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ROLE OF MELANOCORTIN 4 RECEPTOR IN HYPERTENSION INDUCED BY CHRONIC INTERMITTENT HYPOXIA

Jussara M. do Carmo¹, Alexandre A. da Silva^{1,2,3}, Sydney P. Moak¹, Fernanda S. da Silva^{1,2}, Frank T. Spradley^{1,4}, and John E. Hall¹

¹-Department of Physiology and Biophysics, Mississippi Center for Obesity Research, Cardiovascular-Renal Research Center, University of Mississippi Medical Center, Jackson, MS, USA.

²-Barão de Mauá University Center, Ribeirão Preto, São Paulo, Brazil.

³-Universidade Estadual de Minas Gerais, Passos, Minas Gerais, Brazil.

⁴-Department of Surgery, University of Mississippi Medical Center, Jackson, MS, USA

Abstract

Aim: We previously demonstrated that central nervous system (CNS) melanocortin 4 receptors (MC4R) play a key role in regulating blood pressure (BP) in some conditions associated with increased SNS activity, including obesity. In this study we examined if activation of CNS MC4R contributes to chronic intermittent hypoxia (CIH)-induced hypertension and ventilatory responses to hypercapnia.

Methods: Rats were instrumented with an intracerebroventricular (ICV) cannula in the lateral cerebral ventricle for continuous infusion of MC4R antagonist (SHU-9119) and telemetry probes for measuring mean arterial pressure (MAP) and heart rate (HR). Untreated and SHU-9119 treated rats as well as obese and lean MC4R deficient rats were exposed to CIH for 7 to 18 consecutive days.

Results: CIH reduced cumulative food intake by 18 ± 5 g while MAP and HR increased by 10 ± 3 mmHg and 9 ± 5 bpm in untreated rats. SHU-9119 increased food intake (from 15 ± 1 to 46 ± 3 g) and prevented CIH-induced reduction in food intake. CIH-induced hypertension was not attenuated by MC4R antagonism (average increase of 10 ± 1 vs. 9 ± 1 mmHg for untreated and SHU-9119 treated rats). In obese MC4R deficient rats CIH for 7 days raised BP by 11 ± 4 mmHg. However, when MC4R deficient rats were food restricted to prevent obesity, CIH-induced hypertension was attenuated by 32%. We also found that MC4R deficiency was associated with impaired ventilatory responses to hypercapnia independently of obesity.

Conclusion: These results show that obesity and the CNS melanocortin system interact in complex ways to elevate BP during CIH and that MC4R may be important in the ventilatory responses to hypercapnia.

Corresponding author: Jussara M. do Carmo, Ph.D, Department of Physiology & Biophysics, University of Mississippi Medical Center, 2500 North State St, Jackson, MS 39216, Phone: (601) 984-4353, Fax: (601) 984-1833, jdocarmo@umc.edu.

CONFLICT OF INTEREST/DISCLOSURES

None.

Keywords

Obesity; obstructive sleep apnea; hypertension; sympathetic activity; hypercapnia

INTRODUCTION

Obesity is a major risk factor for obstructive sleep apnea (OSA) which, in turn, is associated with periods of intermittent hypoxia.^{1–3} Previous studies showed that OSA affects approximately 50% of obese subjects and strong associations have been established for OSA, sympathetic nervous system (SNS) activation and hypertension.^{4–6}

Induction of chronic intermittent hypoxia (CIH) during the sleeping phase in experimental animals mimics the periodic episodes of hypoxia that occur in humans with OSA. Similar to humans, experimental animals exposed to CIH have activated chemoreceptor reflexes,^{7–9} increased SNS activity, reduced cardiac parasympathetic activity, decreased baroreflex sensitivity, and hypertension.^{5,10–12} However, the precise mechanisms by which CIH raises blood pressure (BP) and SNS activity are still under investigation.

We^{13–16} and others^{17–19} showed that the proopiomelanocortin (POMC) neurons and the brain melanocortin 4 receptor (MC4R) play a pivotal role in controlling food intake, energy expenditure, sympathetic nervous system (SNS) activity, and blood pressure (BP) in normal as well as in obese subjects. Mice and humans with loss-of function mutations of POMC or the MC4R have severe obesity, insulin resistance, and dyslipidemia.^{20,21} However, BP is normal or reduced in mice and humans with deficiency of MC4R signaling despite severe obesity and other characteristics of the metabolic syndrome that typically cause hypertension.^{17,20,21} These observations highlight a potential role for central nervous system (CNS) MC4R as an essential link between obesity and hypertension.

There is evidence that OSA may influence activity of the CNS melanocortin pathway. CIH is associated with increased POMC expression in the arcuate nucleus (ARC) of the hypothalamus,²² and our previous studies in rodents and studies in humans have shown a powerful modulatory role of the brain melanocortin system, in particular MC4R, in regulating sympathetic activity in response to several stimuli. For instance, Sayk et al²³ showed that humans with MC4R loss-of-function mutations exhibited reduced muscle sympathetic nerve activity (SNA) at rest and during acute apnea-induced elevation in SNA when compared to obese controls. Greenfield et al¹⁷ also demonstrated reduced BP and circulating catecholamine levels in humans with MC4R deficiency compared to obese controls. We demonstrated that endogenous MC4R activity contributes importantly to hypertension (about 20–25 mmHg) in spontaneously hypertensive rats (SHRs), a model where hypertension is dependent on increased SNA which may be caused, at least partly, by signals from the carotid body chemoreceptors.²⁴ In addition, Sohn et al²⁵ showed that MC4R are expressed in preganglionic neurons of the NTS, DMV and intermedial lateral medulla where they contribute to the regulation of sympathetic and parasympathetic activity. Based on these studies we hypothesized that brain MC4R may be important in modulating the elevation in BP during CIH.

We also previously showed that MC4R deficient mice have reduced ventilatory responses to hypercapnia.²⁶ This observation suggests that the brain melanocortin system may modulate SNS responses to hypoxia and ventilatory responses to hypercapnia, although its role in mediating the chronic cardiovascular effects of CIH is still unknown. Therefore, we also examined the pulmonary ventilation responses to acute hypercapnia in obese and lean MC4R deficient rats to determine the role of MC4R in regulating respiratory function independent of obesity.

RESULTS

Effect of CIH on Body Weight, Food Intake, Plasma Leptin, Insulin and Glucose Concentrations, and Hematocrit in Lean Rats

In most experiments, each animal served as its own control since control measurements were obtained prior to CIH and post-control measurements were made during several days of recovery from CIH. CIH for 7 consecutive days significantly reduced food intake in Sprague-Dawley rats (Fig. 1A). The reduction in food intake was greater on day 1 of CIH, possibly due to the initial stress effect of hypoxia. This effect is more evident when expressed as cumulative decrease in food intake during the 7 days of CIH (Fig. 1B). This decrease in food intake was associated with a modest reduction in body weight of ~7g (Fig. 1C). After CIH was stopped, food intake and body weight returned to baseline values.

Despite the reduction in food intake, CIH for 7 consecutive days did not alter fasting blood glucose (72 ± 3 vs. 75 ± 3 mg/dL), plasma insulin (20 ± 4 vs. 17 ± 5 μ U/mL) or leptin concentrations (3 ± 1 vs. 4 ± 1 ng/mL) compared to baseline values. Hematocrit was significantly increased on the last day of CIH (57.0 ± 0.6 vs. $52.0 \pm 0.4\%$).

Effect of Chronic MC4R Antagonism and CIH on Food Intake, Body Weight and Plasma Glucose, Insulin and Leptin Concentrations

Chronic MC4R antagonism markedly increased food intake (23 ± 1 vs. 43 ± 1 g/day) on day 14 of SHU-9119 infusion (Fig. 2A) which was accompanied by a 23% increase in body weight (Fig. 2C). In contrast to the effects of CIH in vehicle treated rats, CIH during chronic MC4R antagonism did not significantly reduce food intake, except for the first day (Fig. 2A). The effect of MC4R antagonism on food intake is more clearly demonstrated by the increase in cumulative increase over the 14 days of SHU-9119 infusion (Fig. 2B). After SHU-9119 infusion was terminated, food intake decreased nearly to baseline values on day 6 of the recovery period (Fig. 2A).

Although plasma glucose levels were not altered by chronic MC4R antagonism (86 ± 2 vs. 81 ± 3 mg/dL), SHU-9119 infusion markedly increased plasma insulin (111 ± 23 vs. 12 ± 4 μ U/mL) and leptin concentrations (15 ± 2 vs. 2 ± 1 ng/mL). Exposure to CIH of rats treated with the MC4R antagonist did not alter blood glucose (80 ± 2 vs. 86 ± 2 mg/dL), plasma insulin (99 ± 20 vs. 111 ± 23 μ U/mL) or leptin concentrations (10 ± 1 vs. 15 ± 2 ng/mL) when compared to MC4R antagonism alone. Therefore, CIH did not significantly alter the effects of chronic MC4R antagonist to increase plasma insulin or leptin concentrations.

Food Intake, Body Weight, and Plasma Glucose, Insulin and Leptin Responses to CIH in Obese and Lean MC4R KO Rats

To further examine the role of MC4R in modulating the metabolic responses to CIH, we performed similar experiments in obese MC4R KO rats fed ad libitum and in lean pair-weighted (PW) MC4R KO rats to investigate the impact of MC4R deficiency independent of obesity in altering the responses to CIH. CIH significantly reduced food intake in obese MC4R KO rats by 50% leading to net cumulative reduction of -108 ± 13 g in these rats compared to a -33 ± 3 g food intake reduction in control wild-type (WT) rats (Figs. 3A and B). Food intake was kept at baseline values and was not altered by CIH in PW-MC4R KO rats. Despite significant decreases in food intake, body weight was reduced by 7.5, 12.0 and 3.5 % in obese MC4R KO, WT and lean PW-MC4R KO rats, respectively (Fig 3C).

Baseline blood glucose concentration was slightly higher in obese MC4R KO rats but did not reach statistical significance compared to WT rats (97 ± 7 vs. 85 ± 2 mg/dL). However, baseline plasma insulin and leptin concentrations were significantly higher in obese MC4R KO compared to WT rats (71 ± 14 vs. 15 ± 3 μ U/mL and 18 ± 2 vs. 0.9 ± 0.1 ng/mL, respectively). CIH did not significantly alter plasma glucose concentration in MC4R KO or WT rats compared to baseline values (112 ± 10 vs. 97 ± 7 and 81 ± 4 vs. 85 ± 2 mg/dL). CIH, however, reduced plasma leptin concentration in obese MC4R KO rats (12 ± 1 vs. 18 ± 2 ng/mL) while slightly increasing it in WT rats (1.8 ± 0.5 vs. 0.9 ± 0.1 ng/mL). CIH reduced fasting insulin levels in WT rats (7 ± 2 vs. 15 ± 3 μ U/mL) but did not alter it in MC4R KO rats (67 ± 7 vs. 71 ± 14 μ U/mL). We also observed no significant changes in blood glucose (85 ± 6 vs. 82 ± 3 mg/dL) concentration in lean PW-MC4R KO rats during CIH. However, plasma insulin (6 ± 2 vs. 41 ± 6 μ U/mL) and plasma leptin concentrations (12 ± 2 vs. 18 ± 2 ng/mL) were significantly reduced by CIH in PW-MC4R KO rats.

Pulmonary Ventilation Responses to Hypercapnia in Obese and Lean MC4R KO Rats

To examine the role of MC4R in modulating the respiratory responses to hypercapnia in rats, we measured respiratory responses to hypercapnia (7% CO₂) in WT, obese and lean-M4R KO rats. Under normocapnia (room air), f_R was significantly higher in obese MC4R KO rats compared to lean PW-MC4R KO and WT rats. Obese and lean MC4R KO rats had reduced V_T , however V_E was significantly reduced only in PW-MC4R KO compared to WT rats. The hypercapnia-induced changes in ventilatory responses (V_T and V_E) were markedly diminished in obese and lean MC4R KO compared to WT rats (Table 1). We also observed greater f_R responses to hypercapnia in obese MC4R KO rats compared to lean PW-MC4R KO and control rats. These results suggest that MC4R deficiency is associated with impaired ventilatory responses to hypercapnia independently of obesity.

Effect of CIH on Blood Pressure and Heart Rate in Lean Control Rats

As shown in Figs. 4A and 4B, CIH significantly increased mean arterial pressure (MAP) by approximately 10 mmHg in lean control rats. This increase in MAP was sustained throughout the 7 days of CIH and MAP returned all the way back to baseline levels after CIH was stopped. Heart rate (HR) was not significantly altered by CIH (Fig. 4C).

Combined Effects of Chronic MC4R Antagonism and CIH on Blood Pressure and Heart Rate

Chronic MC4R blockade did not significantly alter blood pressure (BP) at baseline and did not alter the impact of CIH on MAP (Fig. 5A). When MC4R blockade was stopped and CIH was continued for 8 days, MAP did not change significantly. After CIH was stopped, MAP gradually decreased by ~12 mmHg during the recovery period and was not significantly different than baseline MAP. Chronic MC4R antagonism reduced HR by 33 ± 4 bpm, an effect that was completely reversed during CIH (Fig. 5B). After cessation of CIH, HR decreased by 19 ± 4 bpm during the recovery period (Fig. 5B).

Blood Pressure and Heart Rate Responses to CIH in Obese and Lean MC4R KO Rats

Baseline MAP was significantly higher in obese and lean PW-MC4R KO compared to WT rats (Fig. 6A). As shown in Figs. 6A and 6B, CIH significantly increased MAP by approximately 9, 11 and 6 mmHg in control, obese MC4R KO and PW-MC4R KO rats, respectively. The pressor effect of CIH was more pronounced in obese MC4R KO and attenuated in lean MC4R compared to control rats (Figs 6A and B). Baseline HR was not different between obese MC4R KO and control rats, while lean PW-MC4R KO had significantly lower HR compared to obese MC4R KO and lean control rats (Fig. 6C).

Effects of CIH on Spontaneous Baroreflex Sensitivity (sBRS) and Power Spectral Analysis of Systolic Arterial Pressure and RR Interval Oscillations before and after MC4R Antagonism

To assess the impact of CIH before and after MC4R antagonism on sBRS we analyzed the sBRS by time domain during control, day 4 of SHU-9119 infusion, day 14 of combined CIH plus SHU-9119 (day 14), day 18 of CIH alone and recovery period day 6. CIH did not alter baroreflex sensitivity before or after MC4R antagonism. Also, sBRS was not significantly altered by CIH in lean Sprague-Dawley rats, in obese MC4RKO, or in lean PW-MC4RKO rats (Table 2). We did not find significant differences in BRS and power spectral analysis between Sprague-Dawley and WT rats (data not shown).

Spectral analyses data of HR and BP were used to assess sympathetic and parasympathetic tone, where oscillations between 0.2 and 0.7 Hz (low frequency, LF) are associated with sympathetic tone and oscillations of HR in the high frequency (HF) region between 0.7 and 2.0 Hz are associated with parasympathetic tone. Obese MC4R KO rats showed increased cardiac parasympathetic tone as reflected by higher HF component of HR oscillations than to lean PW-MC4R KO or control rats (Table 2). CIH, on the other hand, increased LF component of systolic BP in controls and obese MC4R KO rats whereas it did not reach statistical significance in lean PW-MC4R KO rats (Table 2). CIH in rats treated with the MC4R antagonist significantly increased the LF component of heart rate in SD rats (Table 2). Chronic MC4R blockade alone reduced the LF component of systolic blood pressure by 41% in SD rats (Table 2).

DISCUSSION

In this study we showed that MC4R antagonism or MC4R deficiency did not substantially attenuate CIH-induced hypertension. We also demonstrated that CIH for 7–18 days reduced food intake but did not alter plasma glucose, leptin or insulin concentrations. Although lean PW-MC4R deficient rats exhibited attenuated BP responses to CIH, chronic pharmacological MC4R blockade or MC4R deficiency in ad libitum fed rats did not attenuate the BP responses to CIH. Thus, endogenous CNS MC4R does not appear to play an essential role in elevating BP during CIH, whereas food restriction to prevent excess weight gain in MC4R deficient rats may attenuate the elevation in BP caused by CIH.

In addition to its effects on BP, chronic exposure to periodic hypoxia, as occurs in OSA, has been suggested to contribute to metabolic disorders including type 2 diabetes, insulin resistance, and dyslipidemia.²⁷ One proposed mechanism linking metabolic abnormalities to CIH is sympathetic overactivity^{5,10} which could increase catecholamine levels, causing hyperglycemia and hyperinsulinemia.²⁸ In addition, SNS activation may stimulate release of adipocyte-derived inflammatory mediators and leptin which could induce lipolysis and release of free fatty acids from adipose tissue. The consequences of these metabolic alterations have been suggested to cause dysregulation of glucose uptake by peripheral tissues contributing to hyperglycemia and hyperinsulinemia.^{28,29} Previous studies by Polotsky et al²⁷ showed that obese mice exposed to CIH for 12 weeks developed time-dependent increases in fasting serum insulin levels, glucose intolerance, and insulin resistance. However, in the present study CIH for 7 consecutive days did not alter plasma glucose or insulin concentrations. Although inhibition of CNS MC4R was associated with an eight-fold increase in plasma insulin and five-fold increase in plasma leptin concentrations, these changes were likely caused by hyperphagia, weight gain, and increased adiposity during chronic MC4R blockade. CIH did not significantly amplify the effects of MC4R blockade to increase plasma insulin or leptin concentrations. In fact, we found a significant decrease in plasma leptin concentration and a tendency toward reduced plasma insulin concentration during CIH in obese MC4R KO rats. These results suggest that CIH for 7 consecutive days does not cause insulin resistance or hyperglycemia in lean or obese rats. Whether longer periods of CIH would cause major metabolic abnormalities and the role of MC4R activation in altering metabolism in these chronic hypoxic conditions is unclear.

We also found that CIH had a greater effect to reduce food intake in obese MC4R KO compared to control WT rats, suggesting that MC4R activation is not required for the anorexic effects of CIH. Although food intake was not altered by CIH in PW-MC4R KO rats, this was likely due to the fact that these rats were food restricted prior to induction of CIH. Also, while the anorexic effect of CIH was prevented by infusion of SHU-9119, the MC4R antagonist normally causes marked increases in food intake suggesting that CIH was having an important anorexic effect in these rats. The anorexigenic effect of CIH has also been suggested to be mediated in part by leptin or by CIH-induced alterations of specific oxygen-sensing CNS pathways that control feeding behavior.^{30,31} However, further studies are required to examine the mechanisms responsible for the anorexic effects of CIH.

Our results also suggest that the BP responses to CIH are mediated by mechanisms that do not require functional MC4Rs. We previously found an important contribution of MC4R for other forms of hypertension that are dependent of elevated SNS activity.^{13,16,32,33} In contrast, the impact of MC4R blockade on BP in hypertensive animals with reduced or normal SNS activity is very modest.¹³ Although the precise mechanisms linking MC4R activation with hypertension are still being elucidated, it has been demonstrated that MC4R stimulation increases BP by raising SNS activity. For example, injections of MC4R agonists into the CNS raise SNS activity to various tissues, including the kidneys,^{18,34,35} and adrenergic receptor blockade completely prevents the rise in BP caused by chronic CNS MC4R activation.¹⁴ Thus, our finding that MC4R activation was not required for CIH-induced hypertension is somewhat surprising but indicates that not all forms of hypertension caused by sympathetic activation require a functional MC4R.

Our finding that BP quickly returned to control values after stopping CIH is slightly different than previous reports showing a more delayed return of BP to control values after CIH is stopped.^{36,37} In these previous studies, however, BP was measured acutely 24 hours after surgery and catheterization which may confound BP measurements.³⁸ In contrast, we used state-of-the-art telemetry methods for BP measurement.³⁸ Using telemetry we were able to precisely measure BP before, during and after CIH. In fact, our BP data includes beat-to-beat recordings for 10 seconds every 10 minutes 24 hours a day for the duration of the study. To our knowledge this is the first report that examined BP with telemetry for up to 6 days post-CIH (using 7% O₂). Nevertheless, Zoccal et al¹⁰ measured BP 1 and 15 days post-CIH using acutely implanted catheters and found increased BP only on day 1 and normal BP on day 15 post-CIH. These observations are in agreement of our findings.

The elevated levels of leptin in obesity contribute to increased POMC neuronal activity and release of alpha melanocyte stimulating hormone (α -MSH) to promote stimulation of MC4R.^{39,40} However, other factors besides obesity and leptin can also stimulate POMC neurons and make BP dependent on MC4R activation. Hypoxia may be one of those conditions. Acute intermittent hypoxia protocol is associated with increased POMC mRNA in the arcuate nucleus of the hypothalamus.²² Previous studies also suggest that increases in muscle sympathetic activity during acute inspiratory hypoxia are attenuated in humans with MC4R deficiency.⁴¹ Our current results indicate, however, that MC4R antagonism does not significantly reduce BP in response to CIH. In addition, genetic MC4R deficiency did not attenuate CIH-induced hypertension in obese MC4R KO rats. In contrast, lean PW-MC4R KO rats did exhibit attenuated BP response to CIH compared to controls and obese MC4R KO rats. Thus, our findings suggest that MC4R does not play an essential role in mediating the BP responses to CIH, although there may be a complex relationship between CIH, obesity and MC4R activation. Although baseline plasma leptin levels were higher in obese MC4R KO compared to lean PW-MC4R KO rats it is unlikely their different leptin levels affected BP response to CIH. Other factors associated with obesity may contribute to CIH-induced SNS activation independent of MC4R activation. Additional studies are needed to address this possibility.

In this study, we found that chronic MC4R antagonism significantly reduced HR, although this effect was completely offset by CIH in lean rats. Further studies are needed to unravel

which areas of the brain are most important for modulating cardiovascular function by the melanocortin system in response to CIH.

Previous studies have shown that CIH impairs baroreflex control of SNS activity in rats.¹² In the present study we found that CIH did not alter baroreflex sensitivity in lean or obese rats; baroreflex sensitivity was assessed by changes in HR associated with spontaneous increases and decreases in BP under physiological condition. One potential explanation for absence of impaired sBRS during CIH may be the short period of exposure to intermittent hypoxia (7 days) which may not be long enough to cause significant alterations in the baroreflex. In addition, the sequence method limits the range of fluctuations in systolic BP and HR to those observed under resting conditions and moment-to-moment variability could be underestimated due to significant alterations in BP and HR during intermittent hypoxic episodes. Therefore, it is possible that the method used was not sensitive enough to detect a small difference in baroreceptor sensitivity that may have occurred in this study. The sequence method, however, has been shown to correlate well with baroreflex sensitivity evaluated by other methods including the HR responses observed during phenylephrine injections in humans.⁴² We did find a significant increase in the LF component of systolic blood pressure in lean control rats and in obese MC4R KO rats suggesting increased SNS activity during CIH. We also found a greater reduction in the LF component of systolic BP after CIH was stopped and during MC4R antagonist infusion alone which is consistent with our findings of a significant decrease in BP during the last 4 days of SHU-9119 infusion. The reduction in BP during chronic MC4R blockade occurred despite increases in food intake and rapid weight gain in lean rats which would be expected to increase HR and BP due SNS activation. Overall, our results suggest that CNS MC4R antagonism attenuated the effects of weight gain but not the effect of CIH to cause elevations in BP.

The brain melanocortin system has also been suggested to modulate respiratory function. Rats treated with MC4R antagonists and mice with impaired MC4R signaling exhibit attenuated ventilatory responses to hypercapnia.⁴³ In the present study we also found that MC4R deficiency attenuated baseline pulmonary ventilation as well as the acute ventilatory responses to increased CO₂ levels. We also observed increased baseline respiratory frequency in obese MC4R KO rats that was not observed in lean PW-MC4R KO. This finding suggests that obesity enhances baseline respiratory frequency most likely due to reduced pulmonary compliance and decreased tidal volume in MC4R KO rats, while the respiratory responses to hypercapnia were impaired in MC4R deficient rats independent of obesity. These findings are consistent with previous observations that the melanocortin system is an important modulator of respiratory function.

In summary, these data indicate that the CNS-MC4R does not play an essential role in mediating the pressor effects of CIH. However, chronic MC4R antagonism with SHU-9119 prevented the anorexic effects of CIH. Our results are consistent with the possibility that the CNS melanocortin system may interact in complex ways to influence BP during CIH. Our observations indicate that genetic MC4R deficiency did not attenuate CIH-induced hypertension in obese rats, but it did attenuate CIH-induced hypertension in lean PW-MC4R KO; this finding suggests that other factors associated with obesity may contribute to CIH-induced SNS activation independent of MC4R activation. Additional studies are needed to

determine the areas of the brain where MC4R is most important in modulating cardiovascular function in response to CIH. Unraveling the mechanisms by which CIH alters metabolic and cardiovascular functions in obesity may lead to novel therapeutic approaches to treat respiratory disorders as well as high blood pressure.

METHODS

All experimental procedures 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.

Animals

Male 12 to 14 week-old Sprague Dawley rats (n=12) were purchased from Harlan/Envigo (Houston, TX), and MC4R knockout (n=10) and wild-type (WT) Wistar Hannover rats (n=5) were obtained from a colony maintained at the University of Mississippi Medical Center. Generation and validation of MC4R knockout rats has been described elsewhere.⁴⁴

Animal Surgery

The rats were anesthetized with isoflurane and telemetry blood pressure transmitters (Model TA11PAC40, Data Sciences International, MN) were inserted in the abdominal aorta distal to the renal arteries under sterile conditions as previously described¹⁴. Immediately after telemetry probe implantation, a steel cannula (26 gauge, 10 mm long) was placed in the lateral ventricle.¹⁵ After 8 days of recovery from surgery, control measurements including BP, HR, body weight and food intake were measured. Daily 24-h BP averages were derived from bursts of 10s every 10 min using Dataquest 4.0 software. The intracerebroventricular (ICV) cannula placement was confirmed post-mortem. All rats received water and food (#CA 170955, Harlan/Envigo, WI, USA) ad libitum.

Experimental Protocols

Exposure to chronic intermittent hypoxia.—Rats were exposed to CIH, 8 hrs/day from 8:00–16:00 hours for 7 to 18 consecutive days as previously described.^{45, 46} Briefly, rats were acclimatized to specialized chambers (Biospherix Oxycycler, NY, USA) for 2 days. Then chamber O₂ levels were repetitively cycled from 21% to a nadir of 7% for 2 min followed by a return to 21% over the next 3 min. This 5-minute cycle was repeated 12 times each hour, 8 hours each day. Room temperature was maintained at ~25°C. The CIH protocol resulted in a significant reduction in arterial hemoglobin saturation (SaO₂) to approximately 81% after 3 days of CIH (Fig.7).

Responses to CIH after MC4R antagonism.—Mean arterial pressure (MAP) and HR (average of 24-hour measurements), and food intake were recorded daily. After a 5-day control period, the MC4R antagonist, SHU-9119, was infused ICV (1 nmol/h at 0.5 µl/h) for 14 consecutive days via an osmotic minipump (model 2002, Durect Corp., Cupertino, CA) implanted (under isoflurane anesthesia) subcutaneously between the scapulae and connected to the ICV cannula by a tygon tubing (Cole Parmer, 0.38 mm ID, Vernon Hills, IL). The dose of SHU-9119 was based on previous studies.^{13,15,16} On the 6th day of SHU-9119

infusion, the rats were exposed to CIH 8 hrs/day as previously described⁴⁷ while SHU-9119 infusion was continued for 8 days. On the last day of SHU-9119 infusion, we severed the tygon tubing connecting the pump to the ICV cannula to stop infusion, and CIH was continued for an additional 8 days. Then CIH was stopped and the rats were studied for an additional 6-day post-treatment period (Fig 7). After a 5-hour fast blood samples (200 µl) were collected via a tail snip once during the control period, again on day 10 of SHU-9119 treatment, and day 5 of the recovery post-treatment period.

MC4R knockout rats.—Lean and obese MC4R knockout (KO) rats were used to examine whether genetic deficiency of MC4R would attenuate CIH-induced hypertension. To dissociate the direct effects of MC4R deficiency from effects caused by severe obesity, we also examined the cardiometabolic responses to CIH in lean pair-weighted MC4R KO rats. After weaning, MC4R KO rats were food restricted to match the body weight of age-matched WT rats fed ad libitum (~14 g/day).

Spontaneous Baroreflex Sensitivity (BRS) and Power Spectral Analyses of Systolic Arterial Pressure (SAP) and RR Interval (RRI) Oscillations.

Spontaneous baroreflex sensitivity (BRS) was calculated by the sequence method based on quantification of sequences of at least three heart beats in which systolic arterial pressure (SAP) consecutively increases (up sequence) or decreases (down sequence) accompanied by changes in the same direction of the RR intervals (RRI's) of the subsequent beats as described previously.⁴⁸

Ventilation Measurements.

To determine if MC4R deficiency alters ventilatory responses to acute hypercapnia, pulmonary ventilation (VE) was evaluated by whole-body plethysmography as previously described.²⁶

Plasma Insulin, Leptin and Glucose measurements.

Plasma insulin and leptin concentrations were measured by ELISA (R&D Systems and Crystal Chem Inc.) and plasma glucose levels were measured using a glucose analyzer (Beckman).

Statistical Methods and Experimental Design

In most cases each animal served as its own control since control values were obtained followed by an experimental maneuver and then post-control recovery measurements. To control for obesity in the MC4R KO rats we also studied pair weighted MC4R KO rats that were food restricted to prevent obesity. The results are expressed as means ± SEM, and the data were analyzed by 1-way ANOVA with repeated measures followed by Dunnett's post hoc test when comparing control versus experimental values within each group. Comparisons between different groups were performed by 2-way ANOVA followed by Dunnett's post hoc test. Statistical significance was accepted when $P < 0.05$.

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Figure 1.

Dietary and body weight response to chronic intermittent hypoxia (CIH). Food intake (**A**), cumulative food intake (**B**), and body weight (**C**) in Sprague Dawley rats (n=6) in response to CIH for 7 days. * $p < 0.05$ compared to control period.

Figure 2.

Dietary and body weight response to combined MC4R antagonism and chronic intermittent hypoxia (CIH). Food intake (**A**), cumulative food intake (**B**), and body weight (**C**) in Sprague Dawley rats (n=6) in response to MC4R antagonism (SHU-9119, 1.0 nmol/hr, ICV, 14 days) and CIH (18 days). * $p < 0.05$ compared to control period.

Figure 3.

Dietary and body weight response to chronic intermittent hypoxia (CIH). Food intake (**A**), net cumulative food intake (**B**), and body weight (**C**) in WT (n=5), obese MC4R KO (n=5) and lean PW-MC4R KO rats (n=5) in response to CIH for 7 days. * p<0.05 compared to control period. # p<0.05 compared to WT and lean PW-MC4R KO rats.

Figure 4.

Cardiovascular responses to chronic intermittent hypoxia (CIH). Mean arterial pressure (MAP) (A), delta MAP, compared to control (B) and Heart rate (HR) (C) responses to CIH for 7 days in Sprague-Dawley rats (n=6). * $p < 0.05$ compared to control period.

Figure 5.

Cardiovascular responses to combined MC4R antagonism and chronic intermittent hypoxia (CIH). Mean arterial pressure (MAP) (A), and heart rate (HR) (B) in Sprague Dawley rats (n=6) in response to MC4R antagonism (SHU-9119, 1.0 nmol/hr, ICV, 14 days) and CIH (18 days). * $p < 0.05$ compared to control period.

Figure 6.

Cardiovascular responses to chronic intermittent hypoxia (CIH). Mean arterial pressure (MAP) (A), and heart rate (HR) (B) in WT (n=5), obese MC4R KO (n=5) and lean PW-MC4R KO rats (n=5) in response to CIH for 7 days. * p<0.05 compared to control period. # p<0.05 obese MC4R KO rats compared to WT and lean PW-MC4R KO rats. † Lean MC4R KO rats compared to WT.

Figure 7.

Schematic representation of the experimental protocol used in the study. CIH, chronic intermittent hypoxia; SHU-9119, MC4R agonist.

Table 1.

Respiratory frequency (f_R), tidal volume (VT) and ventilation (V_E) during normocapnia (room air) or hypercapnia (7% CO_2) in WT, obese and lean pair-weighted (PW) MC4RKO rats.

	f_R (breaths/min)			VT (ml/kg/min)			V_E (ml/kg/min)		
	WT	MC4R KO	PW-MC4R KO	WT	MC4R KO	PW-MC4R KO	WT	MC4R KO	PW-MC4R KO
Room Air	113.2±6.0	139.0±6.2 [#]	103.3±3.9	3.2±0.1	2.8±0.3 [#]	2.4±0.1 [#]	362.3±25.1	398.3±50.3	251.1±4.5
7% CO₂	137.4±1.8 [*]	161.2±3.6 ^{*,#}	132.5±3.4 [*]	5.3±0.2 [*]	4.1±0.3 ^{*,#}	3.8±0.2 ^{*,#}	730.0±23.3 [*]	669.3±32.9 [*]	499.6±24.9 ^{*,#}
Delta	24.2±2.5	22.2±1.8	29.2±3.0	2.1±0.1	1.3±0.14 [#]	1.4±0.1 [#]	367.7±13.1	271.0±26.1	248.5±20.1 [#]

Values are mean±SEM.

^{*}
p<0.05 compared to room air values;

[#]
p<0.05 compared to WT rats.

Table 2.

sBRS, spectral analysis data of RR interval (RRi) and systolic arterial pressure (SAP) during control, day 7 and 18 of CIH, day 4 and 14 of SHU-9119 infusion and day 5 recovery period in Sprague-Dawley, WT, obese MC4R and lean PW-MC4R rats.

	Seq All, ms/mmHg	RRI			SAP				
		LF,nu	HF, nu	% LF	% HF	LF, nu	HF, nu	% LF	% HF
<u>SD rats (n=6)</u>									
Control	1.8±0.2	11.4±1.4	39.5±6.3	23.1±2.1	76.9±2.1	25.7±2.2	19.2±4.0	58.6±2.5	40.9±3.5
CIH	1.7±0.3	11.5±1.4	31.2±5.5	27.8±3.2	72.2±3.2	27.3±2.5	13.0±0.2	70.7±0.9*	29.2±1.4*
Recovery	1.8±0.3	9.5±1.4	30.5±7.9	26.7±4.1	73.4±4.1	18.7±1.9	15.0±4.8	62.7±4.1	37.2±6.7
<u>SD rats – MC4R antagonism (n=5)</u>									
Control	2.5±0.6	10.6±2.5	39.3±7.9	21.9±2.1	78.1±2.9	25.5±3.8	14.6±1.4	62.2±3.6	36.8±3.3
SHU (day 4)	1.7±0.3	10.2±1.4	38.5±5.3	21.6±2.1	78.4±2.5	21.6±5.0	16.6±4.2	55.9±5.8	44.1±5.8
SHU (day 14) plus CIH (day 9)	1.8±0.2	19.8±3.5	47.2±8.6	32.8±7.4	67.2±7.4	22.7±1.1	21.7±4.5	52.8±4.5*	47.2±4.5
CIH (day 18)	2.1±0.8	11.6±4.5	42.2±6.5	20.1±3.0	76.9±2.1	30.9±2.3	13.6±0.8	68.4±1.7*	31.6±1.6*
Recovery	1.7±0.3	9.7±2.0	38.7±6.9	19.4±1.2	80.6±1.2	18.9±3.8	18.2±2.9	56.3±2.9	43.7±2.9
<u>Obese MC4R (n=6)</u>									
Control	1.5±0.2	4.0±0.3	34.6±5.8	11.8±2.2	88.2±2.2	10.2±1.1	7.6±1.9	59.0±2.3	41.0±3.8
CIH	1.6±0.6	39.5±3.5	38.3±9.3	58.5±5.2	41.5±5.2	28.9±3.3	10.6±0.8	72.4±1.4*	27.6±2.4*
Recovery	1.9±0.2	5.1±0.6	27.6±6.7	16.2±2.8	83.8±2.8	9.5±1.4	9.3±1.3	50.4±4.0	49.6±6.5
<u>Lean MC4R (n=5)</u>									
Control	1.9±0.1	8.3±2.5	30.0±3.7	22.4±7.2	77.6±7.3	12.9±0.7	9.4±0.5	58.0±1.0	42.0±1.7
CIH	1.6±0.3	38.7±2.3	37.8±5.0	51.3±5.0	48.7±5.0	34.6±2.3	14.5±1.7	70.5±1.7	29.5±2.8
Recovery	1.9±0.2	6.8±1.1	43.1±4.9	14.2±1.2	85.8±2.6	21.0±2.8	15.4±0.9	56.7±2.4	43.3±3.9

Values are expressed as mean±SEM. Seq, sequence; LF, low frequency; HF, high frequency; nu, normalized units (represent the relative value of each power component in proportion to the total power minus VLF (very low frequency) component. Values were obtained on day 5 of control, day 5 or 7 of CIH, SHU-9119 infusion plus CIH and recovery periods. # p<0.05 compared to control period.