Roles of Norepinephrine, Free Fatty Acids, Thyroid Status, and Skeletal Muscle Uncoupling Protein 3 Expression in Sympathomimetic-Induced Thermogenesis

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ABSTRACT

Thyroid hormone (TH) plays a fundamental role in thermoregulation, yet the molecular mediators of its effects are not fully defined. Recently, skeletal muscle (SKM) uncoupling protein (UCP) 3 was shown to be an important mediator of the thermogenic effects of the widely abused sympathomimetic agents 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) and methamphetamine. Expression of UCP3 is regulated by TH. Activation of UCP3 is indirectly regulated by norepinephrine (NE) and is dependent upon the availability of free fatty acids (FFAs). We hypothesized that UCP3 may be a molecular link between TH and hyperthermia, requiring increased levels of both NE and FFAs to accomplish the thermogenic effect. Here, we demonstrate that MDMA (40 mg/kg s.c.) significantly increases plasma FFA levels 30 min after treatment. Pharmacologically increasing NE levels through the inhibition of phe-}

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ulating an inducible, thermogenic proton leak pathway (Krauss et al., 2005). The prototype UCP1 mediates FT in brown adipose tissue (BAT), an organ specialized for heat production in hibernating mammals and rodents (Lin and Klingenberg, 1980). However, because of the absence of BAT in humans, skeletal muscle (SKM) plays a much larger and more significant role in the thermogenic response to sympathetic nervous system (SNS) stimulation (Astrup et al., 1989; Ye et al., 1996; Rose et al., 1999). This FT is thought to be mediated by norepinephrine (NE)-induced activation of a TH-regulated homolog of UCP1 (UCP3), found primarily in SKM (Gong et al., 1997; Lombardi et al., 2002).

The sympathetic nervous system is the major regulator of fat mobilization in human skeletal muscle and white adipose tissue. In a mechanism similar to FT induction in BAT, SKM UCP3 activation is believed to be mediated by concerted activation of α1- and β3-adrenergic receptors (α1AR + β3AR) in SKM, leading to cAMP-dependent liberation of free fatty acids (FFAs), which are required for UCP activation (Zhao et al., 1997). In addition, α1AR, β3AR, and triglyceride lipase mediate white adipose tissue lipolysis and FFA liberation into the bloodstream (for review, see Raynor, 2001). Supported also by adrenoceptor expression studies in human tissues (Revelli et al., 1993; Chamberlain et al., 1999), these findings suggest a model in which the sympathetic nervous system may contribute to SKM UCP3 activation by increasing both circulating and i.m. generated fatty acids. Further support for the role of UCP3 in FT stems from the observation that UCP3-deficient (UCP3−/−) mice show almost complete protection from hyperthermia induced by the potent sympathomimetic agents 3,4-methylenedioxyamphetamine (MDMA) (Mills et al., 2003) and methamphetamine (Sprague et al., 2004). The present study was designed to determine whether a TH-SNS-SKM-UCP3 axis may contribute to MDMA-induced hyperthermia in rats by examining the relationships between fluctuations in FFA, NE, and epinephrine (Epi) levels, acute and chronic changes in thyroid status, SKM UCP3 protein expression, and the extent of MDMA-induced changes in core body temperatures.

Materials and Methods

Animals. Plasma samples for determination of FFA, NE, and Epi levels were obtained from male Sprague-Dawley rats (Harlan, Dublin, VA) weighing 175 to 199 g (7–8 weeks of age). Animals used for NE and Epi evaluation were jugular vein-cannulated and sham and thyroparathyroidectomized (TX) rats of the same strain and weight were obtained from Harlan (Indianapolis, IN). Rats that had undergone surgery were housed in cages (cage size, 21.0 × 41.9 × 20.3 cm) individually; all of the other animals were housed in groups of three. Animals were maintained at an ambient temperature of 22 to 26°C and a 12-h light/12-h dark cycle and were provided ad libitum access to food and water.

Surgical procedures for thyroparathyroidectomy have been described previously (Sprague et al., 2003). Calcium needed to be maintained in these animals because of removal of the parathyroid gland, so TX animals were administered 2 to 4% calcium lactate solution as a drinking source for the duration of the experiment. Experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 80-23, revised 1996) and were approved by the Virginia Tech and Ohio Northern Animal Care and Use Committees.

Drugs and Chemicals. MDMA was generously donated by Dr. David E. Nichols (Purdue University, West Lafayette, IN). Levotyrodone was purchased from AmeriSource-Bergen (Williamston, MI); 1,4:3,6-didehydroxy-α-methylbenzylamine (DCMB) and all other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO).

Study Design. Male Sprague-Dawley rats were used to obtain blood samples via ventricular puncture for analysis of FFA levels. Rats were injected with either saline or MDMA (40 mg/kg s.c.) at time 0; the saline group (n = 6) was immediately sacrificed, and blood samples were obtained, whereas the two MDMA groups (n = 6) were sacrificed at 30 and 60 min post-treatment to procure blood samples in the same manner as the saline group. The dose of MDMA used in the present study is in accordance with previous reports (Mills et al., 2003; Sprague et al., 2003) and represents a severe human poisoning model where the degree change in temperature correlates with human mortality rates of ~45% (Gowing et al., 2002).

A crossover study design was used to investigate plasma NE and Epi levels, so each jugular vein-cannulated male Sprague-Dawley rat served as its own control. On the 1st treatment day, animals received a pretreatment of saline or DCMB (0.4 mmol/kg i.p.) 1 h prior to a main treatment of saline. DCMB is an inhibitor of phenylethanolamine-N-methyltransferase (Fuller et al., 1973) and was administered to prevent elevations in plasma levels of Epi and increase levels of NE. The half-life of DCMB in the adrenal glands is 3.4 h (Fuller et al., 1973), so 24 h were given to allow full clearance before the groups (n = 6) were switched, and the 2nd phase of the study was started, using MDMA (20 mg/kg s.c.) as the main treatment. Because combining DCMB with MDMA treatment was hypothesized to potentiate the hyperthermic response, dosing for these animals was reduced relative to that used in other portions of the study to prevent lethal complications.

For the chronic TH study, control euthyroid (EU) and TX male Sprague-Dawley rats with (HYPER) or without (Hypo) chronic tyroxine supplementation (levothyroxine 100 μg/kg i.p. daily for 7 days) were used to maintain a consistent thyroid status and avoid the confounding effects of autoregulation of plasma TH levels through an intact hypothalamic-pituitary-thyroid axis. To determine effects of acute changes in T4 status on MDMA-induced thermogenesis and UCP3 expression, HYPO and EU subjects were administered levothyroxine (100 μg/kg i.p.) 30 min before challenge with MDMA. TX and corresponding shams were treated with MDMA (40 mg/kg s.c.) or saline 1 week postsurgery. Animals were randomly allocated to the following treatment groups: sham (EU)-MDMA (n = 6), TX (HYPO)-MDMA (n = 9), sham (EU)-saline (n = 6), TX-chronic levothyroxine (HYPER, n = 5), HYPO-acute levothyroxine (n = 6), and sham (EU)-acute levothyroxine (n = 8).

Temperature Measurements. Temperatures of the animals used in the FFA study were taken just before and 30 and 60 min after the administration of saline or MDMA. Rectal temperatures for the plasma NE and Epi study were taken at 30-min intervals beginning just before the pretreatment and continuing until 1 h after the main treatment for a total of five measurements. Core temperatures for the TH study were determined in all animals before saline or MDMA administration and at 1, 2, and 3 h post-treatment as described. All rectal temperatures were measured using a Physiostemp Thermalert TH-8 thermocouple (Physitemp Instruments, Clifton, NJ) attached to a RET-2 rectal probe.

FFA Determination. Plasma nonesterified fatty acid levels were determined by the acyl-CoA synthetase-acyl-CoA oxidase method at the Diagnostic Laboratory of the College of Veterinary Medicine at Cornell University (Ithaca, NY). In brief, acyl-CoA was combined with the plasma to create CoA thiol esters. Acyl-CoA oxidase was then added, generating hydrogen peroxide, which along with included peroxidase oxidatively condensed 3-methyl-N-ethyl-N(β-hydroxy-ethyl)-aniline and 4-aminoantipyrine. This created a purple adduct that allowed for measurement of nonesterified fatty acids due to its absorption at 550 nm.

Plasma NE and Epi Analysis. Plasma was obtained via blood draws amounting to 500 μl each, obtained at three different time points: the time of the pretreatment and 30 and 60 min after the
main treatment. After removal, blood was placed into Microtainer tubes with lithium heparin and centrifuged for extraction of plasma, which was subsequently stored at $-80^\circ$C until analysis could be completed.

Plasma samples (70–100 $\mu$l) were prepared using the methods of Holmes et al. (1994) for analysis of NE and Epi levels. The only modifications were injection of samples and maintenance of the column at room temperature. Mobile phase consisted of 0.05 M sodium phosphate, 0.03 M citric acid buffer (with a pH range of 2.1–3.9), 0.1 mM EDTA, 0.42% sodium octyl sulfate, and 15% methanol. A high-performance liquid chromatography (480 series) with electrochemical detection (LC-44) and a C$_{18}$ reverse-phase analytical column (3.0 mm i.d. $\times$ 10 cm o.d.; 3 $\mu$m) was used (Bioanalytical Systems, Inc., West Lafayette, IN). ChromGraph 2.34 software (Bioanalytical Systems, Inc.) was used to integrate and analyze the raw data for the determination of Epi and NE levels, compared with internal standard curves.

**Determination of $T_4$ Levels.** Immediately following temperature recordings in response to MDMA treatments in HYPO, EU, and HYPER groups, animals were euthanized, blood was drawn for plasma $T_4$ measurements, and gastrocnemius biopsies were obtained for the determination of mitochondrial UCP3 expression by Western blots. Measurement of total plasma $T_4$ levels was conducted using a SNAP T4 (Indexx Laboratories, Sacramento, CA) enzyme-linked immunosorbent assay along with a standard curve as described previously (Sprague et al., 2003). The lower limit of detection was 2.0 $\mu$g/dl.

**Mitochondrial Isolation and Western Blot.** Rat and mouse (UCP3$^{-/-}$ as a negative antibody control) skeletal muscle mitochondrial lysates were prepared as follows. Gastrocnemius biopsies were minced in isolation buffer (100 mM KCl, 50 mM Tris/HCl, and 2 mM EDTA, pH 7.4, 4°C) and homogenized following centrifugation at 500g for 10 min. Supernatants were centrifuged for 10 min at 10,500g to pellet mitochondria. Mitochondria were resuspended in lysis buffer (0.1% Triton X-100, phosphate-buffered saline, and protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN), and lysates were separated (30 $\mu$g of total mitochondrial protein/lane) by gel electrophoresis and transferred to nitrocellulose membranes, which were stained with the reversible water-soluble protein stain Ponceau S to verify equal loading and to identify a suitable control band for densitometric normalizations for determining relative UCP3 expression. Antibodies directed against UCP proteins notoriously recognize nonspecific proteins that migrate in the same range (32–37 kDa) as UCP. In the present study, we verified the specificity of UCP3 detection with several different antibodies by comparing immunoreactivities in mitochondrial lysates from rat gastrocnemius SKM and from wild-type and UCP3-deficient mouse SKM. Only the Abcam antibody specifically recognized both mouse and rat UCP3. Densitometric measurements of UCP3 band densities versus a control band (Ponceau S stain) were performed using UN-SCAN-IT 4.3 scientific digitization software (Orem, UT) and expressed as UCP3/control with the average of the UCP3 intensity obtained from EU animals set to 1.0.

**Statistics and Linear Regression.** Plasma FFA, NE, and Epi levels, TH data, and all respective rectal temperature data were analyzed by GraphPad InStat version 3.05 (InStat; GraphPad Software Inc., San Diego, CA) using an analysis of variance (ANOVA) with a Student-Newman-Keuls post hoc for between-group comparisons and a Dunnett’s post hoc test for within-group comparisons. Statistical significance was set a priori at $p < 0.05$.

Linear regression and correlation coefficients were determined by plotting individual data points for each subject within HYPO, EU, and HYPER treatment groups ($n = 4–6$) for maximal change in temperature following MDMA administration, plasma $T_4$ levels, and $\Delta$TH data. A least-squares line was fit to the regression data. The linearity of relationships between plasma $T_4$ and UCP3 expression and $\Delta$TH data was determined by linear regression analysis. Statistical significance was determined using a linear relationship ANOVA test (InStat).

**Results**

**MDMA Increases Plasma FFA Levels.** Animals treated with MDMA experienced an increase in core body temperature to 40.43 ± 0.27°C, a rise of 2.75 ± 0.31°C above baseline (results not shown). FFA levels significantly increased ($p < 0.05$; 0.93 ± 0.19 mEq/l) to more than double the amount of baseline (0.42 ± 0.10 mEq/l) 30 min after MDMA administration (Fig. 1).

**DCMB Potentiates MDMA-Induced Hyperthermia and NE Release.** Although DCMB alone induced a hyperthermic response after 2 h ($p < 0.01$) compared with other groups (Fig. 2A), when combined with MDMA, it potentiated the characteristic hyperthermic response associated with exposure to MDMA by itself ($p < 0.05$). NE levels were significantly enhanced in the DCMB control group 90 min after administration ($p < 0.01$), whereas Epi levels did not change ($p > 0.05$; data not shown). On the 2nd day of treatment, MDMA alone was able to significantly ($p < 0.05$) increase NE levels to 106.69 ± 33.61 times of baseline after 60 min (Fig. 2B). The combination of DCMB-MDMA enhanced plasma levels of NE 60 min after the main treatment to levels 189.25 ± 43.34 times of baseline, an amount different from all of the other time points that day ($p < 0.05$; Fig. 2B).

**Chronic but Not Acute Changes in Thyroid Status Regulate the Thermogenic Response to MDMA.** Upon MDMA administration, EU animals exhibited a significant elevation in core body temperature (2.1 ± 0.3°C) that was sustained for 3 h from pretreatment baseline temperatures (Fig. 3A). The maximal thermogenic response to MDMA was significantly increased (approximately 32%, $p < 0.001$) in the HYPER group (2.9 ± 0.3°C). It is noteworthy that HYPER animals quickly succumbed to the lethal effects (data not shown) of MDMA administration and hyperthermia, and death was preceded by a rapid decrease in body temperature compared with the EU + MDMA group in which hyperthermia was sustained. In contrast, MDMA administration not only failed to increase body temperatures in HYPO animals but induced a marked and lasting hypothermia (−3.6 ± 0.3°C). Interestingly, acute pretreatment with levothyroxine
in the HYPO or EU animals had no effect on the temperature response patterns induced by MDMA (Fig. 3B).

**Chronic Changes in Thyroid Status Regulate Levels of Skeletal Muscle UCP3 Protein.** Levels of plasma T₄ (Fig. 4A) in the three groups were as follows: HYPO, undetectable; EU, 7.5 ± 0.36 μg/dl; and HYPER, 48.6 ± 7.6 μg/dl. Figure 4A is a representative Western blot showing UCP3 expression in isolated mitochondria (30 μg of mitochondrial protein/lane) in SKM biopsies from two of the six total animals per each of the HYPO, EU, and HYPER groups. UCP3 expression was decreased by approximately 70% in the HYPO (lanes 3 and 4) versus EU (lanes 5 and 6) animals. On the other hand, HYPER (lanes 7 and 8) animals showed an approximate 228% increase in UCP3 expression versus EU animals. Acute levothyroxine (30 min) and MDMA (180 min) treatments failed to change UCP3 SKM protein levels (data not shown).

**Regression Analysis Reveals a Strong Linear Correlation among Levels of Plasma T₄, UCP3, and Magnitude of Hyperthermia Induced by MDMA.** Linear regression analysis was used to compare in each animal maximal temperature change induced by MDMA, levels of skeletal muscle mitochondrial UCP3 protein expression (as determined by densitometry), and plasma T₄ levels. Our data show that there is a direct, statistically significant relationship between levels of plasma T₄ and UCP3 expression (Fig. 5A, p < 0.0001) and between levels of UCP3 expression and maximal change in temperature induced by MDMA (Fig. 5B, p < 0.0002).

**Discussion**

Despite that UCP3 is established to be a mitochondrial uncoupling protein, no consensus exists regarding its physiologic functions, although it is speculated to regulate mitochondrial lipid export, generation of mitochondrial reactive oxygen species (ROS), and thermogenesis under defined conditions (Brand and Esteves, 2005). Controversy exists re-
garding the involvement of the novel UCP isoforms (UCP2, UCP3) in thermoregulation because mice deficient in either homolog show only weak phenotypes; in particular, in each case, animals adapt appropriately to cold (Samec et al., 1998; Gong et al., 2000). Moreover, animals overexpressing SKM UCP3 exhibit normal basal body temperatures. However, these conclusions must be considered in light of substantial evidence showing that regardless of expression levels, proton leak mediated by UCP1–3 is not constitutive but rather requires activation by FFAs and/or ROS (Echtay et al., 2002). Unregulated uncoupling is generally held to occur when UCP is expressed at levels far exceeding the normal physiological range (Harper et al., 2002). Thus, it could be argued that the weak phenotypes observed in UCP3-deficient animals resulted from the lack of sufficient activation of UCP3 in wild-type subjects to resolve a thermoregulatory function and in UCP3-overexpressing animals resulted from a failure to adequately activate transgenic UCP3. The recent observation that UCP3-deficient mice show an almost complete blunting of the SKM thermogenic response to MDMA supports the argument that UCP3 requires activation in vivo (Mills et al., 2003) and that BAT UCP1 may not contribute significantly to FT in response to sympomimetic agents.

The present data support the notion that UCP3 regulates TH-dependent FT in SKM. UCP3 activators FFAs and ROS accumulate in SKM during contraction or exercise (Watt et al., 2003; McArdle et al., 2004), and Curtin et al. (2002) found that SKM UCP3 overexpression enhances the thermogenic response to contraction ex vivo, but no differences in muscle temperatures were apparent at rest. Likewise, for any given amount of mechanical work, EU SKM generates more heat than HYPO (Leijendekker et al., 1987). This increased thermogenesis probably results from activation of an UCP because compared with EU, SKM mitochondria from HYPER rats exhibit increased proton conductance (Brand et al., 1992) along with increased levels of ROS (Gredilla et al., 2001), FFAs (Lombardi et al., 2002), and coenzyme Q, a putative cofactor for UCP activity (Venditti et al., 2003). Moreover, increased mitochondrial proton leak in HYPER SKM is abolished when FFAs are removed (Silvestri et al., 2005). Interestingly, fasting increases SKM UCP3 expression but does not increase SKM proton leak (Boss et al., 1998). However, fasting also decreases mitochondrial coenzyme Q (Moreno et al., 2003) levels in SKM, which probably leads to a decrease in UCP activity, regardless of increased expression. Although it is not clear whether ROS are generally required to activate UCP3, there is considerable agreement that UCP3-induced proton leak is FFA-dependent and that it represents the major ROS-induced target for respiratory uncoupling in SKM. In support of this contention, superoxide production induced by exogenous xanthine-xanthine oxidase activates FFA-dependent proton conductance in isolated wild-type mitochondria that is not observed in SKM mitochondria from UCP3 knockout mice (Talbot et al., 2004). In
the present study, MDMA more than doubled plasma FFA levels, increasing the likelihood of uncoupling.

Insights gained from rodent models of thermoregulation probably underestimate the potential importance of SKM UCP3 to TH-dependent thermogenesis in humans. The most obvious reason is that adult humans lack BAT (and thus UCP1). Several other lines of evidence also support a significant role for SKM and UCP3. First, in the hyperthyroid state, UCP1-dependent thermogenesis is blunted as a result of suppression of adrenergic signaling in brown adipocytes (Golozoubova et al., 2004), but isolated SKM mitochondria from hyperthyroid rats show enhanced FFA-induced uncoupling (Brand et al., 1992). In addition, SKM is the primary contributor to thermogenesis and oxygen consumption induced by NE in animals (Rose et al., 1999), and humans (Astrup et al., 1985), and a 35-fold increase in plasma NE precedes MDMA-induced hyperthermia (Sprague et al., 2005). Interestingly, combined antagonism of α1- and β3-AR, which are expressed in SKM (Martin et al., 1990; Silence et al., 1993), prevents and reverses hyperthermia induced by MDMA (Sprague et al., 2005). β3-ARs have an affinity 30 times greater for NE compared with Epi (Hoffmann et al., 2000), further suggesting that NE is the primary SNS neurotransmitter involved in MDMA-mediated thermogenesis. The role of NE was investigated in the present study by using DCMB to increase plasma NE levels and prevent elevations in circulating Epi. By combining DCMB and MDMA treatments, we were able to elicit an augmented response to the thermogenic effects of MDMA, providing additional support for the role of NE, and not Epi in mediating hyperthermia. These observations reveal for the first time a direct, highly significant, linear relationship among plasma TH, SKM mitochondrial UCP3 levels, and the importance of SKM thermogenesis and oxygen consumption mediated by NE, and not Epi in mediating hyperthermia. The studies presented here demonstrate for the first time that SKM thermogenesis is critically dependent on the beta3-adrenoceptor in human skeletal muscle.

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