BRAIN-MEDIATED ANTIDIABETIC, ANOREXIC AND CARDIOVASCULAR ACTIONS OF LEPTIN REQUIRE MELANOCORTIN-4 RECEPTOR SIGNALING

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Short Title: MC4R, cardiovascular and antidiabetic actions of leptin

Total word count: 2822
Abstract word count: 250
Number of figures: 4

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We previously demonstrated that leptin has powerful central nervous system (CNS)-mediated antidiabetic actions. In this study we tested the importance of melanocortin-4 receptors (MC4R) for leptin’s ability to suppress food intake, increase blood pressure (BP) and heart rate (HR), and normalize glucose levels in insulin-dependent diabetes. MC4R-knockout (MC4R-KO) and control wild-type (WT) rats were implanted with intracerebroventricular (ICV) cannula and BP and HR were measured 24-hrs/day by telemetry. After 5-day control period, an injection of streptozotocin (STZ) (50 mg/kg, ip) was used to induce diabetes. Eight days after injection, an osmotic pump was implanted subcutaneously and connected to the ICV cannula to deliver leptin (15 μg/day) for 7 days. At baseline, MC4R-KO rats were hyperphagic and 40% heavier than WT rats. Despite obesity, BP was similar (112±2 vs. 111±2 mmHg) and HR was lower in MC4R-KO rats (320±6 vs. 347±5 bpm). Induction of diabetes increased food intake (30%) and reduced BP (~17 mmHg) and HR (~61 bpm) in WT rats, while food intake, BP and HR were reduced by ~10%, 7 mmHg, and 33 bpm, respectively, in MC4R-KO rats. Leptin treatment normalized blood glucose (437±10 to 136±18 mg/dl), reduced food intake (40%), and increased HR (+60 bpm) and BP (+9 mmHg) in WT rats. Only modest changes in blood glucose (367±16 to 326±23 mg/dl), food intake (5%), HR (+16 bpm) and BP (+4 mmHg) were observed in MC4R-KO rats. These results indicate that intact CNS MC4R signaling is necessary for leptin to exert its chronic antidiabetic, anorexic and cardiovascular actions.

**Key Words:** leptin, melanocortin, streptozotocin, food intake, blood pressure, insulin, heart rate.
INTRODUCTION

Leptin, an adipocyte derived peptide that circulates in proportion to the amount of body fat, plays a major role in body weight homeostasis by suppressing appetite and by increasing sympathetic nerve activity (SNA) to various tissues including brown adipose tissue to promote thermogenesis (Zhang 1994; Collins 1996; Haynes 1997; Schwartz 2000). In addition to its well-known effect on body weight regulation leptin also plays an important role in glucose homeostasis. Leptin stimulates glucose utilization in peripheral tissues and modulates liver glucose production by regulating activity of key enzymes involved in gluconeogenesis (German et al. 2009, 2011; Kamohara 1997).

At least part of the long-term action of leptin on glucose homeostasis is mediated by activation of leptin receptors in the central nervous system (CNS). Previous studies showed that chronic intracerebroventricular (ICV) infusion of leptin in streptozotocin (STZ)-induced insulin-deficient diabetic rats completely restored blood glucose to normal levels (da silva 2006; Hidaka 2002; Lin 2002), indicating that leptin exerts a powerful CNS-mediated effect on peripheral glucose regulation and that this effect can occur even in the absence of significant amounts of insulin.

In addition to normalizing blood glucose levels in insulin-deficient diabetes, leptin also prevented the hyperphagia and the marked bradycardia that is known to occur in the STZ-induced diabetic model (da Silva 2006; do Carmo 2008). Collectively, these observations indicate a beneficial impact of leptin not only on metabolic parameters but also on cardiovascular function in type-1 diabetes. Although the CNS mechanisms that mediate leptin’s chronic effects on glucose homeostasis and cardiovascular function are still unclear, we have provided evidence that activation of the brain melanocortin system is an important downstream contributor to leptin’s actions. For example, deletion of leptin receptors in proopiomelanocortin (POMC)
neurons markedly attenuated leptin’s ability to increase blood pressure (BP) and to improve glucose regulation as evidenced by a reduction in fasting insulin and blood glucose levels (do Carmo et al. 2011).

Leptin-induced activation of POMC neurons has been shown to stimulate release of alpha-melanocyte stimulating hormone (α-MSH), which is the endogenous agonist of melanocortin receptors type 3 and 4, in various hypothalamic and extra-hypothalamic regions (da silva 2014; Tao 2010). Although previous studies suggest that the melanocortin 4 receptor (MC4R) may play a more important role in the regulation of appetite, metabolic and cardiovascular functions than the melanocortin 3 receptor (MC3R) (Tallam 2005; Tallam 2006, Tao 2010), the specific role of MC4R in mediating the beneficial actions of leptin on food intake, glucose regulation and cardiovascular function observed in diabetic animals has, to our knowledge, not been reported and remains unclear. Therefore, in this study we tested whether lack of MC4R attenuates the effects of leptin to reverse the hyperphagia, bradycardia and hyperglycemia observed in STZ-diabetic rats. For this purpose, we examined the long-term effects of central leptin administration on blood glucose levels, appetite, body weight, BP and heart rate (HR) during STZ-induced diabetes in MC4R knockout versus wild-type rats. Our results indicate that intact CNS MC4R signaling is necessary for leptin to exert its chronic anorexic, cardiovascular and antidiabetic actions.

METHODS

Animal Surgeries

The experimental procedures and protocols of this study conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.
**Blood pressure telemetry probe implantation:** Male 14- to 16-week-old MC4R knockout (MC4R-KO, n=5) and wild-type Wistar Hannover rats (WT, n=5) obtained from a colony maintained at the university of Mississippi Medical Center were anesthetized with 50 mg/kg sodium pentobarbital (Nembutal) and atropine sulfate (0.1 mg/kg) was administered to prevent excess airway secretions. A telemetry BP transmitter (Model TA11PAC40, Data Sciences International, MN) was implanted in the abdominal aorta distal to the kidneys under sterile conditions for determination of mean arterial pressure (MAP) and heart rate (HR), 24-hours per day, using computerized methods for data collection as previously described (da Silva et al. 2008). MAP and HR were obtained from the 24-hour recordings using a sampling rate of 1000 Hz with duration of 10 seconds every 10–minute period.

**Intracerebroventricular (ICV) cannulation:** Immediately after telemetry probe implantation, a stainless steel cannula (26 gauge, 10 mm long) was implanted into the right lateral cerebral ventricle using the coordinates previously described (Kuo et al. 2003). The guide cannula was anchored in place with 3 stainless steel machine screws, a metal cap, and dental acrylic, and a stylet was inserted to seal the cannula until use. During stereotaxic manipulation, anesthesia was maintained with 1.5 to 2.5% vaporized isofluorane. After 7 days of recovery from surgery, accuracy of the cannula placement was tested by measuring the dipsogenic response (immediate drinking of at least 5 ml of water within 10 minutes) to an ICV injection of 100 ng of angiotensin II. After all experiments were conducted, the animals were killed and the brains removed and sectioned to confirm placement of the cannula.

After recovery from anesthesia, the rats were housed individually in metabolic cages for determination of daily water and food consumption. Rats were fed standard chow (Harlan Teklad, WI) and water was provided ad libitum while maintained on a temperature-controlled
(23° C) 12:12 hr light:dark cycle room. The rats were allowed to recover for 8-10 days before control measurements were collected.

**Experimental Protocols**

MAP, HR, urine volume, food and water intakes were measured 24 hours/day and average values were recorded daily.

**Induction of insulin-deficient diabetes.** After 5 days of stable control measurements, insulin-deficient diabetes was induced by a single intravenous injection of streptozotocin (STZ, 50 mg/kg – Sigma-Aldrich, dissolved in 0.5 ml of 0.05M citrate buffer, pH 4.5).

**Chronic ICV leptin infusion in diabetic rats (n=5):** Eight days after STZ injection, leptin (0.62 μg/hr, 1.0 μl/hr) was infused ICV for 7 days via osmotic minipump (model 2001, Durect Corp.) implanted subcutaneously in the scapular region as previously described (Kuo et al. 2003). We have shown that this rate of ICV leptin infusion fully restores euglycemia in STZ-diabetic rats and does not alter plasma leptin levels (da Silva et al. 2006). On day 7 of leptin ICV infusion, the cannula connecting the osmotic pump with the ICV cannula was severed and the animals followed for an additional 5-day recovery (post-treatment) period (Figure 1).

Blood glucose concentration was measured each morning between 9:00 and 11:00 am for determination of blood glucose levels using glucose strips (Reli On).

**Statistical Analyses**

The data are expressed as mean±SEM and analyzed by using 1-factor or 2-factor ANOVA with repeated measures. The Bonferroni post hoc test was used for comparisons between groups. Dunnett’s test was used for comparisons of experimental and control values within each group, when appropriate. Statistical significance was accepted at a level of P<0.05.
RESULTS

Baseline Phenotypes of WT and MC4R-KO Rats

MC4R\(^{-/-}\) rats were heavier and ate more food than control WT rats (Figures 2A and 2B). Despite being 40% heavier than WT controls, MC4R-KO rats exhibited similar MAP (112±2 vs. 111±2 mmHg, Figure 2C) and reduced HR (320±6 vs. 347±5 bpm, Figure 2D). These results confirm our previous studies in mice showing that MC4R deficiency is accompanied by hyperphagia and early-onset severe obesity without elevated BP or HR which are commonly associated with obesity (Tallam 2005; Tallam 2006; do Carmo 2009).

Impact of STZ-Diabetes on Blood Glucose, Food Intake, Body Weight, MAP and HR

Induction of insulin-deficient diabetes with STZ was associated with severe hyperglycemia; blood glucose levels increased from 100±2 to 367±16 mg/dl and from 80±5 to 437±10 mg/dl in MC4R-KO and WT rats, respectively (Figure 3A). It also caused weight loss in both groups (Figure 3B) despite increased food intake in WT rats (Figure 3C).

Induction of STZ-diabetes also reduced MAP and HR in both groups (MAP: by 7±2 and 17±2 mmHg; and HR: by 33±9 and 61±18 bpm in MC4R-/- and WT rats, respectively) (Figures 4A and 4B).

Appetite, MAP and HR Responses to Chronic ICV Leptin Infusion in STZ-Diabetes

Chronic ICV leptin treatment for 7 consecutive days in diabetic WT rats markedly reduced the hyperglycemia (437±10 to 136±18 mg/dl, Figure 3A) and reversed the hyperphagia, causing food intake to drop below baseline values (Figures 3C). Leptin also returned HR back to control non-diabetic values (Figure 4B) and increased MAP by 11±1 mmHg (Figure 4A). Conversely, in MC4R-KO rats, leptin treatment failed to normalize glucose levels (Figure 3A), to reduce food intake (Figures 3C), or to significantly increase MAP and HR (Figures 4A and 4B). These results support the concept that the melanocortin system plays a critical role in
contributing to leptin’s effects on appetite, cardiovascular function and glucose regulation, and that functional MC4R are required for the powerful brain-mediated antidiabetic action of leptin.

After leptin treatment was stopped, food intake, glucose levels and HR rapidly returned to diabetic values during the 5-day recovery period. MAP, however, remained unchanged. All groups exhibited a gradual and continuous weight loss after induction of STZ-diabetes until the end of the study (Figure 3B).

DISCUSSION

In this study we showed that activation of MC4R is a critical downstream event contributing to the powerful CNS-mediated antidiabetic effect of leptin. We also demonstrated that a functional MC4R is required for leptin to prevent hyperphagia and to reverse bradycardia and reduced BP associated with insulin-dependent diabetes. These findings corroborate our previous observation that pharmacological blockade of MC3R and MC4R using a mixed antagonist abolished leptin’s antidiabetic, anorexic and cardiovascular actions (da Silva et al. 2009), and show that MC4R, and not MC3R or an nonspecific effect of the pharmacological antagonist, is indeed important for leptin’s ability to regulate glucose levels, appetite and cardiovascular function in insulin-dependent diabetes.

Although we used a model with whole-body MC4R deficiency to investigate the importance of MC4R for leptin’s effects in diabetic rats, previous studies including our own (da Silva 2004; da Silva 2009; Meek 2014) showed that the CNS is the main site where MC4R exerts many of its physiologic effects on cardiovascular and metabolic functions, and mediates most of leptin’s actions including those examined in the present study. The specific areas of the brain where MC4Rs contribute to the regulation of appetite and body weight as well as peripheral glucose handling and cardiovascular function, however, are only beginning to be elucidated.
Balthasar et al. (2005) demonstrated that MC4Rs in neurons of the paraventricular nucleus of the hypothalamus are important in controlling appetite but not energy expenditure. We also showed that selective rescue of MC4Rs in POMC neurons of whole-body MC4R deficient mice is associated with increased energy expenditure with no changes in food intake (do Carmo et al. 2013). Moreover, MC4Rs expressed in cholinergic (sympathetic and parasympathetic) pre-ganglionic neurons in the brainstem and spinal cord also participate in the control of energy expenditure and body weight, glucose homeostasis and restores nighttime obesity-induced elevations in BP (Rossi 2011; Sohn 2013). These observations suggest a differential control of appetite, energy expenditure, glucose metabolism and cardiovascular function by MC4R, depending on its expression in different neuronal populations. However, the key sites or neuronal types where MC4Rs are most important in mediating the antidiabetic, dietary and cardiovascular actions of leptin are still unclear.

We previously demonstrated that in addition to restoring euglycemia in insulin-dependent diabetic rats leptin also reversed several cardiovascular changes caused by uncontrolled diabetes, including marked bradycardia, reduced cardiac autonomic tone, baroreflex dysfunction and reduced intrinsic HR (do Carmo et al. 2008). In addition, we showed that leptin’s effects on these cardiovascular parameters (except baroreflex sensitivity) were independent of the normalization of blood glucose levels (do Carmo et al. 2008). Moreover, the return of HR to control prediabetic values evoked by leptin was mediated by the restoration of normal cardiac sympathetic tone and intrinsic HR, suggesting that endogenous MC4R activation is required not only for leptin’s well-known effects on sympathetic activity to regulate BP and HR but also to other sympathetic-independent cardiovascular actions of leptin (e.g. intrinsic HR). These observations also highlight the major role of MC4R as a critical step for many of the physiological effects of leptin.
Previous studies also indicate that the MC4R plays a fundamental role in cardiovascular regulation, beyond mediating leptin’s effects. We showed, for example, that blockade of MC4R using a mixed antagonist markedly reduced the elevated BP of lean spontaneously hypertensive rats, a non-obese model of hypertension associated with high sympathetic tone (da silva et al. 2008). We also found that pharmacological blockade of MC4R reduced BP in obese Zucker rats, a model of defective leptin receptor function (do Carmo et al. 2012).

In the present study we found that despite obesity and hyperphagia MC4R-KO rats had similar BP and lower HR compared to WT controls, which corroborates previous studies in MC4R-KO rats (Stepp et al. 2013) and mice (Tallam et al. 2005, 2006). Also, humans with MC4R mutations are markedly obese but exhibit reduced sympathetic response to stress (e.g. inspiratory hypoxia) and lower incidence of hypertension compared to obese controls (Greenfield et al. 2009, 2011). Therefore, clinical and experimental evidence supports the notion that MC4Rs are a major modulator of sympathetic activity, BP and HR, and are critically important for obesity to be associated with increases in sympathetic activity, BP and HR.

Although our results and previous studies show the importance of MC4Rs on appetite and energy balance regulation, cardiovascular function, and glucose homeostasis, the intracellular signaling pathways are still poorly understood. The MC4R is a G protein-coupled receptor that increases cAMP phosphorylation and activates protein kinase A (PKA) (Tao et al. 2010). Pharmacological activation or inhibition of MC4R have been shown to, respectively, increase and reduce brain-derived neurotrophic factor (BDNF) protein content in certain areas of the brainstem of adult rats (Bariohay et al. 2009) while the acute orexigenic effect of a selective MC4R antagonist was blocked by co-administration of BDNF. Nicholson and colleagues (2007) also found that the reduction in 24-hour food intake and acute increase in BP evoked by the MC4R activation were attenuated by central administration of an anti- BDNF antibody. Other
candidates including oxytocin, corticotrophin-releasing hormone (CRH), and melanin-concentrating hormone (MCH), which are modulated by MC4R activity, have also been proposed to contribute to MC4R’s actions on appetite (Tao 2010, Yosten 2010). However, the role of these factors in contributing to the chronic antidiabetic and cardiovascular actions of the leptin-MC4R axis it is still unclear.

Also unclear are the mechanisms by which the brain leptin-MC4R axis regulates peripheral glucose handling even in the absence of adequate insulin levels. Previous acute studies have demonstrated an important contribution of the autonomic nervous system. For instance, adrenergic receptor blockade markedly attenuated the increase in tissue glucose uptake caused by acute microinjection of leptin into the ventromedial hypothalamus (Haque et al. 1999). Skeletal muscle denervation also markedly attenuated the effects of a 6-hour infusion of leptin to increase glucose uptake (Kamohara et al. 1997). In addition, leptin-mediated acute suppression of hepatic glucose production in non-diabetic was prevented by selective hepatic vagal denervation (German 2009; Li 2011). Although these acute studies support a role for the autonomic nervous system in mediating the CNS effects of leptin on glucose regulation, our previous studies provided evidence that a major part of leptin’s chronic antidiabetic effects are independent of the autonomic nervous. We found no impairment of the chronic CNS-mediated antidiabetic effect of leptin in diabetic rats pre-treated with α- and β-adrenergic receptor blockers (da Silva et al. 2006) or in diabetic rats that underwent hepatic vagotomy (da Silva et al. 2012). Thus, despite strong evidence that the acute central effects of leptin on glucose homeostasis are mediated, at least in part, by the autonomic nervous system the mechanisms behind the long-term actions of the leptin-MC4R axis on glucose regulation, including its powerful antidiabetic effect, remain to be elucidated.
In summary, leptin has powerful CNS-mediated antidiabetic effects in insulin-deficient diabetes that makes it a potential adjunct therapy for diabetes and insulin resistance. Here we show that this antidiabetic action of leptin requires activation of the CNS melanocortin pathway (specifically, activation of MC4Rs). Leptin also exerts important anorexigenic and cardiovascular effects in insulin-dependent diabetes that require activation of CNS MC4R. Unraveling the brain areas where MC4R contributes to these effects of leptin, the downstream events beyond MC4R activation, and the mechanisms that transmit the CNS antidiabetic effect of the leptin-MC4R axis to peripheral tissues will contribute to a better understanding of the CNS control of glucose homeostasis, appetite and cardiovascular function and could lead to the development of novel therapeutic strategies to treated obesity, metabolic syndrome, diabetes and cardiovascular diseases.

ACKNOWLEDGMENTS

This research was supported by the National Heart, Lung and Blood Institute Grant PO1HL51971, the National Institute of General Medical Sciences (P20GM104357), an American Heart Association Scientist Development Grant to Jussara M. do Carmo, and by a T32 HL105324 grant.

DISCLOSURES

None.

REFERENCES


FIGURE LEGENDS

Figure 1. Schematic representation of the experimental protocol used in the study. STZ = streptozotocin; MC4R-KO = melanocortin-4 receptor knockout rats; WT = control Wistar Hannover rats; ICV = intracerebroventricular; ip = intraperitoneal.

Figure 2. Baseline characteristics of melanocortin-4 receptor deficient (MC4R-KO) and Wistar Hannover control rats (WT). Body weight (A), food intake (B), mean arterial pressure (MAP) (C), and heart rate (D). Data expressed as mean ± SEM. # indicates p<0.05 versus MC4R-KO rats.

Figure 3. Blood glucose concentration (A), body weight (B) and change in food intake (C) responses to induction of insulin-dependent diabetes and chronic intracerebroventricular (ICV) leptin treatment in melanocortin-4 receptor deficient (MC4R-KO) and Wistar Hannover control rats (WT). Data expressed as mean ± SEM. Results represent the average of the last 3 days of the control period, days 7 and 8 post streptozotocin injection, days 6 and 7 of leptin ICV treatment, and days 4 and 5 of the recovery period. Diab = diabetes. # indicates p<0.05 versus MC4R-KO rats; * indicates p<0.05 versus Control baseline values; & indicates p<0.05 versus Diabetes.

Figure 4. Mean arterial pressure (MAP) (A) and heart rate (B) responses to induction of insulin-dependent diabetes and chronic intracerebroventricular (ICV) leptin treatment in melanocortin-4 receptor deficient (MC4R-KO) and Wistar Hannover control rats (WT). Data expressed as mean ± SEM. Results represent the average of the last 3 days of the control period, days 7 and 8 post streptozotocin injection, days 6 and 7 of leptin ICV treatment, and days 4 and 5 of the recovery period.
period. Diab = diabetes. # indicates p<0.05 versus MC4R-KO rats; * indicates p<0.05 versus Control baseline values; & indicates p<0.05 versus Diabetes.
MC4R KO (n=5)

5 days  7 days  7 days  5 days

STZ (50 mg/kg – ip)

Leptin (15μg/day – ICV)

5 days  7 days  7 days  5 days

WT (n=4)

5 days  7 days  7 days  5 days

STZ (50 mg/kg – ip)
Leptin (15 μg/day, ICV)

MC4R-KO

WT

Baseline FI:

MC4R-KO: 28 ± 3 g

WT: 34 ± 2 g