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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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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, foetal 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, polyvinylidene difluoride;; sem, standard error of the mean; SDS, sodium dodecyl sulphate; TBS, tris buffered saline; UCP, uncoupling protein.

Keywords: uncoupling protein; UCP3; mitochondria; oxygen consumption; MDMA; Ecstasy; 3,4-Methylenedioxymethamphetamine; proton leak

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 (Nicholls and Locke, 1984; Cannon and Nedergaard, 2004; Nicholls, 2006). UCP3 is predominantly found associated with skeletal muscle mitochondria (Boss et al., 1997; Cunningham et al., 2003; Carroll and Porter, 2004; 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 modelling, with known mitochondrial transporters, predict that UCP's 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 (Jabůrek et al., 1999; Echay et al., 2001). 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 (Chapman et al., 2000), yet the resulting increased proton leak in

isolated muscle mitochondria from transgenic mice overexpressing human UCP3 may well be an artefact 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 (Vidal-Puig et al., 2000; Cadenas et al., 2002) 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 suggests that UCP3 (and UCP2) are associated with calcium transport across the mitochondrial inner membrane (Trenker et al., 2007; Trenker et al., 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-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; Mills et al., 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 (Nicholls and Locke, 1984; Cannon and Nedergaard, 2004; Shabillina et al., 2004; Nicholls, 2006). 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 150-200g. Mice were of C57 x 129 F2 background and either UCP3 $-/-$ (homozygotes) or wild-type $(+/-)$ for UCP3, originally from the Jackson laboratories. Mice were males aged between 6-10 weeks weighing approximately 30g. Animals were housed at $25 \pm 1^\circ\text{C}$ 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 NaCNBH₄ and methylamine afforded the MDMA product, which was used as the hydrochloride salt of MDMA in this study (see supplement to Experimental Procedures).

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 x 25 mL). The organic phases were combined and washed with satd. aq. NaHCO₃ (3 x 25 mL). The solution was dried over anhydrous Na₂SO₄, 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 recrystallised from methanol. Yellow crystals (78 %, Method A; 52%, Method B). M.p. 93-94 °C (35). IR_{vmax} 1322, 1505, 1602 cm⁻¹. ¹H NMR δ (CDCl₃) 2.48 (3H, s, CH₃), 6.06 (2H, s, OCH₂O), 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 $\text{C}_{10}\text{H}_{10}\text{NO}_4$: ($\text{M}^+ + \text{H}$) 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 mins, 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 grey/white colour. The reaction was cooled and poured into a mixture of water/ice (100 mL). The product was extracted with dichloromethane (4 x 25 mL) and the organic extracts were washed with 15 % aq. NaOH (3 x 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. Colourless oil (50 %). (Dal Carson, 1990) IR $_{\text{vmax}}$ 1708 cm^{-1} . ^1H NMR δ (CDCl_3) 2.168 (3H, s, CH_3), 3.62 (2H, s, Ar CH_2), 5.96 (2H, s, O CH_2 O), 6.65 (1H, d, J = 8.0 Hz, ArH), 6.67 (1H, s, ArH), 6.79 (1H, 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 $\text{C}_{10}\text{H}_{10}\text{O}_3\text{Na}$: ($\text{M}^+ + \text{Na}$) 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 4M methanolic HCl. When the reaction was complete, excess hydride was quenched using 10 % aq. HCl (30 mL). The aqueous solution was washed with dichloromethane (3 x 25 mL). The aqueous phase was basified with 2M NaOH and extracted with dichloromethane (3 x 25 mL). The organic

phases were combined and dried with anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. No further purification was required. Colourless oil (89 %). (Noggle et al., 1987) IR_{vmax} 3327 cm^{-1} . ^1H NMR δ (CDCl_3) 1.06 (3H, d, $J = 5.9\text{ Hz}$, CH_3), 1.74 (1H, br s, NH), 2.53 (3H, s, HNCH_3), 2.61 (2H, dd, $J = 13.3\text{ Hz}$, $J = 6.3\text{ Hz}$, ArCH_2), 2.72 (1H, dd, $J = 13.0\text{ Hz}$, $J = 7.0\text{ Hz}$, NHCH), 5.94 (2H, s, OCH_2O), 6.65 (1H, d, $J = 8.0\text{ Hz}$, ArH), 6.70 (1H, s, ArH), 6.75 (1H, d, $J = 8.0\text{ Hz}$, ArH). ^{13}C NMR ppm (CDCl_3) 19.09, 33.49, 42.63, 55.98, 100.36, 107.71, 109.04, 121.71, 132.67, 145.68, 147.15. HRMS (EI) calculated for $\text{C}_{11}\text{H}_{16}\text{NO}_2$: ($\text{M}^+ + \text{H}$) 194.1181: found 194.1183.

2.2.4. Synthesis of 2-N-methylamino-1-(3,4-methylenedioxyphenyl)propane hydrochloride salt

MDMA (10mmol) was dissolved in propan-2-ol (3 mL). Conc. HCl (0.5 mL) was added dropwise and the solution was diluted with diethyl ether until turbid. The mixture was allowed to stand overnight at room temperature and the resulting solids were isolated by filtration. The crude HCl salt was recrystallized from a mixture of hexane and ethanol. Colourless crystals (65 %) M.p. $143\text{--}145\text{ }^\circ\text{C}$. IR_{vmax} $2458, 2429\text{ cm}^{-1}$ (Gimeno et al., 2005).

2.3. Administration of MDMA to animals

MDMA dissolved in $200\mu\text{l}$ phosphate-buffered saline (PBS) was administered to male rats at a concentration of 40 mg/kg by subcutaneous injection. $200\mu\text{l}$ PBS alone was administered to control rats. MDMA dissolved in $200\mu\text{l}$ PBS was administered to male mice at a concentration of 20 mg/kg by intraperitoneal injection. $200\mu\text{l}$ PBS alone was administered to control mice. All animals were allowed free access to laboratory chow and water. Two hours after MDMA administration, animals were sacrificed.

2.4. Isolation of mitochondria from liver

Mitochondria were isolated by differential centrifugation essentially as described by Chappell and Hansford (1972). Liver was removed weighed and placed in an ice-cold sucrose based buffer (250mM sucrose, 5mM tris-HCl pH 7.4, 2mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA)). The liver was chopped finely using a scissors and washed several times with the aforementioned sucrose based buffer. The chopped liver was homogenized by hand using a Dounce homogenizer and the homogenate was subjected to centrifugation at 800xg for 3min at 4°C. The resulting supernatant was subjected to centrifugation at 12,000xg for 10min at 4°C yielding a pellet of mitochondria. The pellet was washed by resuspension in sucrose based medium and subjected to centrifugation under the same conditions. The resulting "mitochondrial" pellet was resuspended to the required concentration and stored on ice. Liver mitochondria from MDMA treated and PBS treated animals were prepared in the presence or absence of phosphatase inhibitors (10mM KF and 1mM Na₂VO₃).

2.5. Isolation of skeletal muscle mitochondria

Skeletal muscle mitochondria from the hind leg muscle of rats or mice were isolated by the method described by Bhattacharya et al. (1991). Hindquarter muscle (leg muscle) were removed weighed and placed in ice cold isolation medium (100mM sucrose, 9mM ethylenediamineacetic acid (EDTA), 100mM tris-HCl pH 7.4, 46mM KCl). Skeletal muscle was finely chopped on a pre-cooled glass tile with a razor blade, transferred to isolation medium containing 0.02% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) napsin (Sigma, protease VII) and incubated on ice with stirring, for 5min. The tissue was further disrupted using a Waring blender at half-maximum speed for 15sec. The homogenate was subjected to centrifugation at 1,500xg for 3min at 4°C. The whitish lipid layer on top of the supernatant was removed

and the supernatant was subjected to centrifugation at 12,000xg for 10min at 4°C. The resulting mitochondrial pellet was re-suspended in ice cold isolation medium containing 2% (w/v) essentially fatty acid free BSA and centrifuged at 12,000xg for 10min at 4°C. The resulting mitochondrial pellet was re-suspended in ice cold isolation medium and subjected to centrifugation at 12,000xg for 10min at 4°C and the resulting pellet re-suspended in ice cold isolation medium to the required concentration. Skeletal muscle mitochondria from MDMA treated and PBS treated animals were prepared in the presence or absence of phosphatase inhibitors (10mM KF and 1mM Na₂VO₃).

2.5. Mitochondrial Protein Determination using the Bicinchoninic Acid Assay (BCA)

Quantification of protein concentrations in tissue samples was carried out using the bicinchoninic acid (BCA) assay described by Smith *et al.* (1985). All of the following solutions (standards or samples) were directly prepared in a 96-well plate. A standard protein solution of BSA was suitably diluted in de-ionized water from 0 to 0.25mg/ml. The amount of mitochondrial protein present in the samples was determined by reference to a standard curve derived from the above known concentrations of BSA. Isolated mitochondria were diluted in H₂O before analysis to ensure the final concentration of the samples would fall within the range of the standard curve. Once the samples and the standards were prepared, 200μl of a working solution of BCA (1:49(v/v) BCA: Cu₂SO₄) was added to each well. The plate was then incubated for 30 minutes in an incubator set at 37°C. The absorbance of each sample was measured spectrophotometrically at 550nm.

2.6 Polyacrylamide electrophoresis

Samples were prepared as described in Cunningham *et al.* (2003). Essentially (10-100μg) protein were solubilized in sample buffer, vortexed and pulsed in a minifuge before boiling at 100°C for 5min.

Polyacrylamide electrophoresis was performed as detailed in Cunningham et al. (2003). Essentially mitochondrial samples were run on linear one-dimensional SDS (12%) polyacrylamide denaturing gels under reducing conditions using a Protean III (Bio-Rad) gel system.

2.7 Immunoblotting

Protein was transferred to polyvinylidene difluoride (PVDF) (Immobilin-PS^Q; Millipore) using a semi-dry apparatus for 2h at 110mA or a Bio-Rad Transfer Cell for 1-4h at 20V/cm. After transfer was complete, the PVDF was washed three times for 10min in phosphate-buffered saline (PBS)-Tween (0.14M NaCl, 2.7mM KCl, 11.5mM Na₂PO₄, 1.8mM KH₂PO₄, pH 7.4 with) 0.1%(v/v) Tween-20) and blocked for 1h in Tris-buffered saline (TBS) with 0.1% (v/v) Tween 20 containing 5% (w/v) non-fat dried milk (Marvel). This step was followed by three 10min washes with TBS-Tween. Blots were probed with primary antibody overnight at 4°C in the aforementioned solution containing either (a) a 1:500 dilution of affinity purified anti-UCP 3 peptide antibody (Custom made for R.K. Porter by Eurogentec, detailed in Cunningham et al., 2003) (b) a 1:1,000 dilution of anti-PDH-E1- α antibody (Mitosciences), (c) a 1:1000 dilution of anti-phosphotyrosine antibody (Millipore) or (d) a 1:1000 dilution of anti-phosphoserine antibody (Millipore). Following blocking and overnight primary antibody incubation, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) in TBS-Tween, 0.1% (v/v) Tween 20, 5% (w/v) non-fat dried milk (Marvel) for 1h at room temperature. Blots were developed using an ECL detection system (Amersham Biosciences), and immunoreactions were visualized by exposure to Kodak X-Omat LS film.

2.8 Immunoprecipitation

Mitochondria from skeletal muscle or liver were isolated and protein was immunoprecipitated using either (a) protein G agarose anti-phosphotyrosine antibody or (b) protein A agarose anti-phosphoserine antibody. Mitochondria were lysed in modified RIPA buffer containing 1% NP-40, and freshly added protease inhibitors, at 4°C for 20 minutes with gentle rotation. The lysate was centrifuged at 14,000xg in a pre-cooled centrifuge for 15 minutes. The lysate was pre-cleared with protein G or protein A beads for 15 minutes at 4°C with gentle rotation. The protein G or protein A beads were removed by centrifugation at 4,000xg for 3 minutes in a pre-cooled centrifuge. The pre-cleared lysates were incubated with 5µg of antibody overnight at 4°C with gentle rotation. The next morning the beads were washed 3 x 10 minutes at 4°C with modified RIPA buffer containing 0.1% NP-40. The bound material was eluted by heating beads in 2x sample buffer for 5 minutes at 95°C and the samples were subjected to SDS-PAGE (12% resolving gel) and immunoblot analysis. UCP3 was detected using a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (custom made by Eurogentec).

2.9 Measurement of Oxygen Consumption by Mitochondria

All measurements of respiration were made using an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria), and oxygen flux was resolved using DATLAB software. The oxygraph-2k is a two chamber titration-injection respirometer with a limit of oxygen flux detection of 1pmol s⁻¹ ml⁻¹. Standardized instrumental and chemical calibrations were performed to correct for back diffusion of oxygen into the chamber from the various components; leak from the exterior, oxygen consumption by the chemical medium, and sensor oxygen consumption. The mitochondrial suspension was stirred using a PVDF magnetic stirrer and thermostatically maintained at 37°C. Oxygen consumption rates by mitochondria (0.0625mg/ml) were determined at 37°C in 120 mM KCl, 5 mM Hepes-KOH (pH 7.4), 1mM EGTA, 1 µM atractyloside, 1 µg/ml oligomycin, 0.1% de-fatted BSA, 1 µM rotenone and 5 mM succinate-KOH in a pre-calibrated Oroboros Oxygraph. Steady-state non-phosphorylating oxygen consumption rates due to proton leak were obtained (state 4), before subsequent addition of 1 mM

GDP followed by the addition of 60 μM (~25nM estimated final concentration based González-Barroso et al., 1998) oleate and 0.25 μM FCCP, to obtain the uncoupled (maximal electron transport chain) rate.

2.9 Statistics

All data are expressed as means $\pm$ S.E.M. of at least 3 separate experiments performed in triplicate. Statistical differences were determined by a two-tail unpaired student's t-test. Unless otherwise stated a p value of ≤ 0.05 was taken to indicate significance.

3. Results

3.1. Selectivity of the detection of UCP3

Figure 1A demonstrates the selectivity and sensitivity of the UCP3 peptide antibody. The UCP3 antibody detects UCP3 expressed in yeast lysates of yeast overexpressing UCP3 (Cunningham et al., 2003) and mitochondria isolated from skeletal muscle from wild-type mice, but not in mitochondria isolated from skeletal of UCP3 knock-out mice or in liver of wild-type mice (where there is no UCP3 present). The presence of mitochondria was confirmed using an antibody to pyruvate dehydrogenase subunit E1 α (Fig. 1B). These data are consistent with selectivity and sensitivity determinations using this antibody previously reported by Cunningham et al. (2003). From these data we can conclude that the UCP3 antibody is selective for UCP3.

3.2. UCP3 detection following MDMA and PBS treatment

Injection of animals with either MDMA or PBS, 2 hours before sacrifice, had no effect on the protein expression levels of UCP3 in muscle isolated from animals in the presence or absence of phosphatase inhibitors (Fig. 2A). Confirmation of the presence of mitochondria in the preparations was demonstrated using an antibody to pyruvate dehydrogenase subunit E1 α (Fig. 2B). From these data we conclude that levels of UCP3 protein expression are not affected by MDMA or PBS injection or isolation of skeletal muscle mitochondria in the presence or absence of phosphatase inhibitors.

3.3 Detection of phosphorylated mitochondrial proteins following MDMA and PBS treatment

Figure 3 demonstrates that many proteins in muscle mitochondria are either phosphorylated on tyrosine or serine. Clearly administration of MDMA increases the detectable levels of mitochondrial protein that are tyrosine phosphorylated compared to mitochondria isolated from animals treated with PBS alone (Figure 3A). The differential is less obvious for proteins that are serine phosphorylated in a comparison of skeletal muscle mitochondria from MDMA and PBS treated animals (Figure 3B).

Furthermore, and as might be expected, one can preserve a greater level of mitochondrial protein phosphorylation (serine and tyrosine phosphorylation) when the phosphatase inhibitors (10mM KF and 1mM Na₂VO₃) are included in the mitochondria preparation medium. A similar pattern of mitochondrial protein phosphorylation to that of phosphoserine, was observed for phosphothreonine (results not shown).

We conclude that MDMA administration to animals causes a greater degree of phosphorylation of mitochondrial proteins (particularly evident for phosphotyrosine) than when animals are treated with PBS alone. We further conclude that addition of the phosphatase inhibitors (10mM KF and 1mM Na₂VO₃)

VO₃) to the mitochondrial preparation medium increases chances of preserving the phosphorylation state of mitochondria on isolation.

3.4 Immunoblots detecting phosphorylated UCP3 following MDMA treatment

Figure 4 demonstrates the result of the (A) anti-phosphotyrosine antibody and (B) phosphoserine antibody immunoprecipitation of mitochondrial proteins, cross referenced with the anti-UCP3 antibody on an immunoblot. UCP3 is detectable in mitochondrial protein immunoprecipitated using (A) anti-phosphotyrosine and (B) phosphoserine antibodies but only in skeletal muscle mitochondrial protein immunoprecipitated, from MDMA treated animals, isolated in the presence of phosphatase inhibitors. No UCP3 is detectable in mitochondrial protein immunoprecipitated using (A) anti-phosphotyrosine and (B) phosphoserine antibodies in skeletal muscle mitochondria from PBS treated animals, isolated in the presence of phosphatase inhibitors, in skeletal muscle mitochondria, from MDMA treated animals, isolated in the absence of phosphatase inhibitors or in liver mitochondria (which do not contain UCP3) from MDMA treated animals, isolated in the presence of phosphatase inhibitors. In an equivalent phosphothreonine immunoprecipitation, no UCP3 was detected (results not shown). We conclude that MDMA treatment of animals results in tyrosine and serine (but not threonine) phosphorylation of UCP3 in skeletal muscle mitochondria. We further conclude that addition of the phosphatase inhibitors to the mitochondrial preparation medium preserves the phosphorylation state of UCP3 in skeletal muscle mitochondria.

3.5 State 4 oxygen consumption only increases in skeletal muscle mitochondria isolated in the presence of phosphatase inhibitors from MDMA treated animals

In light of that fact that MDMA treatment of animals causes phosphorylation of UCP3 in skeletal muscle mitochondria, which is preserved when phosphatase inhibitors are included in the mitochondria preparation medium, we looked to see whether state 4 oxygen consumption rate is affected by MDMA treatment of animals compared to PBS treated controls. The data in Figure 5A demonstrate that state 4 oxygen consumption rates only increased significantly in skeletal muscle mitochondria, isolated in the presence of phosphatase inhibitors from MDMA treated animals compared to skeletal muscle mitochondria, isolated in the presence of phosphatase inhibitors from PBS treated control animals (MDMA treated 84.02 ± 0.01 ; PBS treated 63.0 ± 1.5 ; $p=0.002$; $n=3$. Units are nmol dioxygen/min/mg mitochondrial protein). There was no increase in state 4 oxygen consumption rates in skeletal muscle mitochondria, isolated in the absence of phosphatase inhibitors from MDMA treated animals compared to skeletal muscle mitochondria, isolated in the absence of phosphatase inhibitors from PBS treated control animals (Figure 5B), nor was there significant difference in state 4 oxygen consumption rate in a comparison of mitochondria from PBS treated animals isolated in the presence of phosphatase inhibitors (Figure 5A) with mitochondria from PBS or MDMA treated animals isolated in the absence of phosphatase inhibitors (Figure 5B). Using liver mitochondria as controls, as they have no UCP3, we observe no difference in state 4 oxygen consumption rates in liver mitochondria, isolated in the presence (Figure 5C) or absence (Figure 5D) of phosphatase inhibitors from MDMA treated animals compared to liver mitochondria, isolated in the presence or absence of phosphatase inhibitors from PBS treated control animals. Furthermore, we can observe that state 4 oxygen consumption rates, by skeletal muscle mitochondria or liver mitochondria, isolated in the presence or absence of phosphatase inhibitors from animals that are treated with MDMA or PBS are insensitive to addition of the purine nucleotide GDP and subsequent addition of the long-chain fatty acid oleate (Figures 5A, 5B, 5C and 5D). Addition of oleate before addition of GDP had no effect on state 4 oxygen consumption rates under any of the conditions described (results not shown). In addition we observe that the uncoupled rate (+FCCP) of oxygen consumption by skeletal muscle mitochondria or liver mitochondria (of rat), is

greater in the preparations in presence of phosphatase inhibitors compared to those without phosphatase inhibitors (Figures 5A, 5B, 5C and 5D). However, there is no significant difference in uncoupled oxygen consumption rates in by skeletal muscle mitochondria or liver mitochondria, in presence or absence of phosphatase inhibitors in a comparison of MDMA and PBS treated animals (Figures 5A, 5B, 5C and 5D). Similar results were obtained for skeletal muscle mitochondria and liver mitochondria from mice (data not shown). We conclude that state 4 oxygen consumption rate by skeletal muscle mitochondria is only increased in MDMA treated animals and the increase is preserved by the presence of phosphatase inhibitors in the preparation medium.

3.6 Increase in state 4 oxygen consumption (proton leak) of skeletal muscle mitochondria from MDMA treated animals is due to phosphorylated UCP3

In order to determine whether the increased state 4 oxygen consumption rate by skeletal muscle mitochondria from MDMA treated animals compared to PBS treated animals, isolated in the presence of phosphatase inhibitors, was due to UCP3 phosphorylation, we compared state 4 oxygen consumption rates in skeletal muscle mitochondria from wild-type and UCP3 knock-out mice. Our data show that the state 4 oxygen consumption rate, with succinate as substrate, were significantly greater only in skeletal muscle mitochondria isolated from wild-type mice compared to UCP3 knock-out mice in animals treated with MDMA plus phosphatase inhibitors (MDMA treated wild-types 82.0 ± 2.3 ; MDMA treated UCP3 knock-outs 64.0 ± 1.5 ; $p=0.003$; $n=3$). Units are nmol dioxygen/min/mg mitochondrial protein) (Figure 6A). There was no difference in the state 4 oxygen consumption rate with succinate as substrate in skeletal muscle mitochondria, isolated from wild-type mice when compared to UCP3 knock-out mice, from MDMA treated animals prepared in the absence of phosphatase inhibitors (Figure 6B), nor was there significant difference in state 4 oxygen consumption rate in a comparison of mitochondria from UCP3^{-/-} mice isolated in the presence of phosphatase inhibitors (Figure 6A) with

mitochondria from wild-type or UCP3^{-/-} mice isolated in the absence of phosphatase inhibitors (Figure 6B). Furthermore, there was no difference in state 4 oxygen consumption rates of liver mitochondria, respiring on succinate, isolated from wild-type mice when compared to UCP3 knock-out mice, from MDMA treated animals prepared in the presence of phosphatase inhibitors (Figure 6C). Consistent with the data for succinate as substrate, our data show that the state 4 oxygen consumption rate, with glycerol-3-phosphate as substrate, was significantly greater in skeletal muscle mitochondria isolated from wild-type mice when compared to UCP3 knock-out mice in animals treated with MDMA plus phosphatase inhibitors (Figure 6D). No difference in state 4 oxygen consumption rate was observed in liver mitochondria isolated in the presence of phosphatase inhibitors from MDMA treated animals in a comparison of wild-type and UCP3 knock-out mice (Figure 6C). Again we can observe that the state 4 oxygen consumption rates, by skeletal muscle mitochondria or liver mitochondria, isolated in the presence or absence of phosphatase inhibitors from animals that are treated with MDMA, are insensitive to addition of the purine nucleotide GDP or the addition of the long-chain fatty acid oleate (Figures 6A, 6B and 6C). In addition we observe that the uncoupled rate (+FCCP) of oxygen consumption by skeletal muscle mitochondria (of MDMA treated mice), is, if anything slightly less in the preparations in presence of phosphatase inhibitors, compared to those without phosphatase inhibitors (Figures 6A and 6B). Furthermore, there is no significant difference in uncoupled oxygen consumption rates in skeletal muscle mitochondria (figure 6A and 6B) or liver mitochondria (Figure 6C) in presence of phosphatase inhibitors, in a comparison of wild-type and UCP3 knock-out mice treated with MDMA (Figures 6A, 6B and 6C). It should be noted that our comparison of state 4 oxygen consumptions rates for skeletal muscle mitochondria, isolated in the absence of phosphatase inhibitors, from wild-type and UCP3 knock-out mice are consistent with data from the studies of Vidal-Puig et al. (2000) and Cadenas et al. (2002) but differ slightly from those of Gong et al. (2000). We conclude that the increase in state 4 oxygen consumption rate in skeletal muscle mitochondria isolated from MDMA treated animals, when phosphatase inhibitors are present in the preparation medium, is due to phosphorylated UCP3.

Discussion

We have provided evidence that MDMA treatment of animals causes *in vivo* phosphorylation of UCP3 in skeletal muscle mitochondria, the consequence of which is activation of UCP3, to increase proton leak in skeletal muscle mitochondria.

Confidence in antibody specificity is crucially important and we have demonstrated that the UCP3 peptide antibody we are using in this study is selective for UCP3 over other mitochondrial transporters (Figure 1). These data are consistent with previously reported selectivity determinations for this antibody over other mitochondrial transporters including UCP1 and UCP2 (Cunningham et al., 2003) and its use for detection of UCP3, in mitochondria from thymocytes and splenocytes (Carroll et al., 2004). The usefulness of the antibody is also reflected in its sensitivity to detect UCP3 cleanly in whole muscle homogenates (Cunningham et al., 2003).

Using this antibody, we show that the amount of UCP3 protein present in skeletal muscle mitochondria is not affected 2 hours after treatment of animals with MDMA (Figure 2). These data are not too surprising as one might not expect to see a significant change in UCP3 protein abundance within 2 hours after MDMA treatment. Furthermore, as expected, UCP3 abundance is not affected by addition of phosphatase inhibitors to the mitochondrial preparation, and clearly the phosphatase inhibitors are not affecting the ability to detect UCP3.

The data in Figure 3 demonstrate that MDMA administration to animals causes a greater degree of phosphorylation of mitochondrial proteins than when animals are treated with PBS alone, particularly evident for phosphotyrosine. Furthermore, the addition of the phosphatase inhibitors to the

mitochondrial preparation medium, increases the capacity to preserve the in vivo sites of phosphorylation of mitochondrial proteins once mitochondria are isolated.

The phosphorylation protection protocol was inspired by Lee et al. (2009) who demonstrated preservation of labile phosphorylation within cytochrome oxidase, when phosphatase inhibitors were incorporated in to the preparation medium of heart mitochondria with the subsequent demonstration that cytochrome oxidase activity can be regulated by tyrosine phosphorylation of subunit IV.

Our data go on to show that skeletal muscle mitochondrial UCP3 is one of those proteins phosphorylated in vivo, on serine(s) and tyrosine(s) (but not threonine) following treatment of animals with MDMA (Figure 4). UCP3 phosphorylation is not preserved when skeletal muscle mitochondria are prepared in the absence of phosphatase inhibitors, following treatment of animals with MDMA, indicating the labile nature of these covalent modifications. Furthermore, we confirm that the phosphorylation of UCP3 is due to MDMA, as PBS injection of animals did not cause UCP3 phosphorylation of skeletal muscle mitochondria. The detection of phosphorylation of UCP3 following MDMA treatment is a novel finding and is consistent with data from Carroll et al. (2008) demonstrating increased phosphorylation of UCP1 in BAT mitochondria following cold-acclimation. In the case of UCP1, serine2/3 and serine51 phosphorylation were detected following purification of UCP1 from brown adipose tissue mitochondria. The detection of the phosphorylation of serines and tyrosines in UCP3, yet a lack of detection of phosphorylation of threonine in UCP3, may reflect a degree selective activation of UCP3. Future endeavours will identify the phosphorylation sites within UCP3.

In order to discover whether in vivo mitochondrial UCP3 phosphorylation affected mitochondrial proton leak, we first compared the state 4 oxygen consumption rate of skeletal muscle mitochondria isolated from MDMA and PBS treated animals. We could show that the state 4 oxygen consumption rate by skeletal muscle mitochondria is increased only in MDMA treated animals, where the in vivo phosphorylation of the mitochondrial proteins are preserved (Figure 5). Taking this fact together with the observation that MDMA does not affect UCP3 abundance (Figure 2), we can deduce that any

increase in state 4 oxygen consumption rate is due to MDMA treatment and may be due to a change in UCP3 activity. The absence of any differential effect of state 4 oxygen consumption rate in liver mitochondria isolated from the same animals treated with either MDMA or PBS, in the presence or absence of phosphatase inhibitors, again consolidates the fact that the MDMA effect on state 4 oxygen consumption is not universal and not occurring in mitochondria from a tissue (liver) that does not constitutively express UCP3 (Boss et al., 1997; Krauss et al., 2005).

Having established that MDMA treatment increased state 4 oxygen consumption in skeletal muscle mitochondria, we set out to determine the contribution of UCP3 to the increased proton leak by comparing state 4 oxygen consumption rates in mitochondria from wild-type and UCP3 knock-out mice. We were able to demonstrate that state 4 oxygen consumption rates were only increased in wild-type mice treated with MDMA under conditions where the *in vivo* phosphorylation state of the skeletal muscle mitochondria was preserved (Figures 6A and 6D). Furthermore, a comparison of mitochondria from wild-type and UCP3 knock-out mice clearly demonstrate that, all the increased state 4 oxygen consumption rate is due only to UCP3 as a result of MDMA treatment under conditions where we know UCP3 is phosphorylated (Figure 6A and 6D). No increase in state 4 oxygen consumption rate in mitochondria is observed under conditions where UCP3 is present but where we know UCP3 is not phosphorylated (6B and 6E). Our data also show no difference in maximal electron transport chain activity in a comparison of skeletal muscle mitochondria from wild-type and UCP3 knock-out mice (Figures 6A and 6B). Therefore, we conclude that the increase in state 4 oxygen consumption rate, after MDMA treatment, is due to increased proton leak through phosphorylated UCP3.

Our observation that UCP3 needs to be covalently modified is consistent with data from *in vitro* activation studies from other laboratories that have demonstrated that UCP3 needs to be activated to catalyze a proton leak (Lee et al., 2009). Certainly, addition of superoxide activates UCP3 in isolated skeletal muscle mitochondria resulting in increased and GDP-sensitive proton leak, probably as a result of generation of reactive polyunsaturated fatty acid oxidation products that covalently bind to UCP3

(Echtay et al., 2002). This position is endorsed by the observation that in vitro addition of highly reactive aldehydes, such as 4-hydroxy-2-nonenals to isolated mitochondria, increases proton leak of skeletal muscle mitochondria possibly through UCP's (Echtay et al., 2003) or possibly through a less specific mechanism (Shabalina et al., 2006). The other interesting observation in our study, is the lack of purine nucleotide sensitivity of the skeletal muscle mitochondrial proton leak, catalyzed by in vivo activated/phosphorylated UCP3 (Figures 5 and 6). This contrasts with purine nucleotide sensitive nature of the in vitro covalent modification activation by 4-hydroxy-2-nonenals on UCP3 containing mitochondria reported by Echtay et al. (2003), the purine nucleotide sensitive in vitro generated lipid hydroperoxide uncoupling activities reported for mitochondria by Lombardi et al. (2010) and the purine nucleotide sensitive proton leak reported for proteoliposomes containing expressed reconstituted UCP3 (Jabůrek et al., 1999; Echtay et al., 2001). One explanation for the lack of purine nucleotide sensitivity to mitochondria containing activated/phosphorylated UCP3, may be due to the fact that our observations reflect the manifestation of the true in vivo mechanism of UCP3 activation or at least the in vivo mechanism of UCP3 activation as a result of administration of this particular pharmacological agent (MDMA) to animals. Future work will elucidate whether there is UCP3 activation through phosphorylation as a result of physiological stimuli and whether this too is purine nucleotide insensitive. Interestingly, there is an independent observation of UCP3 insensitivity to purine nucleotide binding. Divakaruni et al. (2010) have shown that trypsinolysis of UCP3 in mitochondria, following addition of long-chain fatty acids to soluble protein is unprotected by GDP, unlike the situation in mitochondria that contain UCP1, suggesting that UCP3, unlike UCP1, does not bind GDP.

We should also point out that it is not uncommon, when comparing proton leaks, to measure proton leak kinetics i.e. state 4 oxygen consumption rate as a function of membrane potential driving the proton leak, and compare proton leak at a given membrane potential. In this study we chose to compare state 4 oxygen consumption rates (with reference to maximal electron transport chain activity) as means to compare proton leak. The reason for our strategy has to do with the accuracy of

measuring oxygen consumption rates in state 4. Due to its design, the instrument that we used to measure oxygen consumption, the Oroboros Oxygraph-2k respirometer, has minimal back diffusion of air/oxygen into the oxygen electrode chamber compared with Perspex oxygen electrode chambers (Gnaiger, 2001). Further modification of a Perspex oxygen electrode chambers to accommodate electrodes to detect membrane potential obviously increases the rate of back diffusion of air/oxygen even further, resulting in an underestimation of state 4 oxygen consumption rates/proton leak rates by mitochondria. We wanted to achieve an accurate measure of state 4 oxygen consumption rates as possible and so used an Oxygraph-2k respirometer which as yet has no facility for measuring membrane potential. One can equate state 4 oxygen consumption rate with proton leak rate, as we know state 4 oxygen consumption is predominantly controlled by proton leak across the mitochondrial inner membrane (90%, Brand et al., 1988). The remaining element controlling state 4 oxygen consumption is substrate supply and electron transport chain activity and the fact that we see no significant difference in maximal electron transport chain activity in a comparison of skeletal muscle from wild-type and UCP3 knock-out mice again reassures us that any difference in state 4 oxygen consumption rates are due to phosphorylated UCP3.

To conclude, by MDMA treatment of animals and by preserving the phosphorylation state of mitochondrial proteins we have discovered a novel *in vivo* mechanism by which UCP3 is activated to increase proton leak, namely *in vivo* covalent modification through phosphorylation of as yet unspecified serines and tyrosines on UCP3. It remains to be determined whether phosphorylation of UCP3 in skeletal muscle mitochondria is a primary physiological means of regulating UCP3 function or whether this type of activation is only a consequence of the pharmacological nature of MDMA. We propose that the MDMA induced hyperthermia in skeletal muscle described by Mills et al. (2003) is due to increased proton leak *in vivo* as a result of activation of UCP3 through phosphorylation.

And finally, as mentioned earlier, the physiological significance of UCP3 activation has yet to be determined but UCP3 activation could be involved in cold-induced adaptive-thermogenesis. For instance Wijers et al. (2008) have demonstrated increased state 4 oxygen consumption in skeletal muscle mitochondria isolated from cold-acclimated humans. However, a recent study using cold acclimated wild-type and UCP1 knock-out mice states that "UCP3 is not innately uncoupling, that it cannot be induced to act as an uncoupler by either fatty acids or by endogenous reactive oxygen species, and that effects of GDP on respiration are not mediated by UCP3 study" (Shabalina et al., 2010). Interestingly, Sun et al. (2003) have demonstrated that increased UCP3 protein levels in rat skeletal muscle following lipopolysaccharide/dexamethasone injection, possibly indicating a role for UCP3 involvement in thermogenesis in sepsis. Future work on UCP3 activation will focus on the aforementioned areas of research.

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Figure Legends

Figure 1. Anti-UCP 3 peptide antibody detects UCP 3 in isolated muscle mitochondria from wild-type (WT) but not from UCP 3 $-/-$ mice. Lysate from yeast expressing UCP3, skeletal muscle mitochondria

from UCP 3 ^{-/-} and wild-type mice and liver mitochondria from wild-type mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:500 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of commercial anti-PDH E1- α antibody (B). The ~ 33 kDa band and a ~44 kDa band denote the presence of UCP 3 and PDH E1- α respectively.

Figure 2. Expression levels of UCP 3 protein in muscle mitochondria isolated in the presence and in the absence of phosphatase inhibitors. Skeletal muscle mitochondria were isolated from MDMA and PBS treated animals in the presence and absence of phosphatase inhibitors and subsequently subjected to SDS-PAGE (12% resolving gel) as and immunoblot analysis with a 1:500 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of commercial anti-PDH E1- α antibody (B). The ~ 33 kDa band and a ~44 kDa band denote the presence of UCP 3 and PDH E1- α respectively.

Figure 3. Comparison phosphotyrosine and phosphoserine profiles of skeletal muscle mitochondria isolated in the presence and in the absence of phosphatase inhibitors. Muscle mitochondria were isolated from MDMA and PBS treated muscle in the presence and absence of phosphatase inhibitors and subsequently subjected to SDS-PAGE (12% resolving gel) and immunoblot analysis with a 1:1000 dilution of anti-phosphotyrosine antibody (A), a 1:1000 dilution of anti-phosphoserine antibody (Millipore) (A) and an anti-PDH-E1- α antibody (C and D). The ~44 kDa band denotes the presence of PDH E1- α .

Figure 4. Detection of phosphorylated UCP 3 in skeletal muscle mitochondria isolated from MDMA treated mice in the presence of phosphatase inhibitors. Mitochondria from skeletal muscle or liver were isolated and protein was immunoprecipitated using either (A) protein G agarose anti-phosphotyrosine

antibody or (B) protein A agarose anti-phosphoserine antibody. UCP3 (~33kDa) was detected using a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody.

Figure 5. Comparison of state 4 oxygen consumption rates by skeletal muscle and liver mitochondria, isolated in the presence or absence of phosphatase inhibitors, from MDMA treated and PBS treated animals. Skeletal muscle and liver mitochondria were isolated from MDMA treated and PBS treated animals in the presence and absence of phosphatase inhibitors. Oxygen consumption rates by mitochondria (0.0625mg/ml) from (A) skeletal muscle isolated in the presence of phosphatase inhibitors, (B) skeletal muscle isolated in the absence of phosphatase inhibitors, (C) liver isolated in the presence of phosphatase inhibitors and (D) liver isolated in the absence of phosphatase inhibitors were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH (pH 7.4, 1 mM EGTA, 1 μ M atractyloside, 1 μ g/ml oligomycin, 0.1% de-fatted BSA, 1 μ M rotenone and 5 mM succinate-KOH in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained (state 4). 1 mM GDP was added followed by the addition of 60 μ M oleate (~25nM final conc.). Finally, 0.25 μ M FCCP was titrated to attain the state 4 uncoupled rate. Data are mean $\pm$ sem of three experiments performed in triplicate. *p* values $\leq$ 0.05 on an unpaired Students 't'-test were deemed significant. N.S = not significant.

Figure 6. Comparison of state 4 oxygen consumption rates by skeletal muscle and liver mitochondria, isolated in the presence or absence of phosphatase inhibitors, from MDMA treated wild-type and UCP3 knock-out animals. Skeletal muscle and liver mitochondria were isolated from MDMA treated wild-type and knock-out animals in the presence and absence of phosphatase inhibitors. Oxygen consumption rates by mitochondria (0.0625mg/ml) from (A) skeletal muscle (respiring on succinate) isolated in the presence of phosphatase inhibitors, (B) skeletal muscle (respiring on succinate) isolated in the absence of phosphatase inhibitors, (C) liver (respiring on succinate) isolated in the presence of phosphatase inhibitors (D) skeletal muscle (respiring on glycerol-3-phosphate) isolated in the presence of phosphatase inhibitors and (E) liver (respiring on glycerol-3-phosphate) isolated in the presence of

phosphatase inhibitors, were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH (pH 7.4, 1 mM EGTA, 1 μ M atractyloside, 1 μ g/ml oligomycin, 0.1% de-fatted BSA, 1 μ M rotenone in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained (state 4). 1 mM GDP was added followed by the addition of 60 μ M oleate (~25nM final conc.). Finally, 0.25 μ M FCCP was titrated to attain the state 4 uncoupled rate. Data are mean $\pm$ sem of three experiments performed in triplicate. p values $\leq$ 0.05 on an unpaired Students 't'-test were deemed significant. N.S = not significant.

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(A). Muscle plus phosphatase inhibitors (succinate) (B). Muscle minus phosphatase inhibitors (succinate) (C). Liver plus phosphatase inhibitors (succinate) (D). Muscle plus phosphatase inhibitors (glycerol-3-phosphate) (E). Liver plus phosphatase inhibitors (glycerol-3-phosphate)