Published in final edited form as:

Eur J Pharmacol. 2009 August 1; 615(1-3): 257–261. doi:10.1016/j.ejphar.2009.05.021.

Pharmacodynamic characterization of insulin on MDMA-induced thermogenesis

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Abstract

Sympathomimetic drugs (MDMA; Ecstasy) induce a potentially catastrophic hyperthermia that involves free fatty acid (FFA) activation of mitochondrial uncoupling proteins (UCP). Insulin is an important regulator of plasma FFA levels, although its role in thermogenesis is unclear. The aims of the present study were 1) to characterize the pharmacodynamic effects of MDMA on plasma insulin and glucose, 2) to examine the effects of insulin on MDMA-induced thermogenesis and 3) to examine MDMA-induced thermogenesis in an animal model of insulin resistance, the obese Zucker rat. Insulin levels peaked 15 min. after MDMA (40 mg/kg, sc), which preceded the peak temperature change at 60 min. Plasma glucose levels also peaked 15 min. after MDMA and remained elevated throughout the 90-min. monitoring period. Insulin pretreatment (10 units/kg, sc) 30 min. before a low dose of MDMA (5 mg/kg, sc) potentiated the thermogenic response. Insulin resistant, fa/fa (obese) Zucker rats demonstrated an attenuated thermogenic response to MDMA (40 mg/kg, sc). Consistent with the role for FFA in UCP3 expression, immunoblot analysis showed significantly increased levels of UCP3 protein in obese compared to lean Zucker skeletal muscle. In conclusion, the results of the present study suggest a potential role of insulin signaling in sympathomimetic-induced thermogenesis.

Keywords

3,4-methylenedioxymethamphetamine; thermogenesis; insulin; glucose; Zucker

1. Introduction

The sympathomimetic agent 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) increases body temperature by preventing heat dissipation via peripheral vasoconstriction (Pedersen and Blessing, 2001) and by heat generation via activation of mitochondrial

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uncoupling proteins (UCP3; Mills et al., 2004). Sympathetic nervous system activation by MDMA results in peripheral norepinephrine release and subsequent activation of α_1 - and β_3 -adrenergic receptors (Kuusela et al., 1997; Zhao et al., 1997; Himms-Hagen et al., 1978). β_3 -adrenergic receptor activation leads to cAMP-mediated stimulation of hormone sensitive lipase and subsequent release of free fatty acids (FFA). FFA can initiate facultative thermogenesis (Mills et al., 2004), a process which ATP synthesis is “uncoupled” from substrate oxidation by FFA forming a proton-conductive pore in mitochondrial UCP3 located in skeletal muscle (Echtay et al., 2001; Brand and Esteves, 2005). Plasma FFA levels have been shown to increase substantially after MDMA, increasing the probability of ‘uncoupling’ (Sprague et al., 2007). Additionally, increasing plasma FFA levels with a high-fat diet enhances the thermogenic response to MDMA (Mills et al., 2007). These findings support a role for FFA in UCP3 activation and subsequent heat generation induced by MDMA (Mills et al., 2007).

FFA and glucose, the cell's main energy sources, are postulated to cross cell membranes by binding to their respective transport proteins. Glucose enters via GLUT-4 proteins, whose translocation to cellular membranes follows insulin stimulation or muscle contraction (Birnbaum, 1992). FFA can enter skeletal muscle and adipose cells through carrier-mediated proteins, such as fatty acid translocase (FAT/CD36), fatty acid binding protein (plasma membrane) (FABPpm) and fatty acid transport proteins (FATP1-6; Campbell et al., 2004; Bonen et al., 2007). Similar to GLUT4, the translocation of FFA transport proteins such as FAT/CD36 has been shown to be insulin-induced (Bonen et al., 2007). Insulin signaling therefore appears vital to the uptake of FFA into skeletal muscle and adipose cells.

The aims of the present study were 1) to characterize the pharmacodynamic effects of MDMA on plasma insulin and glucose levels, 2) to examine the effects of insulin on MDMA-induced thermogenesis and 3) to examine the thermogenic effects of MDMA in a rodent model type 2 diabetes insulin resistance. Obese Zucker rats are widely accepted as a genetic model of insulin resistance (Kasiske et al., 1992; Houseknecht et al., 1996). We hypothesized that MDMA administration might induce an increase in insulin levels to facilitate FFA transport into the mitochondrial membrane and subsequent heat generation via “uncoupling.” Furthermore, we hypothesized that if insulin signaling is involved in MDMA-induced thermogenesis, then insulin pretreatment before a low dose of MDMA should enhance the thermogenic response. These expected results would suggest a role of insulin signaling and insulin-mediated FFA transport in MDMA-induced thermogenesis. Lastly, obese Zucker rats have elevated plasma FFA, which could potentiate the thermogenic effects of MDMA; however, insulin resistance could blunt the thermogenic effects of MDMA. This animal model creates both a paradox and a unique opportunity to elucidate the roles of FFA and insulin in MDMA-mediated thermogenesis.

2. Methods

2.1 Animals

31 adult, male Sprague-Dawley rats (175-200g) with or without jugular vein catheters (JVC) were obtained from Harlan (Indianapolis, IN). For JVC animals, the following standard protocol at Harlan (Indianapolis, IN) was utilized. Anesthetized animals were placed in dorsal recumbency and the ventral cervical area was shaved and swabbed with surgical scrub (iodine and alcohol). A 1 to 1.5 cm skin incision was made over the right jugular vein. The fascia and underlying cervical muscles were separated by blunt dissection to reveal the jugular vein. Using forceps, a length of doubled sterile silk suture was passed under the jugular vein and the loop cut to provide two suture pieces. The more distal suture is tied tightly to occlude blood flow from the head region. The proximal suture was loosely tied around the jugular vein. Using microscissors, a small cut was made in the jugular vein between the two ligatures. A microrenathane 40 catheter was filled with heparin glycerol and inserted into the vein towards

the heart at a predetermined distance dependent upon the size of the rat. The proximal ligature was tightened around the vein and catheter to prevent dislodgement. The ends of the distal ligature were tied to the dumbbell on the catheter for additional anchorage. After confirming patency, the catheter was tunneled subcutaneously and exteriorized at the nape of the neck. The catheter was made accessible via a rat jack and capped with a stainless steel wire plug. The initial surgical incision was closed with tissue adhesive. Furthermore, 12 male homozygous obese (fa/fa) and 12 male homozygous/heterozygous lean (Fa/-) Zucker rats were obtained from Harlan (Indianapolis, IN). Zucker rats were 7-9 weeks of age and weighed between 200-250 g. Animals were housed in groups of 3 (cage size: 21.0×41.9×20.3 cm) on a 12-h light-dark cycle at an ambient temperature of 22-26°C. *Ad libitum* access to food (Harlan Teklad 22/5 Rodent Diet, Harlan, Indianapolis, IN, 15% kcal from fat, 28% kcal from protein, 57% kcal from carbohydrates) and water was provided. Experimental procedures were carried out 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 approved by the Ohio Northern University Animal Care and Use Committee.

2.2 Drugs and chemicals

MDMA was generously donated by Dr. David E. Nichols of Purdue University (West Lafayette, IN). Pentobarbital and all other chemicals or reagents were obtained from Sigma Chemical (St. Louis, MO).

2.3 Temperature measurements

All temperatures were measured using a Physiotemp Thermalert TH-8 thermocouple (Physiotemp Instruments, Clifton, NJ) with an attached RET-2 rectal probe. White petrolatum was applied to the probe prior to gentle insertion 3 cm into the rectum while rat was gently restrained. Stable measurements were obtained within 30 sec of probe insertion.

2.4 Pharmacodynamic characterization of MDMA effects on plasma insulin and blood glucose levels

Seven, JVC Sprague-Dawley rats were treated with MDMA (40 mg/kg, sc) and blood draws and rectal temperatures were taken under light hand restraint at 0, 15, 30, 60, and 90 min. after treatment. Blood glucose was analyzed using a glucose analyzer (OneTouch Ultra Smart blood glucose monitor, Lifescan, Milpitas, CA). A rat insulin radioimmunoassay (Millipore Corporation) using a ¹²⁵I detection method was used to measure plasma insulin levels.

2.5 Effects of Insulin on low-dose MDMA-induced body temperature effects

Twenty-four, Sprague-Dawley rats were randomly allocated into 4 treatment groups to receive insulin (10 units/kg, sc) 30 min. before MDMA (5 mg/kg, sc); insulin (10 units/kg, sc) 30 min. before MDMA (5 mg/kg, sc) plus glucose (1 g/kg, ip) 30 minutes after MDMA; saline followed by MDMA; and finally two dose of saline.

2.6 Effects of MDMA on core body temperature in insulin-resistant Zucker rats

12 obese and 12 lean (controls) Zucker rats were weighed, numbered and randomly assigned to receive either saline or MDMA (40 mg/kg, sc) such that each treatment condition was n=6. The dose of MDMA used in this study coincides with doses used in previous studies (Sprague et al., 2007) and is representative of the archetypal dose considered to cause severe poisoning in humans, resulting in a temperature change with an approximate 45% mortality rate (Gowing et al., 2002). Rectal temperatures were taken just before administration of either saline or MDMA, and at 30 and 60 min after administration. After 60 min, animals were euthanized with pentobarbital (100 mg/kg, ip) and blood samples collected via cardiac puncture for FFA and glucose analysis.

2.7 Non-esterified fatty acid (NEFA) level determination

Plasma NEFA levels were analyzed at the Diagnostic Laboratory at Cornell University (Ithaca, NY) as previously described (Sprague et al., 2007). 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 peroxidase, oxidatively condensed 3-methyl-N-ethyl-N-(β -hydroxy-ethyl)-aniline and 4-aminoantipyrine. This created a purple adduct, which enabled measurement of NEFA by spectrophotometry at 550 nm.

2.8 Western blot analysis

The right gastrocnemius was removed, *in toto*, and immediately flash frozen in liquid N₂. Gastrocnemius biopsies (100 mg samples) were minced in isolation buffer (100mM KCl, 50mM Tris-HCl, 2mM EGTA, pH 7.4, 4°C), homogenized and centrifuged at 500g (4°C). Mitochondria were pelleted at 10 500g for 10 min and resuspended in 25 μ L lysis buffer (0.1% Triton-100, phosphate-buffered saline pH 7.4) and incubated on ice for 60 min. 50 μ g (skeletal muscle) or 15 μ g (brown adipose tissue) of mitochondrial protein were resolved by SDS-PAGE and transferred to nitrocellulose. Following transfer, the SDS-PAGE gels were stained overnight with Gelcode Blue (Pierce Biotechnology) for total mitochondrial protein loading. Membranes were probed with anti-UCP3 (Abcam ab3477) 1:500. Bands were visualized with secondary anti-rabbit or mouse antibodies (Amersham) linked to horseradish peroxidase using standard chemiluminescence (Pierce Biotechnology).

2.9 Statistical analysis

Data within a treatment group were evaluated by an analysis of variance (ANOVA) with a Dunnett post hoc test, while data between groups were evaluated by an ANOVA with a Student-Newman-Keuls post hoc test. When only two groups were present, a student's t-test was used. All data were analyzed by GraphPad InStat® v.3.05. Statistical significance was set a priori at $P < 0.05$.

3. Results

3.1 Pharmacodynamic characterization of MDMA effects on plasma insulin and glucose levels

Fifteen min. after MDMA (40 mg/kg, sc) administration, insulin levels were significantly ($P < 0.01$) increased and returned to baseline at 30 min. This increase in insulin levels preceded the peak rise in core body temperature at 60 min. (Fig 1A). Plasma glucose levels were significantly ($P < 0.01$) elevated fifteen min. after MDMA and remain elevated throughout the 90-min. monitoring period (Fig. 1B).

3.2 Effects of insulin on body temperature effects of a low-dose of MDMA

A 5 mg/kg (sc) dose of MDMA induced a slightly, albeit, significant increase in body temperature at 30 and 60 min. (from 37.6 ± 0.1 °C to 38.5 ± 0.3 °C; $P < 0.05$; Fig 2). Animals treated with insulin (10 units/kg, sc) 30 min. before MDMA potentiated this thermogenic response resulting in a peak temperature of 40.0 ± 0.1 °C at 60 min. A one g/kg (ip) glucose dose plus insulin (10 units/kg, sc) attenuated the enhanced thermogenic response to MDMA (5 mg/kg, sc) induced by insulin (Fig. 2). Insulin (10 units/kg, sc) alone did not significantly alter body temperature (data not shown).

3.3 Effects of MDMA on body temperature in insulin resistant Zucker rats

Baseline core temperatures were not significantly different between the treatment groups. Both obese and lean MDMA-treated groups demonstrated a significant increase ($P < 0.05$) in core temperature at 30 min and remained elevated at 60 min compared to the saline-treated groups

(Fig. 3). Post-hoc analysis demonstrated that the obese/MDMA group displayed an attenuated ($P < 0.05$) thermogenic response compared to the lean/MDMA group at both 30 and 60 min time intervals.

3.4 Effects of MDMA on non-esterified fatty acid (NEFA) levels in insulin-resistant Zucker rats

NEFA levels were analyzed 60 min after MDMA or saline administration in all groups. In the saline-treated group, obese rats had significantly ($P < 0.001$) higher NEFA levels (0.73 ± 0.10 mEq/L) compared to lean controls (0.27 ± 0.05 mEq/L; Fig. 4A). In the MDMA-treated group, obese rats had significantly ($P < 0.001$) higher NEFA levels (0.96 ± 0.18 mEq/L) compared to lean controls (0.12 ± 0.05 mEq/L; Fig. 4A). However, there was no significant difference between the MDMA and saline-treated groups within the obese or lean groups.

3.5 Effects of MDMA on blood glucose levels in insulin resistant Zucker rats

Blood glucose levels were analyzed 60 min after MDMA or saline administration in all treatment groups. Glucose levels were not significantly different between obese and lean Zucker rats after saline administration. MDMA significantly ($P < 0.01$) increased plasma glucose levels in only the Obese Zucker rats (Fig. 4B).

3.6 UCP3 expression in insulin resistant Zucker rats

Western blot analysis of UCP3 expression in isolated gastrocnemius muscle mitochondria demonstrated an increase in UCP3 levels in obese versus lean Zucker rats (Figure 5). UCP3 protein expression in brown adipose tissue appears similar in both obese and lean controls.

4. Discussion

Here, we demonstrate that insulin levels peak 15 min. after MDMA (40 mg/kg, sc), which preceded the peak temperature change at 60 min. Blood glucose levels also peaked 15 min. after MDMA and remained elevated throughout the 90-min. monitoring period. Increases in plasma epinephrine levels have been noted following a challenge dose of MDMA (Sprague et al., 2005). This increase in plasma epinephrine levels may account for the increase in plasma glucose levels and subsequent increase in insulin release. Insulin pretreatment (10 units/kg, sc) 30 min. before a low dose of MDMA (5 mg/kg, sc) potentiated the thermogenic response. When glucose (1 g/kg, ip) was administered with insulin as a pretreatment, the enhanced thermogenic response to MDMA was attenuated. Insulin-resistant, obese Zucker rats (a rodent model of type 2 diabetes) displayed an attenuated hyperthermic response to MDMA compared to lean controls. This attenuated thermogenic response to MDMA was in spite of obese Zucker rats displaying increased plasma FFA levels and skeletal muscle UCP3 expression compared to lean controls. Overall, these results suggest a role for insulin signaling in the thermogenic effects of sympathomimetic agents such as MDMA.

Activation of the sympathetic nervous system releases both norepinephrine and epinephrine, which subsequently inhibits insulin release via direct action on postsynaptic α_2 -adrenoceptors on the pancreatic islet beta cells (Ismail et al., 1983; Niddam et al, 1990). The results of the present study demonstrating that the MDMA induced a significant acute increase in insulin levels may seem somewhat of a paradox. However, McMahon et al. (1971) reported that methamphetamine acutely increased insulin levels *in vivo* and subsequent *in vitro* tests suggested that the insulin release was a direct effect of methamphetamine on the pancreas. Thus, the acute increase in insulin levels may be a direct effect of MDMA on the pancreas that is not sensitive to sympathetic nervous system activation of postsynaptic α_2 -adrenoceptors on the pancreatic islet beta cells.

Insulin is a known mediator of FAT/CD36 translocation to the cellular membrane (Campbell et al., 2004; Bonen et al., 2007) and the subsequent uptake of FFA into skeletal muscle cells. Moreover, we have previously shown that MDMA increases plasma FFA levels 30 min. after administration (Sprague et al., 2007). Thus, the acute insulin increase induced by MDMA demonstrated in the present study may help drive FFA activation of UCP3 in skeletal muscle and subsequent heat generation. Furthermore, the enhanced thermogenic response to a low dose of MDMA induced by insulin pretreatment in the present study supports the hypothesis for a role of insulin signaling and insulin-mediated FFA transport into the mitochondrial membrane.

The results of the insulin resistant Zucker rat experiment may seem somewhat contradictory to previous studies examining the roles of FFA and UCP3 in MDMA-induced thermogenesis. We have previously shown that exposure to a high-fat diet increases plasma FFA levels and potentiates the thermogenic effects of MDMA without altering UCP1 and UCP3 protein expression (Mills et al., 2007). UCP3-deficient mice displayed a blunted thermogenic response to MDMA compared to wild-type mice (Mills et al., 2003). In contrast to our previous results, the present study found that insulin-resistant obese Zucker rats displayed an attenuated thermogenic response to MDMA despite having increased plasma FFA levels and UCP3 expression compared to lean controls. Obese Zucker rats are insulin- and contraction-resistant to the translocation of FAT/CD36 to the cell membrane (Han et al., 2007) and insulin resistant to the translocation of GLUT-4 to the cell membrane (Brozinick et al., 1992; 1994) rendering these animals hyperinsulinemic and a rodent model of type 2 diabetes (Kasiske et al., 1992; Houseknecht et al., 1998). Without proper insulin signaling, as in the obese Zucker rats, plasma FFA cannot enter the mitochondria as efficiently. This could inhibit FFA initiated UCP3-facilitated proton leak in mitochondria and subsequent heat generation (Brand and Esteves, 2005; Echtay et al., 2001). Thus, the results of the present study taken together with previous work in our laboratory suggests that insulin signaling may be a major contributor in mediating sympathomimetic-induced thermogenesis.

A possible alternative explanation for the attenuated thermogenic response to MDMA in the obese Zucker rats could be related to the reduced sympathetic nervous system tone. Obese Zucker rats display an impaired compensatory response to cold-(Planche et al., 1983) and diet-induced stress factors (Rothwell et al., 1981). Since MDMA-induced thermogenesis involves both peripheral vasoconstriction (Pedersen and Blessing, 2001) and sympathetic nervous system activation of UCP3 in skeletal muscle (Mills et al., 2003), either of these factors could potentially explain the attenuated thermogenic response in the obese Zucker rats.

Another possible alternative explanation for the partial blockade of MDMA-induced thermogenesis in the obese Zucker rat could be reduced mitochondrial oxygen consumption. Obese Zucker rats are hyperphagic resulting in elevated adipocyte triglyceride levels (Phillips et al., 1996). Increased triglyceride storage has been implicated in pancreatic beta cell lipotoxicity (DeFronzo, 2004) and subsequent decreased insulin secretion. A complication of insulin resistance is reduced mitochondrial oxygen consumption, which has been shown in obese Zucker rats (Levin et al., 1984).

In conclusion, the results of the present study suggest a role of insulin signaling in the thermogenic effects of the sympathomimetic MDMA. MDMA transiently increased plasma insulin levels 15 min. after administration, which preceded the peak thermogenic effect at 60 min. Unfortunately, the molecular mechanisms by which a sympathomimetic agent increases, not decreases, insulin levels is presently unclear and further research is warranted to elucidate this mechanism. Pretreatment with insulin potentiated the thermogenic response to a low dose of MDMA; suggesting that insulin-induced FFA uptake in thermogenic skeletal muscle might be involved in mediating MDMA-induced hyperthermia. Finally, in a rodent model of type 2

diabetes (obese Zucker rat), MDMA-induced thermogenesis was attenuated. This attenuated hyperthermic response was in spite of several factors that have been shown to enhance MDMA-induced thermogenesis (increased plasma FFA and/or increased skeletal muscle UCP3 expression) suggesting that while plasma FFA and skeletal muscle UCP3 are important components of sympathomimetic-induced thermogenesis, other factors such as insulin sensitivity may have a more prominent role.

Acknowledgments

This research was supported by National Institutes of Health grant [DA022712]. We declare that there is no conflict of interest for any of the authors.

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A.

B.

Fig. 1. Effects of MDMA (40 mg/kg, sc) on plasma insulin levels and rectal (core) temperature (A) and blood glucose levels (B). Each point represents the mean $\pm$ S.E.M. (n=6-7). *Indicates significantly different from all other time points ($P<0.01$).

Fig. 2.

Effects of insulin on body temperature effects of a low MDMA dose (5 mg/kg, sc). Insulin (10 units/kg, sc) was administered 15 min. before MDMA. Glucose (1 g/kg, ip) was administered 15 min. after MDMA. Each point represents the mean $\pm$ S.E.M. (n=6). *Indicates significantly different from all other treatment groups ($P < 0.001$); **indicates significantly different from normal saline (NS)-NS only ($P < 0.01$); and ^ indicates significantly different from NS-NS and insulin-glucose-MDMA ($P < 0.05$).

Fig.3.

Effects of MDMA (40 mg/kg, sc) on rectal temperature in fa/fa (obese) and Fa/- (lean) Zucker rats. MDMA was administered at time 0. Each point represents the mean $\pm$ S.E.M. (n=6).

*Indicates significantly different from all other time points ($P < 0.05$).

A.

B.

Fig.4.

Effects of MDMA (40 mg/kg, sc) on plasma FFA (A) and glucose (B) levels in fa/fa (obese) and Fa/- (lean) Zucker rats. FFA and glucose levels were determined 60 min. post- MDMA or saline administration. Each column represents the mean $\pm$ S.E.M. (n=5-6). *Indicates significantly different from saline controls within a treatment group ($P < 0.05$).

Fig.5.

UCP3 protein levels in fa/fa (obese) and Fa/- (lean) Zucker rats. Gastrocnemius skeletal muscle (SKM) and brown adipose tissue (BAT) were harvested and lysates prepared from isolated mitochondria. UCP3 protein was detected via immunoblotting (upper panels). SDS-PAGE gels were stained for protein loading controls (lower panels).