS treatment) of animals results in both in vivo serine and tyrosine phosphorylation of UCP3 in skeletal muscle mitochondria, isolated in the presence of phosphatase inhibitors to preserve in vivo phosphorylation. In addition, proton leak is only increased in skeletal muscle mitochondria isolated from MDMA treated animals (in the presence of phosphatase inhibitors) and the increased proton leak is due to phosphorylated UCP3. UCP3 abundance in skeletal muscle mitochondria is unaffected by MDMA administration. Preservation of UCP3 phosphorylation and increased proton leak is lost when skeletal muscle mitochondria are isolated in the absence of phosphatase inhibitors. We conclude that MDMA treatment of animals increases proton leak in skeletal muscle mitochondria by activating UCP3 through in vivo covalent modification of UCP3 by phosphorylation. Furthermore, we deduce that the MDMA induced hyperthermia in skeletal muscle is due to increased proton leak in vivo as a result of activation of UCP3 through phosphorylation.

1. Introduction

Uncoupling protein 3 (UCP3) was first discovered by Boss et al. (1997) and was predicted to be involved in heat production in muscle (Samec et al., 1998) due to its homology (57%) with the well characterized uncoupling protein, UCP1, found in brown adipose tissue (Cannon and Nedergaard, 2004; Nicholls, 2006; Nicholls and Locke, 1984). UCP3 is predominantly found associated with skeletal muscle mitochondria (Boss et al., 1997; Carroll and Porter, 2004; Cunningham et al., 2003; Krauss et al., 2005) and initially, great interest was focused on UCP3 as an anti-obesity gene (Comuzzie and Allison, 1998) and as a potential anti-obesity/anti-type 2 diabetes drug target (Campfield et al., 1998). However, that focal point waned somewhat, but not completely (Harper et al., 2001), with the publication of two independent papers demonstrating that UCP3 knock-out mice were not obese and indeed had no significant difference in body mass compared to wild-type animals (Gong et al., 2000; Vidal-Puig et al., 2000). It is still a matter of investigation as to what UCP3 actually catalyzes, although predictions from sequence analysis, and (pseudo)symmetry modeling, with known mitochondrial transporters, predict that UCPs are keto-acid transporters (Robinson et al., 2008).

Despite these predictions, direct studies on UCP3 function have yielded a variety of conclusions. Evidence from in vitro expression and reconstitution of UCP3 into vesicle membranes suggest that UCP3 facilitates a UCP1-like proton leak in a fatty acid dependent, purine nucleotide-sensitive manner (Echtay et al., 2001; Jabůrek et al., 1999). Early experiments on yeast expressing UCP3 showed increased mitochondrial proton leak (Hagen et al., 1999), yet others reported that UCP3 uncoupling in yeast mitochondria was artifactual (Harper et al., 2002). On the other hand, work with transgenic mice overexpressing human UCP3 in mouse skeletal muscle resulted in marked physiological consequences of weight loss and hyperphagia

Abbreviations: BAT, brown adipose tissue; BCA, bicinchoninic acid; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; FBS, fetal bovine serum; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HEPES, (*N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid)); MDMA, 3,4-methylenedioxymethamphetamine (aka ecstasy); PDH, pyruvate dehydrogenase; PMK, 1-(3,4-methylenedioxyphenyl)-2-propanone; PVDF, polyvinyldifluoride; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; TBS, tris buffered saline; UCP, uncoupling protein.

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(Chapman et al., 2000), yet the resulting increased proton leak in isolated muscle mitochondria from transgenic mice overexpressing human UCP3 may well be an artifact of protein abundance in the inner membrane, rather than a subtly regulated proton leak through UCP3 (Cadenas et al., 2002). Data from UCP3 knock-out mice have yielded ambiguous results, in that the absence of UCP3 has been shown to have no effect on state 4 oxygen consumption (Cadenas et al., 2002; Vidal-Puig et al., 2000) or proton leak (Cadenas et al., 2002) in some studies, while having an effect on state 4 oxygen consumption (Gong et al., 2000) or proton leak (Vidal-Puig et al., 2000) in others. Other data where UCP2/3 was over-expressed in cells suggest that UCP3 (and UCP2) are associated with calcium transport across the mitochondrial inner membrane (Trenker et al., 2007, 2008), although these data are strenuously contested (Brookes et al., 2008). Lombardi et al. (2010) have recently provided evidence that UCP3 translocates in vitro generated lipid hydroperoxides from the matrix of isolated mitochondria, with the net manifestation of uncoupling.

The observation that physiological modulation of UCP3 abundance through starvation, which is known to increase UCP3 protein expression and abundance in mitochondria (2–3-fold increase per mg mitochondrial protein) but does not manifest itself as increased proton leak in isolated skeletal muscle mitochondria from starved animals (Cadenas et al., 1999) suggests that if UCP3 facilitates proton leak it needs to be activated in vivo to perform that function. Certainly, others have shown that UCP3 can act to facilitate a purine nucleotide-sensitive proton leak, on addition of superoxide (Echtay et al., 2002) or polyunsaturated fatty acid oxidation products (such as 4-hydroxy-2-nonenals) to isolated mitochondria (Echtay et al., 2003), although the specificity of this activation by fatty acid oxidation products has been questioned by Shabalina et al. (2006). A most fascinating piece of evidence for the in vivo activation of UCP3 comes from the observations of Mills et al. (2003) who demonstrated that UCP3 was essential for 3,4-methylenedioxymethamphetamine (MDMA aka ecstasy) induced hyperthermia in skeletal muscle of mice. The most probable explanation for this pharmacological observation, is that MDMA administration activates constitutively expressed UCP3, resulting in uncoupled skeletal muscle mitochondria with the consequent uncoupling of skeletal muscle metabolism. One possible explanation put forward for the activation process stems from the fact that, apart from its central psychotropic effect, MDMA is known to trigger the sympathetic nervous system. Thus the activation of UCP3 in skeletal muscle on administration of MDMA to animals may result via release and binding of free fatty acids to UCP3 (Mills et al., 2004, 2007), in an analogous way to activation of UCP1 by free fatty acids in brown adipose tissue following release of noradrenalin under conditions of cold-acclimation (Cannon and Nedergaard, 2004; Nicholls, 2006; Nicholls and Locke, 1984; Shabalina et al., 2004). However, in light of the observation from our laboratory that phosphorylation of UCP1 is augmented under conditions of cold-acclimation (Carroll et al., 2008), we set out to investigate whether in vivo phosphorylation of UCP3 was the means by which UCP3 was activated to increase proton leak following MDMA administration.

2. Material and methods

2.1. Animals

Wistar rats were 8–12 weeks old, a mix of male and females and weighed between 150 and 200 g. Mice were of C57 × 129 F2 background and either UCP3−/− (homozygotes) or wild-type (+/+) for UCP3, originally from the Jackson laboratories. Mice were males aged between 6 and 10 weeks weighing approximately 30 g. Animals were housed at 25 ± 1 °C in individually ventilated cages. All animals were allowed free access to food [Harlan 2018 Teklad Global 18% Protein Rodent diet] and water and a 12-hour light/dark cycle was in place. All mice were killed by cervical dislocation and rats by CO_2 asphyxiation. All animals were housed in the Bioresources unit, in Trinity College Dublin.

2.2. Synthesis of 3,4-methylenedioxymethamphetamine (MDMA)

MDMA was synthesized in a three-step reaction via reductive amination of the 1-(3,4-methylenedioxyphenyl)-2-propanone (PMK) precursor with modifications to the previously reported procedure by Gimeno et al. (2005). 1-(3,4-methylenedioxyphenyl)-1-nitropropene was obtained from a Henry–Knoevenagel condensation of 3,4-methylenedioxybenzaldehyde and nitromethane, using either potassium fluoride and *N*,*N*-dimethylamine hydrochloride or cyclohexylamine as organic base. The nitrostyrene was then reduced with iron powder in glacial acetic acid to afford PMK. Reductive amination of the ketone with $NaCNBH_4$ and methylamine afforded the MDMA product, which was used as the hydrochloride salt of MDMA in this study.

2.2.1. Synthesis of 1-(3,4-methylenedioxyphenyl)-2-nitro-1-propene

To a solution of piperonal (10.0 mmol, 2.07 g) in glacial acetic acid (15 mL; Method A) or toluene (15 mL; Method B) was added nitroethane (20.0 mmol) followed by potassium fluoride(1.5 mmol, 87 mg) and *N*,*N*-dimethylamine hydrochloride (20.0 mmol, 1.63 g) (Method A) or cyclohexylamine (10.00 mmol, 0.99 g), (Method B). The reaction was heated in a water bath for 6 h, water (50 mL) was added and the reaction mixture was allowed to stand overnight. The precipitated nitroethene was isolated by filtration. The filtrate was further diluted with water (50 mL) and extracted with dichloromethane (3 × 25 mL). The organic phases were combined and washed with satd. aq. $NaHCO_3$ (3 × 25 mL). The solution was dried over anhydrous Na_2SO_4, filtered and all solvent removed in vacuo, to give an oil which was purified by column chromatography over silica gel (eluent: 7:3 hexane/diethyl ether). The chromatographed and precipitated nitroethene fractions were combined and recrystallized from methanol. Yellow crystals (78%, Method A; 52%, Method B). M.p. 93–94 °C (Lukaszewski, 1982). IRν_{max} 1322, 1505, 1602 cm^{-1}. ^1H NMR δ ($CDCl_3$) 2.48 (3H, s, CH_3), 6.06 (2H, s, OCH_2O), 6.91 (1H, d, J = 8.0 Hz, ArH), 6.96 (1H, s, ArH), 6.99 (1H, d, J = 8.0 Hz, ArH). ^{13}C NMR ppm ($CDCl_3$) 13.71, 101.34, 108.42, 109.07, 125.52, 125.77, 133.22, 145.69, 147.80, 148.85. HRMS (EI) calculated for $C_{10}H_{10}NO_4$: ($M^+ + H$) 208.0610: found 208.0602.

2.2.2. Synthesis of 1-(3,4-methylenedioxyphenyl)-2-propanone

A suspension of iron powder (10 mmol, 0.55 g) in glacial acetic acid (30 mL) was heated on a steam bath for 20 min, stirring occasionally. To this mixture, a solution of 1-(3,4-methylenedioxyphenyl)-2-nitro-1-propene (2.2 mmol, 0.45 g) in glacial acetic acid (20 mL) was added over 20 min. The reaction was heated for 2.5 h until the mixture became a gray/white color. The reaction was cooled and poured into a mixture of water/ice (100 mL). The product was extracted with dichloromethane (4 × 25 mL) and the organic extracts were washed with 15% aq. NaOH (3 × 25 mL). The organic phase was then dried over anhydrous Na_2SO_4 and concentrated in vacuo, to afford the product, which required no further purification. Colorless oil (50%). (Dal Cason, 1990) IRν_{max} 1708 cm^{-1}. ^1H NMR δ ($CDCl_3$) 2.168 (3H, s, CH_3), 3.62 (2H, s, $ArCH_2$), 5.96 (2H, s, OCH_2O), 6.65 (1H, d, J = 8.0 Hz, ArH), 6.67 (1 H, s, ArH), 6.79 (1 H, d, J = 8.0 Hz, ArH). ^{13}C NMR ppm ($CDCl_3$) 28.70, 50.09, 100.60, 108.02, 109.30, 122.06, 127.34, 146.23, 147.38, 206.15. HRMS (EI) calculated for $C_{10}H_{10}O_3Na$: ($M^+ + Na$) 201.0519: found 201.0528.

2.2.3. Synthesis of 2-N-methylamino-1-(3,4-methylenedioxyphenyl) propane (MDMA)

To a solution of 1-(3,4-methylenedioxyphenyl)-2-propanone (10 mmol, 1.78 g) in dry methanol (30 mL) was added methylamine HCl (80 mmol, 5.40 g) and $NaCNBH_4$ (14 mmol, 0.52 g). The mixture was stirred at room temperature for 72 h and monitored by TLC. The pH was adjusted occasionally to pH 5–6 using 4 M methanolic HCl. When the reaction was complete, excess hydride was quenched using