

**Roles of norepinephrine, free fatty acids, thyroid status and skeletal muscle
uncoupling protein 3 expression in sympathomimetic-induced thermogenesis***

Jon E. Sprague, Xianmei Yang, Joni Sommers, Tracy L. Gilman, and Edward M. Mills

The Department of Pharmaceutical and Biomedical Sciences, The Raabe College of
Pharmacy, Ohio Northern University, Ada, Ohio 45810 (J.E.S., J.S.)

Virginia College of Osteopathic Medicine, and Department of Biomedical Sciences and
Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060
(J.E.S., T.L.G.)

Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at
Austin, Austin, TX 78712 (X.Y., E.M.M.)

Running Title: Determinants of sympathomimetic-induced hyperthermia

Corresponding Author

Edward M. Mills, Ph.D.

Assistant Professor of Pharmacology and Toxicology

College of Pharmacy

2409 University Ave., PHR 5.218

1 University Station A1915

Austin, TX 78712

Phone: 512-471-6699

Fax: 512-471-5002

Email: ted_mills@mail.utexas.edu

Number of text pages: 16

Number of tables: 0

Number of figures: 5

Number of references: 40

Number of words in Abstract: 243

Number of words in Introduction: 516

Number of words in Discussion: 902

Abbreviations: MH, malignant hyperthermia; TH, thyroid hormone; T₃, 3,5,3'-triiodo-L-thyronine; T₄, 3,5,3',5'-tetraiodothyronine, thyroxine; FT, facultative thermogenesis;

UCP, uncoupling protein; BAT, brown adipose tissue; SKM, skeletal muscle; SNS, sympathetic nervous system; NE, norepinephrine; AR, adrenergic receptors; cAMP, cyclic adenosine monophosphate; FFA, free fatty acid; MDMA, 3,4-methylenedioxymethamphetamine; Epi, epinephrine; JVC, jugular vein-cannulated; TX, thyroparathyroidectomized; DCMB, \pm 2,3-dichloro- α -methylbenzylamine; PNMT, phenylethanolamine N-methyltransferase; EU, euthyroid; HYPER, with chronic thyroxine supplementation; HYPO, without chronic thyroxine supplementation; ACS-ACOD, acyl-CoA synthetase – acyl-CoA oxidase; acyl-CoA, acyl-coenzymeA; BAS, Bioanalytical Systems, Inc.; HPLC, high-performance liquid chromatography; Kd, kilodaltons; WT, wild type; ANOVA, analysis of variance; ROS, reactive oxygen species; CoQ, coenzyme Q.

Recommended section assignment: Toxicology

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 3 (UCP3) 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, sc) significantly increases plasma FFA levels 30 minutes after treatment. Pharmacologically increasing NE levels through the inhibition of phenylethanolamine N-methyltransferase with \pm 2,3-dichloro- α -methylbenzylamine (DCMB) potentiated the hyperthermic effects of a 20 mg/kg dose of MDMA. Using Western blots and regression analysis, we further illustrated that chronic hyperthyroidism in rats potentiates the hyperthermic effects of MDMA and increases levels of SKM UCP3 protein in a linear fashion according to levels of circulating plasma TH. Conversely, chronic hypothyroidism results in a hypothermic response to MDMA which is directly proportionate to decreased UCP3 expression. Acute TH supplementation did not change the skeletal muscle UCP3 expression levels or temperature responses to MDMA. These findings suggest that, although MDMA-induced hyperthermia appears to result from increased NE and FFA levels, susceptibility is ultimately determined by TH regulation of UCP3-dependent thermogenesis.

Introduction

Hyperthermia results from a severe, unregulated rise in core body temperature induced by high ambient temperature, strenuous exercise, endocrinopathy, or drug exposure. To our knowledge, no drug treatment has been established through controlled trials as an efficacious therapy for conditions which involve severe hyperthermia, with the exception of malignant hyperthermia (MH), which is effectively reversed with dantrolene (Blank and Boggs, 1993). The lack of therapeutic options for the management of hyperthermia likely results from an inadequate understanding of the basic molecular mechanisms of thermogenesis.

The principal active thyroid hormone (TH) 3,5,3'-triiodo-L-thyronine (T_3) is formed from the precursor 3,5,3',5'-tetraiodothyronine (T_4 , thyroxine) by deiodinases present in various tissues (Bianco and Larsen, 2005). Though TH has been established as the primary endocrinologic regulator of body temperature (Silva, 2005) and facultative thermogenesis (FT; reviewed by Lowell and Spiegelman, 2000), the mechanisms involved are complex and incompletely characterized. One family of genes regulated by TH (Gong et al., 1997) and believed to play a significant role in FT encodes for the mitochondrial uncoupling proteins (UCPs).

UCPs “uncouple” free energy stored in the mitochondrial electrochemical proton gradient from ATP synthesis by regulating 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 homologue 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 α_1 - and β_3 adrenergic receptors (α_1 AR + β_3 AR) in SKM, leading to cAMP-dependent liberation of free fatty acids (FFAs), which are required for UCP activation (Zhao et al., 1997). Additionally, α_1 AR, β_3 AR and triglyceride lipase mediate white adipose tissue lipolysis and FFA liberation into the bloodstream (reviewed in Rayner, 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 intramuscularly 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-methylenedioxymethamphetamine (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.

Methods

Animals. Plasma samples for determination of FFA, NE and Epi levels were obtained from male Sprague-Dawley rats (Harlan; Dublin, VA) weighing 175-199g (7-8 weeks of age). Animals used for NE and Epi evaluation were jugular vein-cannulated (JVC). Sham and thyroparathyroidectomized (TX) rats of the same strain and weight were obtained from Harlan (Indianapolis, IN). Rats which had undergone surgery were housed (cage size: 21.0x41.9x20.3cm) individually; all other animals were housed in groups of three. Animals were maintained at an ambient temperature of 22-26°C and a 12:12h light/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 2-4% 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 No. 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). Levothyroxine was purchased from Ameriasource-Bergen (Williamston, MI); 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 either injected with saline or MDMA (40 mg/kg, sc) at time 0; the saline group (n=6) was immediately sacrificed and

blood samples were obtained, while the two MDMA groups (n=6) were sacrificed at 30 and 60 minutes post-treatment to procure blood samples in the same manner as the saline group. The dose of MDMA utilized 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 cross-over study design was used to investigate plasma NE and Epi levels, so each JVC male Sprague-Dawley rat served as its own control. On the first treatment day animals received a pretreatment of saline or \pm 2,3-dichloro- α -methylbenzylamine (DCMB; 0.4 mmol/kg, ip) one hour prior to a main treatment of saline. DCMB is an inhibitor of phenylethanolamine N-methyltransferase (PNMT; Fuller et al., 1973) and was administered in order 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 hours (Fuller et al., 1973), so 24 hours were given to allow full clearance before the groups (n=6) were switched and the second phase of the study was began, using MDMA (20mg/kg, sc) 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 thyroparathyroidectomized (TX) male Sprague-Dawley rats with (HYPER) or without (HYPO) chronic thyroxine supplementation (levothyroxine 100 μ g/kg, ip daily X 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, ip) 30 minutes prior to challenge with MDMA. TX and corresponding shams were treated with MDMA (40mg/kg sc) or saline 1 week post-surgery. 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 prior to, and 30 and 60 minutes after, the administration of saline or MDMA. Rectal temperatures for the plasma NE and Epi study were taken at 30 minute intervals, beginning just prior to the pretreatment and continuing until 1 hour after the main treatment, for a total of 5 measurements. Core temperatures for the TH study were determined in all animals before saline or MDMA administration and at hours 1, 2, and 3 post-treatment as described. All rectal temperatures were measured using a Physiotemp Thermalert TH-8 thermocouple (Physitemp Instruments, Clifton, NJ) attached to a RET-2 rectal probe.

Free fatty acid (FFA) determination. Plasma non-esterified fatty acid levels were determined by the acyl-CoA synthetase – acyl-CoA oxidase (ACS-ACOD) method at the Diagnostic Laboratory of the College of Veterinary Medicine at Cornell University (Ithaca, NY). Briefly, acyl-coenzymeA (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

which allowed for measurement of non-esterified fatty acids due to maximal absorption at 550 nm.

Plasma norepinephrine (NE) and epinephrine (Epi) analysis. Plasma was obtained via blood draws amounting to 500 μ L each, obtained at three different time points: the time of the pretreatment, and 30 and 60 minutes after the main treatment. Following removal, blood was placed into Microtainer® tubes with lithium heparin and centrifuged for extraction of plasma, which was subsequently stored at -80° C until analysis could be completed.

Plasma samples (70-100 μ 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 BAS HPLC (480-series) with electrochemical detection (BAS LC-44) and a C_{18} reverse-phase analytical column (BAS 3.0 mm ID x 10 cm ODS, 3 μ m) was used (West Lafayette, IN). BAS ChromGraph® 2.34 software was used to integrate and analyze the raw data for the determination of Epi and NE levels, as compared to internal standard curves.

Determination of thyroxine (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 T_4 (Indexx Laboratories, Sacramento, CA) enzyme linked immunosorbent assay along with

a standard curve as described (Sprague et al., 2003). The lower limit of detection was 2.0 $\mu\text{g}/\text{dL}$.

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 followed by centrifugation at 500 g for 10 minutes. Supernatants were centrifuged for 10 minutes at 10,500 g to pellet mitochondria. Mitochondria were resuspended in lysis buffer (0.1 % Triton X-100, PBS, protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN) and lysates were separated (30 μg 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 (See Ponceau S, Fig. 4A) and to identify a suitable control band for densitometric normalizations for determining relative UCP3 expression. Antibodies directed against UCP proteins notoriously recognize non-specific proteins that migrate in the same range (32-37 Kd) as UCP (Fig. 4B). 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 (WT) and UCP3-deficient mouse SKM (Fig. 4A-B). Only the Abcam antibody specifically recognized both mouse and rat UCP3 (Fig. 4B). 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® v.3.05 (InStat) 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 (a) maximal change in temperature following MDMA administration, (b) plasma T₄ levels, and (c) SKM UCP3 protein expression. The linearity of relationships between plasma T₄ and UCP3 expression, and UCP3 expression and maximal change in temperature, were 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 minutes after MDMA administration (Fig. 1).

DCMB potentiates MDMA-induced hyperthermia and NE release. While DCMB alone induced a hypothermic response after 2 hours ($p < 0.01$) as compared to 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 minutes after administration ($p < 0.01$), while Epi levels did not change ($p > 0.05$; data not shown). On the second 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 minutes (Fig. 2B). The combination of DCMB-MDMA enhanced plasma levels of NE 60 minutes after the main treatment to levels 189.25 ± 43.34 times of baseline, an amount different from all 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) which was sustained for 3 hr 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). Of note, 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 to 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 both 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). Fig. 4A is a representative Western blot showing UCP3 expression in isolated mitochondria (30 µg mito-protein / lane) in SKM biopsies from 2 of the 6 total animals per each of HYPO, EU, and HYPER group. UCP3 levels among all subjects within each group showed little variability. Based upon normalized band densities, UCP3 expression was decreased by approximately 70 % in the HYPO (Lanes 3-4) versus EU (lanes 5-6) animals. On the other hand, HYPER (lanes 7-8) animals showed an approximate 228% increase in UCP3 expression versus EU animals. Acute levothyroxine (30 minutes) and MDMA (180 minutes) treatments failed to change UCP3 SKM protein levels (data not shown).

Regression analysis reveals a strong linear correlation between 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, though it is speculated to regulate (a) mitochondrial lipid export, (b) generation of mitochondrial reactive oxygen species (ROS) and (c) thermogenesis under defined conditions (Brand and Esteves, 2005). Controversy exists regarding 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 (a) UCP3 deficient animals resulted from the lack of sufficient activation of UCP3 in wild type subjects to resolve a thermoregulatory function, and in (b) 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 sympathomimetic 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. Similarly, for any given amount of mechanical work, EU SKM generates more heat than HYPO (Leijendekker et al., 1987). This increased thermogenesis likely results from activation of an UCP, because compared to 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 (CoQ), 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 CoQ (Moreno et al., 2003) levels in SKM, which likely leads to a decrease in UCP activity, regardless of increased expression. Though 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-acid 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 likely 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 α_1 - and β_3 -AR, which are expressed in SKM (Martin et al., 1990; Sillence et al., 1993), prevents and reverses hyperthermia induced by MDMA (Sprague et al., 2005). β_3 -AR have an affinity 30 times greater for NE as compared to Epi (Hoffmann et al., 2004), further suggesting that NE is the primary SNS neurotransmitter involved in MDMA-mediated hyperthermia. 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 between plasma TH, SKM mitochondrial UCP3 expression and extent of sympathomimetic-mediated hyperthermia and suggest that UCP3 may subserve an

important function in FT regulated by TH, NE and FFAs. This evidence also supports the notion that derangements in TH signaling may give rise to changes in UCP3 activity that may explain the idiosyncratic, non-dose responsive nature of sympathomimetic hyperthermia in humans. Important areas for future research will be to address the physiologic significance of UCP3 in thermoregulation in general, and to elucidate the signal transduction mechanisms by which UCP3 is activated in SKM, and the importance of SKM UCP3 in myolytic responses associated with hyperthermia.

Acknowledgements

We are grateful for the generous gift of MDMA from Dr. David E. Nichols.

References

- Astrup A, Bulow J, Madsen J and Christensen NJ (1985) Contribution of BAT and skeletal muscle to thermogenesis induced by ephedrine in man. *Am J Physiol* 248:E507-515.
- Astrup A, Simonsen L, Bulow J, Madsen J and Christensen NJ (1989) Epinephrine mediates facultative carbohydrate-induced thermogenesis in human skeletal muscle. *Am J Physiol* 257:E340-345.
- Bianco AC and Larsen PR (2005) Cellular and structural biology of the deiodinases. *Thyroid* 15:777-786.
- Blank JW and Boggs SD (1993) Successful treatment of an episode of malignant hyperthermia using a large dose of dantrolene. *J Clin Anesth* 5:69-72.
- Boss O, Samec S, Kuhne F, Bijlenga P, Assimacopoulos-Jeannet F, Seydoux J, Giacobino JP and Muzzin P (1998) Uncoupling protein-3 expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature. *J Biol Chem* 273:5-8.
- Brand MD and Esteves TC (2005) Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab* 2:85-93.
- Brand MD, Steverding D, Kadenbach B, Stevenson PM and Hafner RP (1992) The mechanism of the increase in mitochondrial proton permeability induced by thyroid hormones. *Eur J Biochem* 206:775-781.
- Chamberlain PD, Jennings KH, Paul F, Cordell J, Berry A, Holmes SD, Park J, Chambers J, Sennitt MV, Stock MJ, Cawthorne MA, Young PW, Murphy GJ. (1999) The tissue distribution of the human beta3-adrenoceptor studied using a monoclonal

antibody: direct evidence of the beta3 adrenoceptor in human adipose tissue, atrium, and skeletal muscle. *Int J Obes Relat Metab Disord.* 10: 1057-1065.

Curtin NA, Clapham JC and Barclay CJ (2002) Excess recovery heat production by isolated muscles from mice overexpressing uncoupling protein-3. *J Physiol* 542:231-235.

Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC and Brand MD (2002) Superoxide activates mitochondrial uncoupling proteins. *Nature* 415:96-99.

Fuller RW, Roush BW, Snoddy HD and Molloy BB (1973) Inhibition of phenylethanolamine *N*-methyltransferase by benzylamines. 2. *In vitro* and *in vivo* studies with 2,3-dichloro- α -methylbenzylamine. *J Med Chem* 16:106-109.

Golozoubova V, Gullberg H, Matthias A, Cannon B, Vennstrom B and Nedergaard J (2004) Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. *Mol Endocrinol* 18:384-401.

Gong DW, He Y, Karas M and Reitman M (1997) Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta3-adrenergic agonists, and leptin. *J Biol Chem* 272:24129-24132.

Gong DW, Monemdjou S, Gavrilova O, Leon LR, Marcus-Samuels B, Chou CJ, Everett C, Kozak LP, Li C, Deng C, Harper ME and Reitman ML (2000) Lack of obesity and normal response to fasting and thyroid hormone in mice lacking uncoupling protein-3. *J Biol Chem* 275:16251-16257.

- Gowing LR, Henry-Edwards SM, Irvine RJ and Ali RL (2002) The health effects of ecstasy: A literature review. *Drug Alcohol Rev* 21:53-63.
- Gredilla R, Lopez Torres M, Portero-Otin M, Pamplona R and Barja G (2001) Influence of hyper- and hypothyroidism on lipid peroxidation, unsaturation of phospholipids, glutathione system and oxidative damage to nuclear and mitochondrial DNA in mice skeletal muscle. *Mol Cell Biochem* 221:41-48.
- Harper JA, Stuart JA, Jekabsons MB, Roussel D, Brindle KM, Dickinson K, Jones RB and Brand MD (2002) Artfactual uncoupling by uncoupling protein 3 in yeast mitochondria at the concentrations found in mouse and rat skeletal-muscle mitochondria. *Biochem J* 361:49-56.
- Hoffmann C, Leitz MR, Oberdorf-Maass S, Lohse MJ and Klotz KN (2004) Comparative pharmacology of human β -adrenergic receptor subtypes - characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* 369:151-159.
- Holmes C, Eisenhofer G and Goldstein DS (1994) Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection. *J Chromatogr B Biomed Appl* 653:131-138.
- Krauss S, Zhang CY and Lowell BB (2005) The mitochondrial uncoupling-protein homologues. *Nat Rev Mol Cell Biol* 6:248-261.
- Leijendekker WJ, van Hardeveld C and Elzinga G (1987) Heat production during contraction in skeletal muscle of hypothyroid mice. *Am J Physiol* 253:E214-220.

- Lin CS and Klingenberg M (1980) Isolation of the uncoupling protein from brown adipose tissue mitochondria. *FEBS Lett* 113:299-303.
- Lombardi A, Silvestri E, Moreno M, De Lange P, Farina P, Goglia F and Lanni A (2002) Skeletal muscle mitochondrial free-fatty-acid content and membrane potential sensitivity in different thyroid states: Involvement of uncoupling protein-3 and adenine nucleotide translocase. *FEBS Lett* 532:12-16.
- Lowell BB and Spiegelman BM (2000) Towards a molecular understanding of adaptive thermogenesis. *Nature* 404:652-660.
- Martin WH, 3rd, Tolley TK and Saffitz JE (1990) Autoradiographic delineation of skeletal muscle alpha 1-adrenergic receptor distribution. *Am J Physiol* 259:H1402-1408.
- McArdle A, van der Meulen J, Close GL, Pattwell D, Van Remmen H, Huang TT, Richardson AG, Epstein CJ, Faulkner JA and Jackson MJ (2004) Role of mitochondrial superoxide dismutase in contraction-induced generation of reactive oxygen species in skeletal muscle extracellular space. *Am J Physiol Cell Physiol* 286:C1152-1158.
- Mills EM, Banks ML, Sprague JE and Finkel T (2003) Pharmacology: Uncoupling the agony from ecstasy. *Nature* 426:403-404.
- Moreno M, Lombardi A, De Lange P, Silvestri E, Ragni M, Lanni A and Goglia F (2003) Fasting, lipid metabolism, and triiodothyronine in rat gastrocnemius muscle: Interrelated roles of uncoupling protein 3, mitochondrial thioesterase, and coenzyme Q. *FASEB J* 17:1112-1114.

- Raynor DV (2001) The sympathetic nervous system in white adipose tissue regulation. *Proceedings of the Nutrition Society*. 60: 357-364.
- Revelli JP, Muzzin P, Paoloni A, Moinat M, and Giacobino JP (1993) Expression of the beta 3-adrenergic receptor in human white adipose tissue. *J Mol Endocrinol* 2:193-197.
- Rose RW, West AK, Ye JM, McCormick GH and Colquhoun EQ (1999) Nonshivering thermogenesis in a marsupial (the tasmanian bettong *Bettongia gaimardi*) is not attributable to brown adipose tissue. *Physiol Biochem Zool* 72:699-704.
- Samec S, Seydoux J and Dulloo AG (1998) Role of UCP homologues in skeletal muscles and brown adipose tissue: Mediators of thermogenesis or regulators of lipids as fuel substrate? *FASEB J* 12:715-724.
- Sillence MN, Moore NG, Pegg GG and Lindsay DB (1993) Ligand binding properties of putative beta 3-adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes. *Br J Pharmacol* 109:1157-1163.
- Silva JE (2005) Thyroid hormone and the energetic cost of keeping body temperature. *Biosci Rep* 25:129-148.
- Silvestri E, Moreno M, Lombardi A, Ragni M, de Lange P, Alexson SE, Lanni A and Goglia F (2005) Thyroid-hormone effects on putative biochemical pathways involved in UCP3 activation in rat skeletal muscle mitochondria. *FEBS Lett* 579:1639-1645.
- Sprague JE, Banks ML, Cook VJ and Mills EM (2003) Hypothalamic-pituitary-thyroid axis and sympathetic nervous system involvement in hyperthermia induced by

- 3,4-methylenedioxymethamphetamine (Ecstasy). *J Pharmacol Exp Ther* 305:159-166.
- Sprague JE, Mallett NM, Rusyniak DE and Mills E (2004) UCP3 and thyroid hormone involvement in methamphetamine-induced hyperthermia. *Biochem Pharmacol* 68:1339-1343.
- Sprague JE, Moze P, Caden D, Rusyniak DE, Holmes C, Goldstein DS and Mills EM (2005) Carvedilol reverses hyperthermia and attenuates rhabdomyolysis induced by 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) in an animal model. *Crit Care Med* 33:1311-1316.
- Talbot DA, Lambert AJ and Brand MD (2004) Production of endogenous matrix superoxide from mitochondrial complex I leads to activation of uncoupling protein 3. *FEBS Lett* 556:111-115.
- Venditti P, De Rosa R and Di Meo S (2003) Effect of thyroid state on susceptibility to oxidants and swelling of mitochondria from rat tissues. *Free Radic Biol Med* 35:485-494.
- Watt MJ, Stellingwerff T, Heigenhauser GJ and Spriet LL (2003) Effects of plasma adrenaline on hormone-sensitive lipase at rest and during moderate exercise in human skeletal muscle. *J Physiol* 550:325-332.
- Ye JM, Edwards SJ, Rose RW, Steen JT, Clark MG and Colquhoun EQ (1996) Alpha-adrenergic stimulation of thermogenesis in a rat kangaroo (Marsupialia, *Bettongia gaimardi*). *Am J Physiol* 271:R586-592.

Zhao J, Cannon B and Nedergaard J (1997) alpha1-Adrenergic stimulation potentiates the thermogenic action of beta3-adrenoreceptor-generated cAMP in brown fat cells. *J Biol Chem* 272:32847-32856.

Footnotes

*This work was funded in part by a Bowyer, J. Bennett, H. Bennett Chair of Pharmacy Research Grant (JES) and institutional startup funds by the College of Pharmacy, University of Texas at Austin (EMM).

Person to receive reprint requests:

Edward M. Mills, Ph.D.

Assistant Professor of Pharmacology and Toxicology

College of Pharmacy

2409 University Ave., PHR 5.218

1 University Station A1915

Austin, TX 78712

Phone: 512-471-6699

Fax: 512-471-5002

Email: ted_mills@mail.utexas.edu

Legends for Figures

Figure 1: MDMA increases plasma FFA levels. Plasma levels of FFAs significantly increased 30 minutes after MDMA administration (40 mg/kg, sc). MDMA was administered at time 0. Each value is the mean \pm SEM (n=6). *indicates significantly different from all other time points ($p < 0.05$).

Figure 2: DCMB potentiates MDMA-induced hyperthermia and NE release. (A) Change in rectal temperature from time 0 to time 60. Animals were given a pretreatment of saline or DCMB (0.4mmol/kg, ip) 60 minutes before a main treatment of saline or MDMA (20 mg/kg, sc). Temperatures were measured up to one hour following administration of the main treatment. Each column is the mean \pm SEM (n=5-6). *indicates significantly different from all other groups ($p < 0.05$). (B) Percent change from baseline levels of plasma NE. Animals were given a pretreatment of saline or DCMB (0.4mmol/kg, ip) 60 minutes before a main treatment of MDMA (20 mg/kg, sc). Plasma samples were obtained at the time of pretreatment and 30 and 60 minutes after administration of the main treatment. Each time point is the mean \pm SEM (n=3-5). Basal NE levels for the saline-MDMA group was 7.42 ± 0.13 pg/ μ L and 5.93 ± 0.65 pg/ μ L for the DCMB-MDMA treatment group. ^aindicates significantly different from respective baseline ($p < 0.05$). ^bindicates significantly different from all other time points ($p < 0.05$).

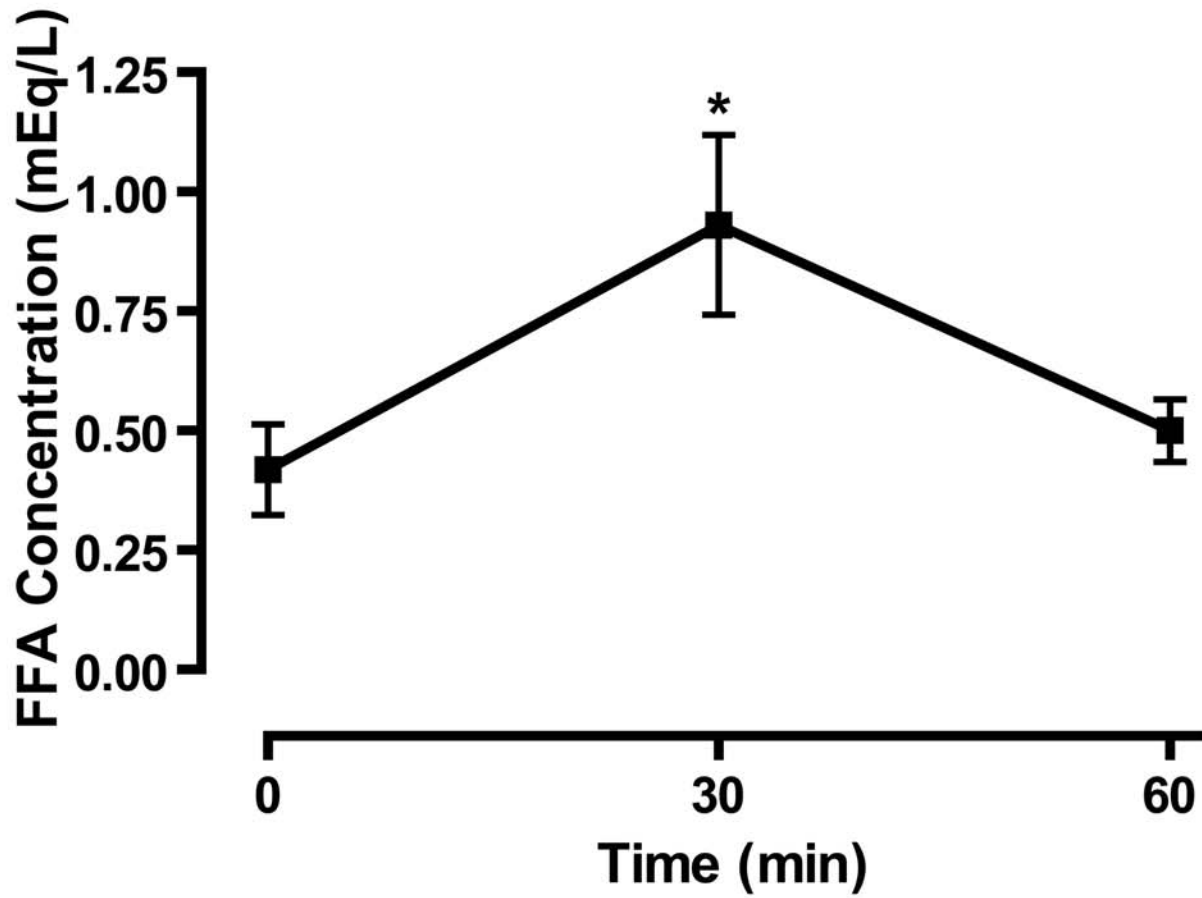
Figure 3: Chronic but not acute changes in thyroid status regulate the thermogenic response to MDMA. (A) MDMA was administered (40mg/kg, sc) to EU (●, n=6), HYPO (□, n=9), and HYPER (■, n=5) animals after measurement of baseline

temperature (time 0) and temperatures were determined in all animals at one, two, and three hour treatment time points. Sham-saline control (○, n=6) were treated in the same manner. Hyperthyroidism was produced by daily administration of levothyroxine (100µg/kg, sc.) for 7 days. (B) Lack of thermoregulatory effect of acute levothyroxine pretreatment (100µg / kg, sc, 30 minutes) on MDMA thermogenesis. 30 minutes following pretreatment, baseline temperature was determined in each animal. Saline (○, n=6) or MDMA was administered (40mg/kg, sc) to EU (●, n=6), or euthyroid animals acutely treated with levothyroxine (▲, n=8) and to HYPO (□, n=9), or HYPO animals acutely treated with levothyroxine (Δ, n=6). Temperatures were determined in all animals at one, two, and three hour post-treatment time points. Each value is the mean ± SEM. *** indicates significantly different from all other treatment groups, $p < 0.001$. EU animals treated acutely with levothyroxine exhibited the same characteristic hyperthermic response to MDMA as EU animals not receiving levothyroxine. Conversely, the hypothermic response to MDMA observed in HYPO animals was not significantly different than the response of HYPO animals treated acutely with levothyroxine.

Figure 4: Chronic changes in thyroid status regulate levels of skeletal muscle UCP3 protein. (A) Anti-UCP3 antibody specificity was confirmed by immunoblotting in mitochondrial lysates from WT and UCP3-deficient mice (KO). UCP3 expression was determined in SKM mitochondrial lysates from HYPO, EU, and HYPER animals (n=4-6) and expression from two representative animals per treatment group is shown. Densitometric analysis of UCP3 expression versus a control reference band (Ref) was performed as described in the Methods section, and values of UCP3 expression are expressed as the mean ± SEM. The mean ± SEM of plasma T₄ (ng/dL) from the same

animals in which levels of UCP3 were determined are shown. (B) Determination of rodent UCP3 antibody specificity. Only Abcam ab3477 specifically recognizes rat (R) and mouse UCP3.

Figure 5: Regression analysis reveals a linear correlation between levels of plasma T₄, UCP3 and magnitude of hyperthermia induced by MDMA. Maximal temperature response to MDMA and levels of plasma T₄ and SKM UCP3 were determined in each animal (n=4-5) in HYPO (■), EU (●), and HYPER (▲) treatment groups. (A) Linear correlation between levels of plasma T₄ and SKM UCP3. (B) Linear correlation between levels of SKM UCP3 and maximal thermogenic response to MDMA. Significance as determined by linear regression ANOVA analysis is shown, along with the correlation coefficients.



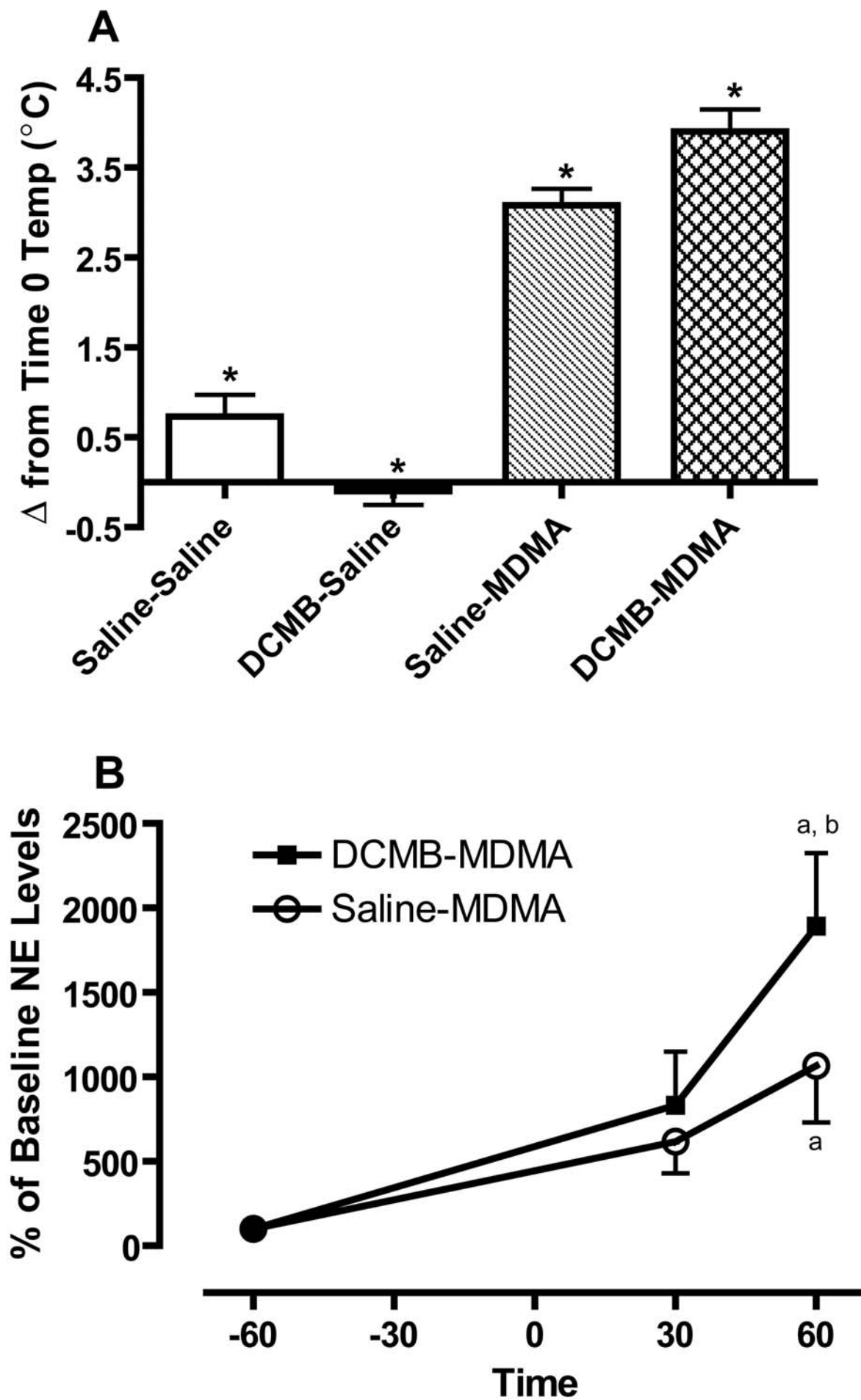
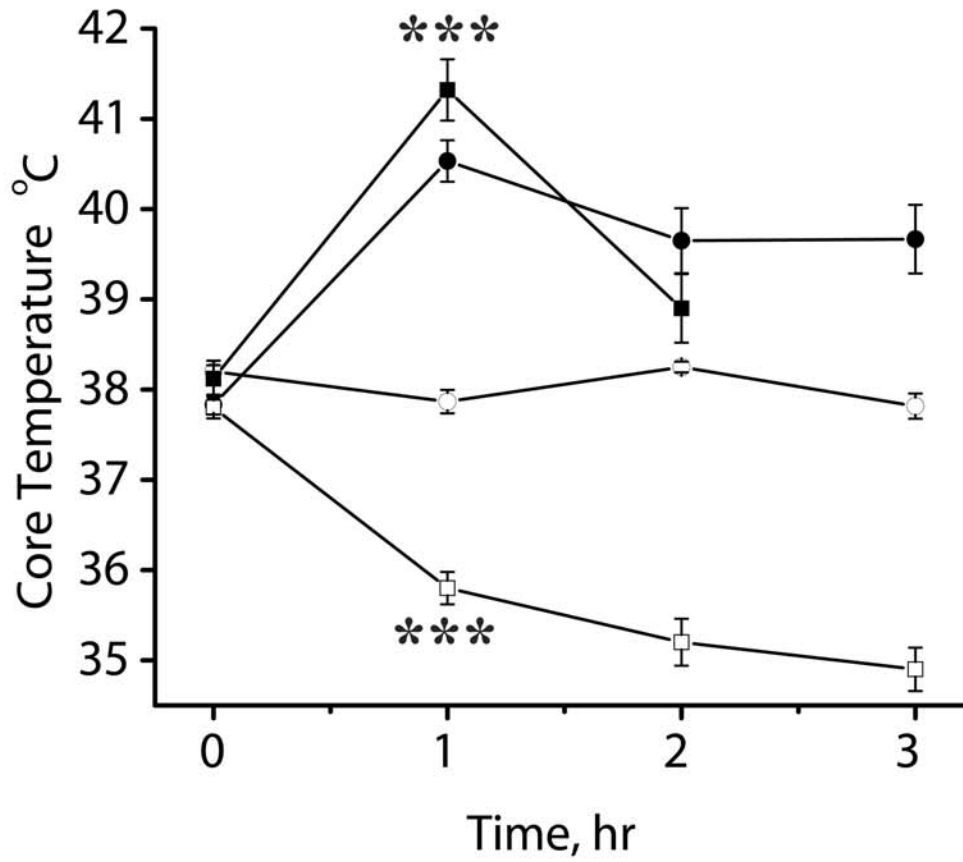


Fig. 3

A.



B.

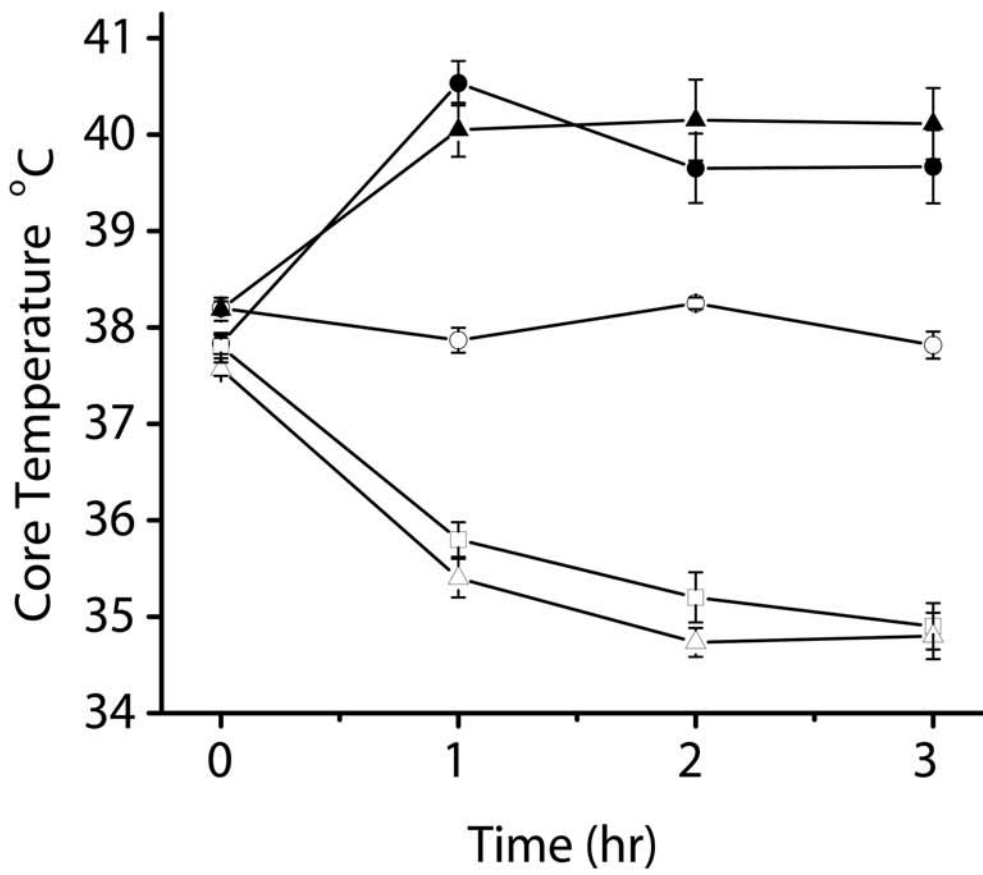
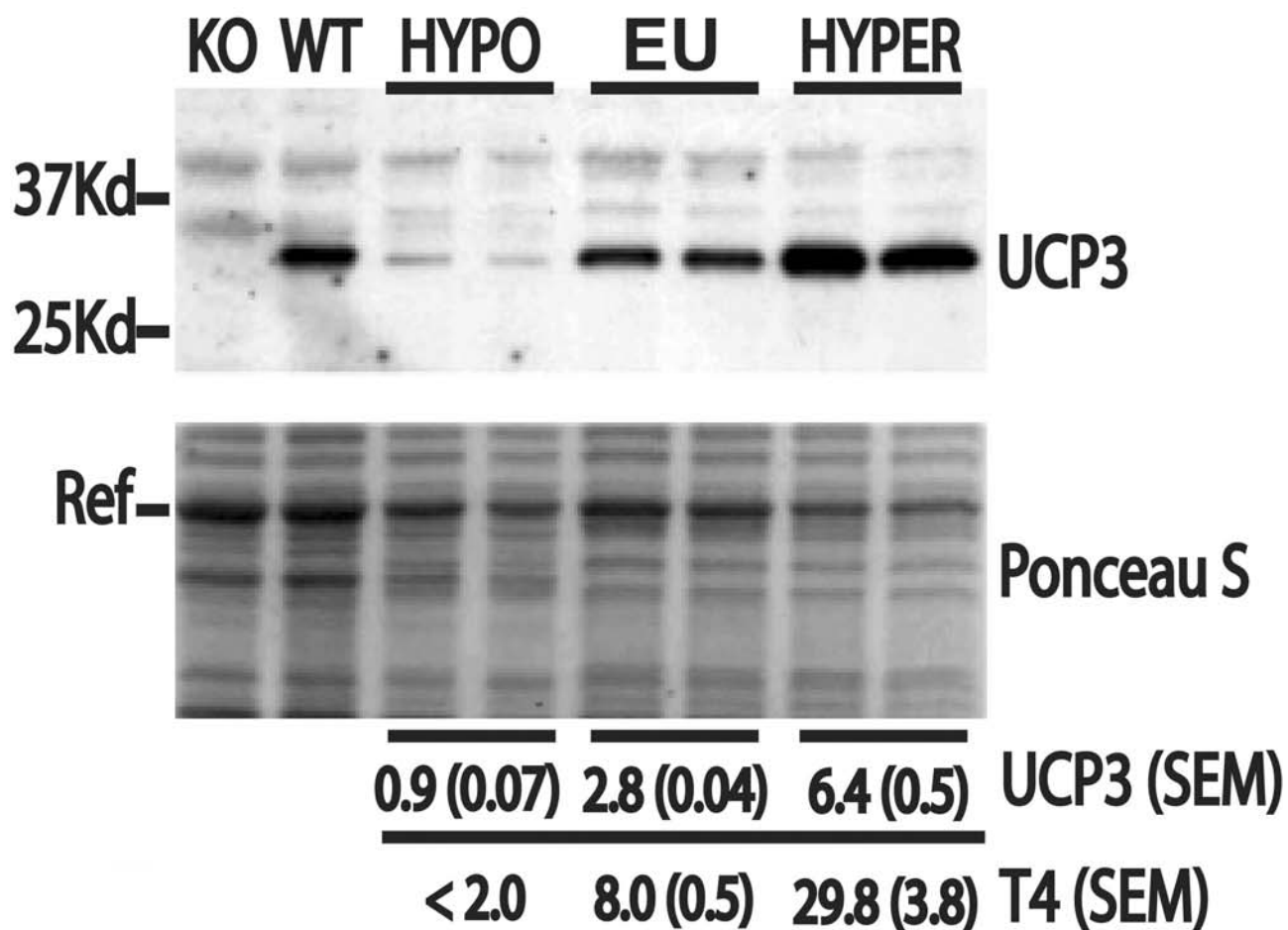
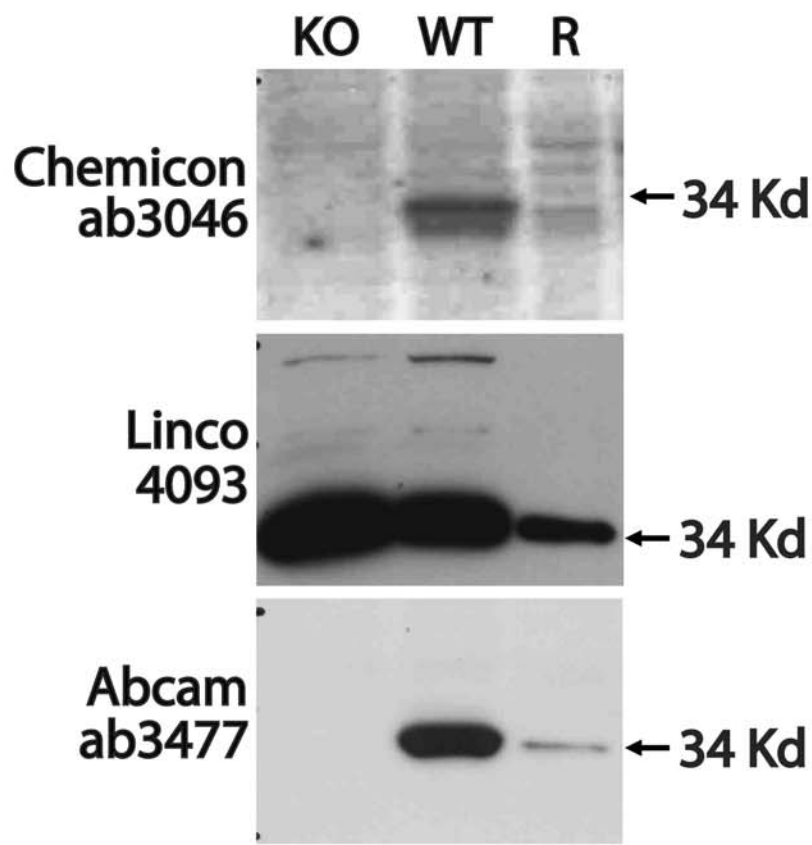
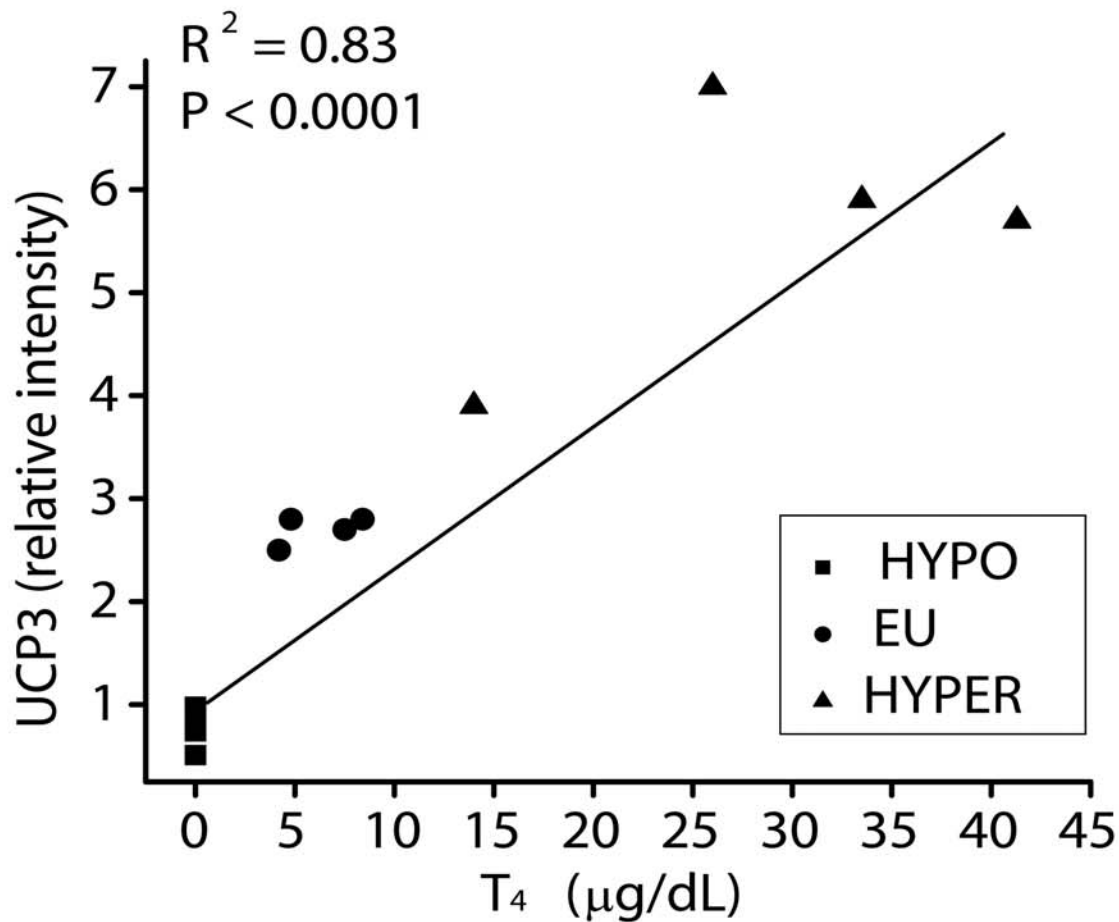


Fig. 4

JPET Fast Forward. Published on September 29, 2006 as DOI: 10.1124/jpet.106.107755
This article has not been copyedited and formatted. The final version may differ from this version.

A.**B.**

A.



B.

