Chapter 3

Intracerebroventricular administration of melanotan II increases insulin sensitivity of glucose disposal in mice.

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Abstract

Aims/hypothesis. The present study was conducted to evaluate the effects of central administration of melanotan II (MTII), a MC3/4 receptor agonist, on hepatic and whole body insulin sensitivity, independent of food intake and body weight.

Methods. 225 ng of MTII was injected in 3 aliquots over 24 hours into the left lateral ventricle in male C57Bl/6 mice without access to food. The control group received 3 distilled water injections. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic-euglycaemic clamp in combination with $^3$H-glucose infusion. GLUT-4 mRNA expression was measured in skeletal muscle.

Results. Plasma glucose and insulin concentrations during basal and hyperinsulinaemic conditions were similar in MTII- and placebo-treated mice. Endogenous glucose production (EGP) and glucose disposal in the basal state were significantly higher in MTII-treated mice compared to the control group (71±22 vs. 43±12 µmol/min/kg, p<0.01). During hyperinsulinaemia, glucose disposal was significantly higher in MTII-treated mice (151±20 vs. 108±20 µmol/min/kg, p<0.01). In contrast, the inhibitory effect of insulin on EGP was not affected by MTII (relative decrease of EGP: 45±27 vs. 50±20%, p<0.01). GLUT-4 mRNA expression in skeletal muscle was significantly increased in MTII-treated mice (307±94 vs. 100±56%, p<0.01).

Conclusions/interpretation. Intracerebroventricular administration of MTII acutely increases insulin-mediated glucose disposal, whereas it does not affect insulin’s capacity to suppress EGP in C57Bl/6 mice. These data indicate that central stimulation of MC3/4 receptors modulates insulin sensitivity in a tissue specific manner, independent of its well-known impact on feeding and body weight.

Introduction

The hypothalamus integrates a multitude of behavioural and metabolic adaptations to food intake and starvation, necessary to maintain fuel homeostasis despite profound environmental variations in nutrient availability. Two types of neurons in the arcuate nucleus of the hypothalamus are of major importance for the control of these processes: neurons co-expressing Agouti related protein (AgRP) and neuropeptide Y (NPY), and neurons expressing pro-opiomelanocortin (POMC), the molecular precursor of alpha-melanocyte stimulating hormone (α-MSH). α-MSH binds to and stimulates melanocortin (MC) receptors. AgRP can bind to MC receptors as well, and
thereby inhibit the POMC pathway. NPY and POMC neuropeptides exert opposing effects on food intake and fuel homeostasis. NPY acts to promote anabolic pathways, whereas \(\alpha\)-MSH counteracts the effects of NPY. For instance, during food deprivation NPY/AgRP neuronal activity is high, whereas POMC/\(\alpha\)-MSH expression levels are low, and this setting of the arcuate neuronal circuitry strongly stimulates food intake and reduces energy expenditure.

Apart from its impact on food intake, intracerebroventricular (icv) administration of NPY acutely hampers insulin’s capacity to inhibit hepatic glucose and VLDL production in C57Bl/6 mice, whereas insulin sensitivity of muscle and adipose tissue remains unaffected. Conversely, chronic (7 days) icv infusion of \(\alpha\)-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the MC3/4 receptor and POMC gene overexpression ameliorates insulin resistance in leptin-deficient mice via a mechanism that is independent of its effects on food intake and body weight. In the latter studies, the effects on insulin sensitivity occur in the presence of a concomitant reduction in food intake and fat mass, which precludes distinction of putative direct effects of \(\alpha\)-MSH or MC4 receptor on insulin sensitivity from indirect effects via feeding and body composition.

In addition to the effect of MC4 receptor activation on insulin sensitivity, Fan et al documented decreased insulin concentration after central activation of the melanocortin neuronal circuitry and increased levels of insulin in MC4 receptor knockout mice, even before the onset of detectable hyperphagia or obesity. In humans, MC4 receptor mutations are associated with obesity.

The aim of the present study was to document the direct effects of activation of MC3/4 receptors on insulin sensitivity (i.e. via other mechanistic routes than feeding and fat mass). Therefore, we injected melanotan II (MTII), an agonist of the MC3/4 receptor icv, and quantified hepatic and peripheral insulin sensitivity of glucose metabolism during a hyperinsulinaemic euglycaemic clamp in mice without access to food.

**Research designs and methods**

**Animals.** Male, 3 months old C57Bl/6 mice (originated from the Jackson Laboratories and bred in our own animal facility) were housed in a temperature and humidity controlled room on a 12-hour-light-dark cycle with free access to standard.
mouse chow and water. All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

**Surgical procedures.** Mice were anaesthetised with 0.5 mg/kg Medetomidine (Pfizer, Capelle a/d IJssel, the Netherlands), 5 mg/kg Midazolam (Roche, Mijdrecht, the Netherlands), and 0.05 mg/kg Fentanyl (Janssen-Cilag, Tilburg, the Netherlands). A 25-gauge guide cannula was stereotactically implanted into the left lateral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral end 2.2 mm ventral. The guide cannula was secured with two screws and dental cement (AgTho’s, Lidingö, Sweden) to the skull surface. After a recovery period of 1 week, adequate placement of the cannulae was tested by measuring the feeding response to an acute icv injection of NPY (5 µg dissolved in 1 µl sterile water) (Bachem, Bubendorf, Germany).

**Hyperinsulinaemic euglycaemic clamp.** Mice fasted for 24 hours (with food withdrawn at 09.00 am the day before the experiment) were used. At 9.00 hours and 17.00 hours the day before the experiment and at 8.45 hours on the day of the experiment mice were given 75 ng (in 1.5 µl distilled water) MTII (PhoenixEurope GmbH, Karlsruhe, Germany) or 1.5 µl distilled water (control group) icv. This dose of MTII was based on data from Murphy et al, who showed inhibition of food intake using this dose. During icv injections, mice were lightly anaesthetised with isoflurane. All experiments were performed at 09.00 hours. Hyperinsulinaemic euglycaemic clamps were performed as described earlier. During the experiments, mice were sedated with 6.25 mg/kg Acepromazine (Sanofi sante animale, Libourne Cedex, France) 6.25 mg/kg Midazolam (Roche, Mijdrecht, the Netherlands), and 0.3125 mg/kg Fentanyl (Janssen-Cilag, Tilburg, the Netherlands).

Basal rates of glucose turnover were measured by giving a primed (0.7 µCi) continuous (1.2 µCi/h) infusion of $^3$H-glucose (Amersham, Little Chalfont, UK) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU) continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours to attain steady state circulating insulin levels of about 4 ng/ml. The $^3$H-glucose infusion (1.2µCi/h) was continued. A variable infusion of 12.5% D-glucose (in PBS) solution was also started and adjusted to maintain blood euglycaemia (measured at 10 minute intervals via tail bleeding, Freestyle,
Blood samples (60 µl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels in the blood were stable and 20 and 40 minutes later) for determination of plasma glucose, NEFA, insulin and \(^3\)H-glucose specific activity.

**mRNA expression of GLUT-4.** A real-time polymerase chain reaction (RT-PCR) was used to measure mRNA expression levels of GLUT-4 in skeletal muscle. Skeletal muscle was taken out in additional groups of mice directly at 10.30 hours after a 24h fast and 3 icv injections with either MTII or vehicle (injections at the same time-points as in the hyperinsulinaemic euglycaemic clamp experiment). Muscle was homogenised in 1.2 ml RNA-Bee (Tel-Test, Inc, Texas, US) and total RNA was extracted according to Chomczynsky and Sacchi. The amount of RNA was determined by spectrophotometry (ND-1000 spectrophotometer, Nanodrop®) at a wavelength of 260 nm. The quality was checked by the ratio of absorption at 260 nm and absorption at 280 nm. Complementary DNA (cDNA) was obtained of total RNA. For RT-PCR, forward and reverse primers and TaqMan probe were designed from mouse specific sequence data (Entrez, National Institutes of Health; and Ensembl, Sanger Institute) using computer software (Primer Express, Applied Biosystems). For each of the genes a Blast Search was done to reveal that sequence homology was obtained only for the target gene. Forward, reverse primers and TaqMan probe (5’ CCATGAGATCTGAGGCCACA 3’; 5’ GTATTTTGCCGAAGTTGTAGCCG 3’; 5’ CAAGGGCAAGATCATCATGCACGACC 3’) of GLUT-4 were used. The TaqMan probe was 5’-6-carboxyfluorescein (FAM) and 3’- (BHQ1) labelled. GAPDH (5’ VIC and 3’ BHQ1, Applied Biosystems) and Cyclophilin (5’ TET and 3’ BHQ1, 5’ CAAATGCTGGACCAAACAA 3’; 5’ GCCATCCAGCCATTCAGTCT 3’; 5’ CCGGTTCAGTTTTATCTGACTGCC 3’) were used as housekeeping genes. PCR amplification was performed in a total reaction volume of 12.5 µl. The reaction mixture consisted of qPCR™ MasterMix (Eurogentec, Belgium), the optimal primer and probe concentrations of GLUT-4 and the endogenous control, nuclease free water and cDNA. An identical cycle profile was used for all genes: 50°C for 2 min + 95°C for 10 min + [95°C for 15 sec + 60°C for 1 min] * 40 cycles. Data were analysed using a comparative critical threshold (Ct) method where the amount of target normalised to the amount of endogenous control (GAPDH/cyclophilin) and relative to
the control sample is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems). All samples were run together allowing relative comparisons of the samples.

**Analytical procedures.** Plasma glucose and NEFA levels were determined using a commercially available kit (Instruchemie, Delfzijl, The Netherlands; Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin and corticosterone concentrations were measured by Elisa (both ALPCO Diagnostics, Windham, NH, USA). For the determination of plasma $^3$H glucose, plasma was deproteinised with 20% trichloroacetic acid, dried to remove water, resuspended in distilled water and counted with scintillation fluid (Ultima Gold, Packard, Meridien, Connecticut, USA).

**Calculations.** Turnover rate of glucose ($\mu$mol/min/kg) was calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of $^3$H-glucose (dpm/$\mu$mol). The ratio was corrected for body weight. EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

**Statistical analysis.** Data are presented as mean ± standard deviation. Differences between groups were determined by Mann-Whitney $U$ test for 2 independent samples. A P-value <0.05 was considered statistically significant.
Results

**Plasma parameters.** Body weight, plasma corticosterone, glucose, NEFA and insulin concentrations in basal and hyperinsulinaemic conditions are shown in Table 1. In the basal state, these parameters did not differ between MTII- and vehicle treated animals. In steady state hyperinsulinaemic conditions, plasma NEFA levels decreased ~2-fold while insulin concentrations increased ~10 fold as expected. No differences were observed in plasma glucose, insulin and NEFA levels between MTII- and vehicle-treated mice during hyperinsulinaemia.

**Table 1.** Body weight, plasma corticosterone, NEFA, glucose and insulin concentration in vehicle (n=10) and MTII (n=8) mice. Values are expressed as means ± SD. n.d. is not determined.

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<th>Basal</th>
<th>Hyperinsulinemic</th>
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<tr>
<td></td>
<td>Vehicle (g)</td>
<td>MTII (g)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>17.9 ± 1.7</td>
<td>18.9 ± 1.6</td>
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<tr>
<td>Corticosterone (mmol/l)</td>
<td>33.3 ± 17.5</td>
<td>37.8 ± 12.4</td>
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<td>Glucose (mmol/l)</td>
<td>5.8 ± 1.0</td>
<td>6.7 ± 1.2</td>
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<tr>
<td>NEFA (mmol/l)</td>
<td>0.55 ± 0.17</td>
<td>0.62 ± 0.17</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.31 ± 0.11</td>
<td>0.31 ± 0.07</td>
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**Glucose turnover.** In basal conditions, EGP (and thereby glucose disposal) was significantly higher in MTII treated animals compared to vehicle treated mice (71 ± 22 vs. 43 ± 10 µmol/min/kg, respectively, p<0.01). During the hyperinsulinaemic period, the rate of glucose infusion necessary to maintain euglycaemia was significantly higher in MTII- than in vehicle-treated animals (114 ± 23 vs. 85 ± 20 µmol/min/kg, p<0.05). Accordingly, the glucose disposal rate was significantly higher in MTII treated animals (151 ± 20 vs. 108 ± 20 µmol/min/kg, resp., p<0.01, Figure 1a). In contrast, hyperinsulinaemia suppressed EGP to a similar extent in MTII- vs. vehicle-treated animals (45 ± 27% vs. 50 ± 20%, ns, Figure 1b).
mRNA expression of GLUT-4. GLUT-4 mRNA expression in skeletal muscle was higher in the MTII treated group compared to vehicle-treated mice (307 ± 94 vs. 100 ± 56 %, p<0.01, Figure 2).

**Figure 1.** Insulin mediated glucose disposal (a) and inhibition of endogenous glucose production (b) by insulin in 24 hours fasted mice that received icv injections of MTII (n=10) or vehicle (n=8). Values represent mean ± SD. *P<0.01 vs. vehicle.

**Figure 2.** GLUT4 mRNA expression levels in 24 hours fasted mice that received injections of MTII (n=6) or vehicle (n=7) in basal conditions. *P<0.01 vs. vehicle.

**Discussion**

This study shows, that activation of MC3/4 receptors enhances whole body sensitivity of glucose metabolism for insulin action in mice via other mechanistic routes than feeding and fat mass. In particular, MTII promotes insulin mediated glucose disposal, whereas it leaves the capacity of insulin to suppress EGP unaffected. These observations are in line with the emerging notion, that neural circuits control insulin action in peripheral tissues.
Interestingly, GLUT-4 mRNA was increased in muscle of MTII treated animals, which suggests, that activation of MC3/4 receptors enhances GLUT-4 gene-expression to promote glucose uptake. The downstream mechanisms that actuate the effects of hypothalamic neuronal circuits on muscle GLUT-4 mRNA expression remain to be fully elucidated. It cannot be ruled out that MTII increased locomotor activity and subsequently GLUT-4 mRNA expression in muscle. However, this seems unlikely since other studies did not observe any increase in locomotor activity after central administration of MTII. Additional studies are required to elucidate the mechanisms involved in the modulation of insulin sensitivity by central administration of MTII.

Glucose production in the basal state was higher in mice treated with MTII, whereas MTII did not affect the capacity of insulin to suppress EGP. Thus, central melanocortin pathways appear to directly impact endogenous glucose output. Although Fan et al. documented decreased plasma insulin concentrations after icv administration of MTII, we did not find significant changes in basal plasma insulin concentrations as a potential explanation for the observed increase in basal glucose production. As Fan et al. injected more than ten times the amount we injected, this discrepancy may be explained by the difference in doses used. Since MTII has been shown to activate the sympathetic nervous system and sympathetic outflow can promote glucose production, it is conceivable that the autonomic nervous system relays signals from the hypothalamus to peripheral tissues to modulate glucose metabolism. Unfortunately, our study does not provide data to evaluate this possibility. Alternatively, the brain interacts with the liver via the hypothalamo-pituitary-adrenal axis (HPA). Corticosteroids stimulate glucose production and HPA activity is under strict control of the hypothalamus. In fact, corticotrophin releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus express MC4 receptors and central MTII injection acutely enhances CRH and corticosterone release in rats. However, MTII did not modify circulating corticosterone concentrations in our experimental setting, which refutes the thesis that central melanocortin pathways modulated the HPA axis to enhance glucose output in this study. Interestingly, short-term fasting is accompanied by a decrease in EGP, and POMC/α-MSH mRNA expressions decrease concomitantly. It is therefore tempting to speculate, that blunted melanocortin signalling is involved in the
decrease in EGP during fasting. In this scenario, administration of MTII may have prevented the normal decline in EGP associated with fasting in the present study.

Although MTII increased basal EGP, it did not appear to affect insulin's capacity to suppress it. A previous paper reports that chronic (7 days) icv infusion of α-MSH reinforces insulin action on glucose production (as well as on glucose disposal) in rats. However, this effect occurred in the presence of concomitant diminutions of food intake and body adiposity and both of these long-term sequelae of MTII administration can impact insulin sensitivity. Our data indicate that activation of melanocortin circuits, through a mechanism that is independent of food intake and body weight, enhances insulin sensitivity and that insulin action on glucose disposal is more sensitive to manipulation of MC3/4 receptors than its capacity to suppress EGP.

We recently showed that icv infusion of NPY in C57Bl/6 mice acutely hampers insulin's inhibitory effect on EGP, whereas it does not appear to affect insulin mediated glucose disposal. We now show, that activation of melanocortin circuits reinforces insulin action on glucose disposal, while suppression of glucose production remains unaffected. NPY and melanocortin circuits in the arcuate nucleus play critical roles in the control of fuel homeostasis in the face of fluctuations in nutrient availability. NPY neurons, active during fasting, stimulate feeding and inhibit energy expenditure, whereas melanocortin circuits, suppressed in fasting conditions, counteract NPY to exert opposing effects on energy balance. These behavioural and metabolic actions serve to protect the body against the perils of famine. Our data suggest that the brain also modulates glucose metabolism to further reinforce the line of defence: enhanced activity of NPY neurons promotes glucose production, whereas reduced melanocortin activity hampers glucose disposal in fasting conditions, keeping glucose available as pivotal fuel for the brain. Conversely, diminished NPYergic and increased melanocortin signalling allow insulin to appropriately suppress glucose output and promote glucose disposal in response to food intake.

The current findings may imply that MC-3/4 receptor agonists can serve as "insulin sensitisers" in the treatment of the metabolic syndrome and type 2 diabetes mellitus. However, tachyphylaxis to chronic MTII administration has been observed in mice and rats. In addition, the present study shows that MTII increases basal EGP. Thus, the impact of chronic MTII administration on glucose metabolism in (insulin resistant) animal models and humans remains to be established.
In conclusion, the present study shows that activation of central melanocortin-3/4 receptors by melanotan II enhances insulin sensitivity of whole body glucose disposal, independent of food intake and fat mass, whereas it does not affect insulin's ability to suppress EGP. These observations are in line with the emerging notion, that neural circuits, apart from their effects on feeding, modulate insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability.

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