

## RESEARCH PAPER

# Influence of dietary fats on ecstasy-induced hyperthermia

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**Background and purpose:** Studies were designed to examine the effects of dietary fats on metabolic effects of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy). These effects included hyperthermia, expression of uncoupling protein (UCP1 and 3) in brown adipose tissue or skeletal muscle and plasma free fatty acid (FFA) levels.

**Experimental approach:** Male Sprague-Dawley rats were fed either a high-fat diet (HFD, 60% kcal) or a lower fat isocaloric controlled diet (LFD, 10% kcal) for 28 days before MDMA challenge.

**Key results:** No significant differences were observed between LFD and HFD groups in terms of body weight, plasma thyroxine (T4) levels and expression of brown fat UCP1 or skeletal muscle UCP3 protein. HFD significantly raised levels of circulating FFA and potentiated the thermogenesis induced by MDMA (10 mg kg<sup>-1</sup>, s.c.), compared to the effects of the LFD. Moreover, 30 and 60 min after MDMA administration, plasma FFA levels decreased in HFD animals, but were markedly elevated in the LFD group.

**Conclusions and implications:** These results indicate that high-fat feeding regulates MDMA-induced thermogenesis by augmenting the activation of UCP rather than its expression.

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**Keywords:** MDMA; hyperthermia; free fatty acids; uncoupling proteins

**Abbreviations:** BAT, brown adipose tissue; G6PDH, glucose-6-phosphate dehydrogenase; HFD, high-fat diet; LFD, low-fat diet; MDMA, 3,4-methylenedioxymethamphetamine; SNS, sympathetic nervous system; T4, thyroxine; T<sub>c</sub>, core body temperatures; UCP, uncoupling protein

## Introduction

Increased production and accumulation of body heat in response to activation of the sympathetic nervous system (SNS) induced by sympathomimetic agents, involves a combination of vasoconstriction-induced loss of heat dissipation (Pedersen and Blessing, 2001) and the induction of inducible or 'facultative' metabolic thermogenic mechanisms (Mills *et al.*, 2004). Mitochondrial uncoupling proteins (UCPs) are the most prominent direct mediators of facultative thermogenesis. UCPs generate heat by regulating inducible mitochondrial proton leak, a highly exergonic process, which 'uncouples' the free energy stored in the electrochemical proton gradient from ATP synthesis. First identified three decades ago, UCP1 in brown adipose tissue (BAT) is both necessary and sufficient for the thermogenic adaptation to cold (Nicholls, 1974). In this complex

physiological response, cold-induced activation of the SNS leads to noradrenaline release and activation of  $\beta$ -adrenoceptors on brown adipocytes, which in turn initiate the 3',5'-cyclic adenosine monophosphate (cAMP) and protein kinase A-dependent activation of intracellular lipases and the accumulation of cellular free-fatty acids (FFA). Although the direct mechanisms and the relevance to *in vivo* conditions remain controversial, abundant *in vitro* evidence from isolated mitochondria and recombinant liposomal reconstitution systems show that FFA are required for UCP-dependent proton transport and respiratory uncoupling.

UCP1 is expressed exclusively in BAT; like most other large animals, adult humans have little or no BAT (nor UCP1). Thus, UCP1 is thought to play an insignificant, negligible role in human metabolic physiology. However, the recently identified UCP1 homologue UCP3 is enriched in skeletal muscle and similarly regulates mitochondrial proton leak and respiratory efficiency. Moreover, UCP3, like UCP1, is strongly regulated by SNS-dependent noradrenaline release,  $\beta_3$ -adrenoceptors, cAMP, and FFA (see Brand and Esteves, 2005). Consistent with its potential involvement in the

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regulation of human thermogenesis and metabolism, UCP3 has been implicated in clinical studies as a candidate susceptibility gene in hypometabolic diseases including obesity (Walder *et al.*, 1998) and type II diabetes mellitus (Gable *et al.*, 2006). Although its relevance to normal thermoregulatory physiology remains to be determined, strong support for the hypothesis that UCP3 is a candidate thermogenic mediator in intact animals stems from recent observations in mice indicating that the hypermetabolic hyperthermic responses to treatment with the amphetamines, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) and methamphetamine, are almost entirely lost in UCP3 knock-out mice (Mills *et al.*, 2003; Sprague *et al.*, 2004). Clinical case reports have demonstrated that MDMA and methamphetamine often induce a catastrophic hypermetabolic hyperthermic response that leads to skeletal muscle breakdown and death (Fahal *et al.*, 1992; Sreaton *et al.*, 1992; Mueller and Korey, 1998), suggesting that pathological mitochondrial uncoupling and heat production within muscle may contribute to human fatalities to these widely abused drugs.

A variety of different FFAs bind and activate proton transport in recombinant UCP3 liposomal preparations (Zackova *et al.*, 2003) and in isolated muscle mitochondria (Echtay *et al.*, 2002). These *in vitro* studies, showing that FFA are second messengers for UCP3 activation, are supported by recent findings in animal models that show that MDMA-induced peak thermogenesis (which is >80% dependent upon UCP3) is preceded by a significant elevation in circulating FFA (Sprague *et al.*, 2007). Strong support for the notion that FFA act as mediators of thermogenic signalling comes from the very recent observation that cold-induced thermogenesis, a response thought to be exclusively mediated by brown fat UCP1, is lost in animals that lack the plasma membrane fatty acid transport protein 1 (Wu *et al.*, 2006). High-fat feeding is known to increase significantly the levels of circulating and intramyocellular triglyceride FFA precursors (Hoeks *et al.*, 2006). Therefore, to test the hypothesis that FFA may similarly act as UCP-activating thermogenic second messengers *in vivo*, we compared several physiological metabolic parameters, along with the acute thermogenic responses to MDMA administration in rats subjected to isocaloric high and control, low fat and dietary regimens.

## Materials and methods

### Animals

All studies were carried out in accordance with the protocols approved by the Virginia Tech Animal Use and Care Committees. Male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were obtained weighing between 175 and 200 g and were housed in groups of two and given access *ad libitum* to either the high-fat diet (HFD) or low-fat diet (LFD), depending on treatment group, and water. Housing conditions were maintained at a temperature of 22–24°C and a 12:12 light–dark cycle. Rats were randomly allocated to either HFD ( $n=12$ ) or LFD ( $n=12$ ) groups. All animals were allowed 1 week of acclimation before dietary treatment. Each treatment group was fed the appropriate diet 4 weeks before MDMA treatment and blood sampling.

Weights were recorded once every week for 4 weeks following the start of dietary treatment.

### High- and low-fat diets

Both HFD and LFD were purchased from Research Diets Inc. (New Brunswick, NJ, USA). The HFD was composed of 60% kcal fat, 20% kcal carbohydrate, and 20% kcal protein. The LFD was composed of 10% kcal fat, 70% kcal carbohydrate and 20% kcal protein.

### MDMA treatment and blood sampling

Baseline, 30 and 60 min post-MDMA (10 mg kg<sup>-1</sup>, subcutaneous (s.c.)) blood samples were obtained by retro orbital sinus bleeds under isoflurane anaesthesia. Owing to possible interactions between isoflurane anaesthesia and MDMA, MDMA blood samples were taken 24 h after baseline samples and HFD rats and LFD rats were divided into two groups: 30 min blood draw ( $n=6$ ) and 60 min blood draw ( $n=6$ ). Each was divided so that core body temperatures ( $T_c$ ) and FFA samples would not be affected by isoflurane anaesthesia.

### Core temperature assessment

$T_c$  values were measured in all animals before and after MDMA administration, using a Thermalert TH-8 (Physitemp Instruments, Clifton, NJ, USA) monitor with a (RET-2) rectal probe attached to the thermocouple and white petrolatum was applied to the probe before insertion. The probe was inserted 3 cm into the rectum while the rat was gently restrained. A steady readout was obtained within 30 s of probe insertion.  $T_c$  values were recorded at 0, 30 and 60 min, as 60 min has been shown as the peak thermogenic response associated with MDMA (Sprague *et al.*, 2007).

### Thyroxine analysis

Plasma thyroxine (T4) levels were determined by the DRI Thyroxine Assay method at the College of Veterinary Medicine (Blacksburg, VA, USA). The homogenous enzyme used in this immunoassay contains ready-to-use liquid reagents. This assay uses 8-anilino-1-naphthalene sulphonic acid to dissociate T4 from plasma-binding proteins. The dissociated T4 is then allowed to compete with glucose-6-phosphate dehydrogenase (G6PDH) labelled T4 within the sample for a fixed amount of anti-T4 specific antibody binding sites in the solution. A lack of T4 within the sample allows the enzymatic activity of T4-labelled G6PDH to be inhibited, creating a relationship between T4 concentration in the sample and enzymatic activity. G6PDH activity was determined spectrophotometrically at 340 nm by measuring its ability to convert nicotinamide adenine dinucleotide to NADH, which proportionally represents T4 levels present within the plasma sample.

### Free-fatty acid determination

Plasma non-esterified fatty acid (NEFA) 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, USA). 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-( $\beta$ -hydroxy-ethyl)-aniline and 4-aminoantipyrine. This created a purple adduct, which enabled measurement of NEFA by spectrophotometry at 550 nm.

#### Mitochondrial isolation and Western blotting

Skeletal muscle mitochondrial lysates were prepared as described (Sprague *et al.*, 2007). Briefly, gastrocnemius and BAT biopsies were minced in isolation buffer (100 mM KCl, 50 mM Tris-HCl, 2 mM ethylene glycol bis( $\beta$ -aminoethyl-ether)-N,N,N',N'-tetraacetic acid, pH 7.4, 4°C), homogenized and centrifuged at 500 g (4°C). Mitochondria were pelleted at 10 500 g for 10 min and resuspended in 20  $\mu$ l lysis buffer (0.1% Triton-100, phosphate-buffered saline pH 7.4) and incubated on ice for 60 min. BAT samples required additional centrifugation to clear adequately the lysates. A total of 40  $\mu$ g of mitochondrial protein were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Filters were probed with rabbit polyclonal antibodies to UCP1 or UCP3 (Abcam ab10983 and ab3477, respectively). Levels of cytochrome *c* were evaluated with anti-cytochrome *c* (Santa Cruz catalogue no. sc-7159) as a control for total mitochondrial protein loading. Bands were visualized with secondary anti-rabbit antibodies linked to horseradish peroxidase using standard chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

#### Statistical analysis

Data within a treatment group were analysed by an analysis of variance (ANOVA) with a Dunnett's *post hoc* test. Data between groups were analysed by an ANOVA with a Student–Newman–Keuls *post hoc* test. When only two groups were compared, a Student's *t*-test was used. All statistical analysis was done using GraphPad Prism (Version 4.0). Significance was set *a priori* at  $P < 0.05$ .

#### Drugs

MDMA was generously donated by Dr David E Nichols, Purdue University (West Lafayette, IN, USA).

## Results

#### Characterization of the physiological effects of the HFD and LFD – thyroid hormone (T4) and body weight

No significant differences were observed between the LFD and HFD groups in either the initial or final body weights over the 4 weeks of dietary treatment. Similarly, levels of plasma T4 – the principal endocrine thermoregulatory hormone and transcriptional regulator of UCP3 – were identical in LFD and HFD groups at the conclusion of the study (Table 1).

**Table 1** Initial weight, final weight, baseline NEFA and baseline T4 levels

Diet	Initial weight (g)	Final weight (g)	NEFA levels (mEq l <sup>-1</sup> )	T4 levels ( $\mu$ g l <sup>-1</sup> )
LFD (n = 12)	182.92 $\pm$ 1.48	356.92 $\pm$ 4.17	0.50 $\pm$ 0.04	49.2 $\pm$ 2.4
HFD (n = 12)	180.08 $\pm$ 1.83	361.58 $\pm$ 6.87	0.71 $\pm$ 0.06*	44.2 $\pm$ 3.0

Abbreviations: NEFA, non-esterified free fatty acids; T4, thyroxine.

The values in the table are means  $\pm$  s.e.m. from the number of animals shown in parentheses.

\*Significance between groups ( $P < 0.03$ ).

#### Core body temperature assessment

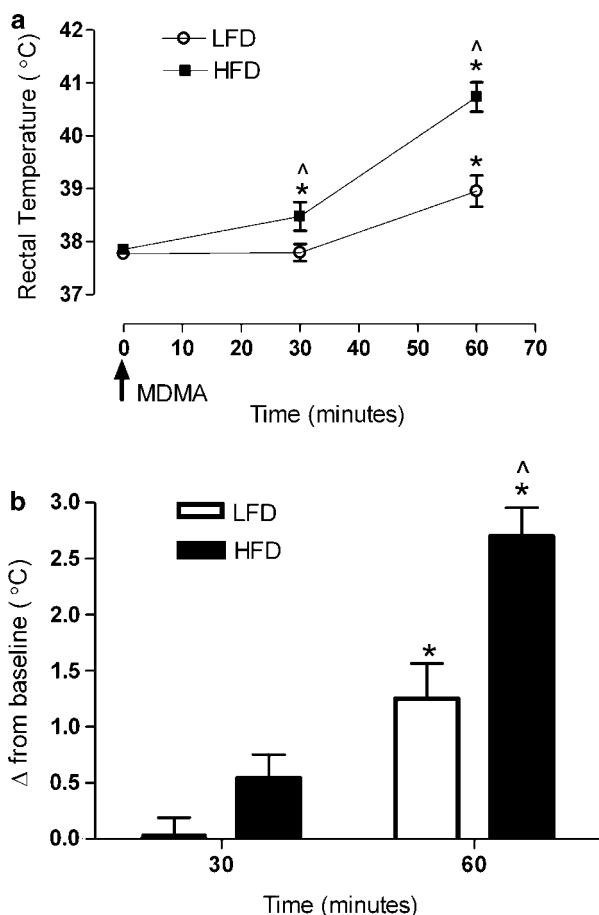
Basal  $T_c$  values did not significantly differ between the HFD and LFD group (Figure 1a). However, subsequent to stimulation of the SNS with MDMA, the rise in body temperature exhibited in the LFD group was significantly augmented in the HFD animals. At 30 min post-MDMA treatment,  $T_c$  values in the HFD were significantly higher than baseline temperatures ( $P < 0.05$ ) and temperatures of the LFD group ( $P < 0.05$ ). At 60 min, the HFD group differed significantly from both its own baseline ( $P < 0.001$ ) and from the LFD group at 60 min post treatment ( $P < 0.001$ ). At 60 min post treatment, both the HFD and LFD groups had significantly higher  $T_c$  values compared to baseline ( $P < 0.01$ ). Moreover, the HFD group maintained a significantly higher temperature 60 min post-MDMA compared to LFD ( $P < 0.01$ ). These data indicate that high fat feeding significantly potentiates the peak (60 min) acute thermogenic/hyperthermic response to MDMA both in terms of the change from baseline and the absolute temperature response comparisons between groups.

#### Free-fatty acid levels

Baseline non-esterified FFA levels before MDMA challenge were significantly higher in HFD versus LFD animals levels 28 days after the start of feeding regimens (Figure 2;  $P < 0.05$ ). To determine if any differences in FFA utilization occurred in response to diet, animals were challenged with MDMA, and FFA levels were measured 30 and 60 min after treatment. At 30 min, no differences were apparent in each group when FFA levels were compared to baseline; however, LFD plasma FFA levels at this time point were significantly higher than those of the HFD group ( $P < 0.01$ ). At 60 min post-MDMA treatment, the LFD group demonstrated a significant elevation in FFA compared to baseline and relative to FFA levels in the HFD group ( $P < 0.001$ ; Figure 2). These data indicate that LFD and HFD fed animals mobilize and/or utilize FFA differently after acute stimulation of the SNS with MDMA.

#### UCP1 and UCP3 levels in gastrocnemius and BAT samples

HFD or LFD feeding for 28 days did not alter the expression of UCP3 protein in mitochondria isolated from skeletal muscle nor did it change levels of UCP1 in brown fat mitochondria. We confirmed the specificity of the Abcam UCP1 antibody (ab10983) by comparing UCP1 immunoreactivity in mitochondria isolated from gastrocnemius (GAST), heart (HRT), and BAT (Figure 3a). This antibody

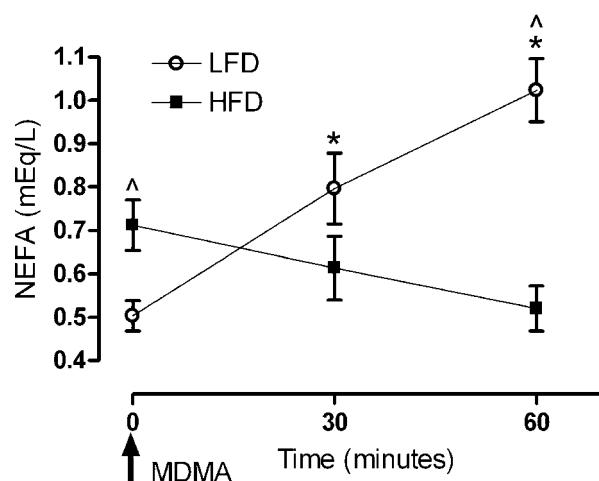


**Figure 1** (a) Effects of MDMA ( $10 \text{ mg kg}^{-1}$ , s.c.) on  $T_c$  values at an ambient temperature of  $23^\circ\text{C}$  in Sprague–Dawley rats consuming either HFD or LFD diets for 28 days. (b) Effects of MDMA ( $10 \text{ mg kg}^{-1}$ , s.c.) on  $T_c$  change from baseline at  $23^\circ\text{C}$  in Sprague–Dawley rats consuming either HFD or LFD diets for 28 days.  $T_c$  values were taken post-MDMA treatment in both LFD and HFD. \*Significance from baseline ( $P < 0.01$ ) and ^ Significance between groups ( $P < 0.02$ ). Each value is the mean  $\pm$  s.e.m. ( $n = 12$  for 0 and 30 min;  $n = 6$  for 60 min). HFD, high-fat diet; LFD, low-fat diet; MDMA, 3,4-methylenedioxymethamphetamine.

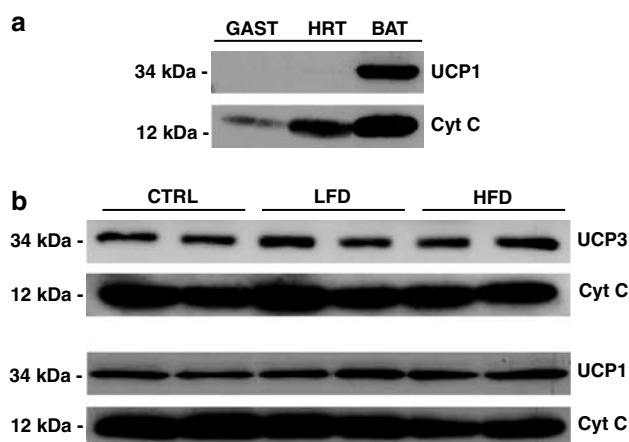
detected a  $\sim 34 \text{ kDa}$  immunoreactive band only in BAT. We have previously used skeletal muscle mitochondria from UCP3 knockout mice to confirm the specificity of the anti-UCP3 antibody (Abcam no. 3477) used in these studies (Sprague *et al.*, 2007). No differences were observed in levels of BAT UCP1 (lower) and skeletal muscle UCP3 (upper) protein in response to chronic changes in dietary fat content (Figure 3b). These data indicate that this particular HFD dietary regimen has no effect on the expression of UCP1 and UCP3 at the protein level in mitochondria.

## Discussion

Recent evidence indicates that the acute thermogenic responses to stimulation of the SNS with MDMA and methamphetamine are largely UCP3-dependent (Mills *et al.*, 2003; Sprague *et al.*, 2004). The peak rise in body temperature induced by these agents ranges from 1 to 2 h in



**Figure 2** Effects of MDMA ( $10 \text{ mg kg}^{-1}$ , s.c.) on non-esterified FFA levels (NEFA) in Sprague–Dawley rats consuming either HFD or LFD diets for 28 days. NEFA levels were taken at baseline and post-MDMA treatment in both LFD and HFD. \*Significance from baseline ( $P < 0.01$ ) and ^ Significance between groups ( $P < 0.05$ ). Each value is the mean  $\pm$  s.e.m. ( $n = 12$  for 0 min;  $n = 6$  for 30 min and  $n = 5$  for 60 min). HFD, high-fat diet; LFD, low-fat diet; MDMA, 3,4-methylenedioxymethamphetamine; NEFA, non-esterified fatty acid.



**Figure 3** Effects of chronic manipulations in dietary fat on UCP expression. (a) Specificity of the Abcam UCP1 antibody (ab10983) comparing UCP1 immunoreactivity in gastrocnemius (GAST), heart (HRT), and BAT. The antibody detected a  $\sim 34 \text{ kDa}$  immunoreactive band only in BAT. (b) No differences were observed in levels of BAT UCP1 (lower) and skeletal muscle UCP3 (upper) protein in response to chronic changes in dietary fat content. BAT, brown adipose tissue; UCP, uncoupling protein.

rodents and is preceded by a significant increase in plasma levels of noradrenaline and FFA (Sprague *et al.*, 2005, 2007). Noradrenaline stimulates thermogenesis in target tissues by binding most avidly to  $\alpha_1$ - and  $\beta_3$ -adrenoreceptors (Kuusela *et al.*, 1997; Zhao *et al.*, 1997).  $\beta_3$ -Adrenoreceptors activate the cAMP-dependent, hormone-sensitive lipase mediated conversion of stored intracellular triacylglycerols into FFA in brown adipocytes and skeletal muscle. Noradrenaline also stimulates the release of FFA into the general circulation from triglyceride depots in white adipose tissue (Garofalo *et al.*, 1996). On the basis of the abundant *in vitro* data, FFAs

activate UCP1-3-dependent respiratory uncoupling through thermogenic proton leak, but the mechanisms by which fatty acids and UCPs interact to mediate proton leak are highly controversial (Garlid *et al.*, 1996). Dietary manipulations have been shown to modify UCP1-dependent thermoregulatory responses to cold. However, these data may have little or no relevance to thermoregulation in adult humans thought not to express metabolically relevant amounts of UCP1 and brown fat. Moreover, there is a lack of studies to support a role for FFA in the regulation of an UCP3-mediated thermogenic function in animals. Here, we demonstrate for the first time that thermogenesis induced by MDMA administration is significantly enhanced by high-fat feeding. We also ruled out the possibilities that the enhanced thermogenic responses exhibited in HFD fed animals were due to changes in body weight, plasma thyroid hormone levels (T4), or changes in the protein levels of UCPs 1 and 3 in brown fat and skeletal muscle, respectively. Following MDMA administration, the HFD animals displayed a reduction in FFA levels and a significant elevation in body temperature. Conversely, animals maintained on a LFD demonstrated a rise in FFA levels, which was also correlated with a rise in core temperature; however, the LFD group never reached the same magnitude of elevation in core temperature as the HFD group.

FFAs are the required ligand activators of UCPs. FFA-induced uncoupling of oxidative phosphorylation within skeletal muscle mitochondria has been shown to play an important role in regulating body temperature in animals exposed to cold environmental conditions (Brustovetsky *et al.*, 1992). FFAs also activate UCP3 and accumulate in skeletal muscle during contraction or exercise (Tsutsui *et al.*, 2001; Watt *et al.*, 2003; McArdle *et al.*, 2004). Curtin *et al.* (2002) found that skeletal muscle UCP3 overexpression enhances the thermogenic response to contraction *ex vivo*, but no differences in muscle temperatures were apparent at rest. Moreover, nonhuman primate studies have demonstrated that increases in muscle contractions (namely locomotor activity) are not required for MDMA to induce hyperthermia (Crean *et al.*, 2006). Skeletal muscle mitochondria from hyperthyroid rats exhibit increased proton conductance (Brand *et al.*, 1992) along with increased levels of FFAs (Lombardi *et al.*, 2002); moreover, increased mitochondrial proton leak in hyperthyroid skeletal muscle is abolished when FFAs are removed (Silvestri *et al.*, 2005). We have previously shown that hyperthyroid animals are more sensitive to the thermogenic effects of MDMA and have elevated levels of skeletal muscle UCP3 (Sprague *et al.*, 2007). Together, these observations strongly suggest that FFAs are likely to be required for uncoupling-mediated thermogenesis.

Previous studies have shown that a HFD can increase plasma FFA levels (Chou *et al.*, 2001) along with skeletal muscle triacylglycerides, and that these differences in skeletal muscle FFA accumulation are greatly enhanced in the absence of UCP3 (Hoeks *et al.*, 2006). In obese and lean rats, fasting has been demonstrated to increase FFA levels (Cha *et al.*, 2005) and in these rats, central administration of C75, an inhibitor of fatty acid synthase, and peripheral administration of phentolamine, a nonselective  $\alpha$ -adreno-

receptor blocker, antagonized fatty acid oxidation in the periphery significantly more than when C75 was used alone (Cha *et al.*, 2005). These latter findings confirm that the SNS and  $\alpha$ -adrenoreceptors are directly involved in fatty acid breakdown.

Cha *et al.* (2005) further demonstrated that the combination of C75 and phentolamine decreased mRNA levels of UCP3 present in skeletal muscle. Studies on the expression of UCP3 protein in muscle in response to dietary fat are equivocal. HFD feeding has been shown to apparently increase UCP3 protein expression in skeletal muscle (Chou *et al.*, 2001), but others have observed no change in mRNA expression (Kusunoki *et al.*, 2005). In the present study, we have demonstrated that a HFD versus an isocaloric control LFD affected neither UCP3 nor UCP1 expression levels in skeletal muscle and brown fat, respectively. However, we confirmed that HFD increased plasma FFA compared to LFD, and significantly augmented hyperthermia induced by MDMA. Furthermore, UCP1 and UCP3 expression in both BAT and skeletal muscle are highly regulated by thyroid hormone (Sprague *et al.*, 2007). Consistent with a lack of effect of HFD on UCP levels in mitochondria from BAT and skeletal muscle, we found that high-fat feeding had no effect on levels of circulating T4. We have recently verified two antibodies/detection methodologies that are appropriate for UCP3 detection in mouse and rat tissues by western blotting (Sprague *et al.*, 2007). In the same article, we report that another purchased UCP3 antibody detects a strong 34 kDa non-specific immunoreactive band in UCP3 knockout mitochondria and wild type equally well. We anticipate that many of the discrepancies reported in the literature are due to the use of methodologies and reagents not verified for specificity, using knockout systems. Furthermore, the lack of change in UCP1 and UCP3 proteins we observed between LFD and HFD animals may also reflect the duration (28 days) and/or the quality of nutritional components and fats in various dietary regimens.

Together, these data demonstrate that dietary fat is a significant and positive regulator of inducible thermogenesis and suggest that the thermoregulatory effects of high-fat feeding involve changes in UCP activity, most likely as the result of elevated FFAs.

## Conflict of interest

The authors state no conflict of interest.

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