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Role of excitatory amino acid input in rostral ventrolateral medulla neurons in rats with obesity-induced hypertension

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Abstract

Obesity is intimately associated with hypertension; increases in blood pressure are closely related to the magnitude of weight gain. The present study aims to determine whether the excitatory amino acid input to rostral ventrolateral medulla (RVLM) contributes to elevated blood pressure in rats with diet-induced obesity. Male Sprague-Dawley rats weighing 280 to 300 grams were fed with a low-fat diet (10% kcal from fat) or moderately high-fat diet (32% kcal from fat) for 16 weeks. At week 16, rats on the moderate high-fat diet were segregated into obesity-prone and obesity-resistant rats based on body weight distribution. Baseline mean arterial pressure (MAP) was significantly higher in obesity-prone rats as compared to obesity-resistant and rats on a low-fat diet. Bilateral injection of kynurenic acid (KYN) (40 nM) into the RVLM of the obesity-prone rats reduced MAP to levels significantly different from those observed in rats on a low-fat diet and obesity-resistant rats (no change in MAP). At a lower concentration (4 nM), KYN injection did not produce any change in MAP in any group. The results obtained suggest that excitatory amino acid input to the RVLM does contribute to the development of hypertension in rats with diet-induced obesity.

Key words: blood pressure, excitatory amino acid, hypertension, obesity, rostral ventrolateral medulla.

Introduction

The rostral ventrolateral medulla (RVLM) plays a pivotal role in the tonic and phasic regulation of cardiovascular function (Araujo *et al.*, 1999; Ito *et al.*, 2001; 2002; Menezes and Fontes, 2007). RVLM neurons that project directly to sympathetic preganglionic neurons in the spinal cord are a major source of basal tonic sympathetic vasomotor activity (Yang and Coote, 1998; Tagawa and Dampney, 1999; Potts *et al.*, 2000; Hu *et al.*, 2002; Oshima *et al.*, 2006). These tonic excitations of sympathetic vasomotor outflow are important in maintaining normal arterial blood pressure (Horiuchi *et al.*, 2004). Thus any neuronal inhibition of the RVLM will result in marked decreases in arterial pressure.

The role of excitatory amino acids (EAA) in mediating neural transmission in the RVLM has been well established. Moreover, the presence of major categories of EAA receptors in the RVLM further supports the role of EAA in this brain region (Sved *et al.*, 2002). A previous study reported that the injection of kynurenic acid (KYN), an EAA antagonist, into RVLM of normotensive rats did not produce any effect on arterial blood pressure (Ito *et al.*, 2000). This indicated that direct excitatory and indirect inhibitory influences of EAA are in a perfect balance since sympathetic vasomotor outflow from RVLM neurons is maintained by a balance of excitatory and inhibitory input (Sved *et al.*, 2001). However, in models of experimental hypertension, the excitatory and inhibitory balance is altered (Ito *et al.*, 2000; 2001). In the Dahl salt-sensitive model, the balance of EAA input was disrupted: the balance is shifted towards excitation in Dahl salt-sensitive rats as compared with Dahl salt-resistant rats. A significant drop in MAP was recorded after microinjection of KYN into the RVLM (Ito *et al.*, 2001). In addition, disruption in the balance of excitatory and inhibitory input was also observed in a spontaneously hypertensive rat model (Ito *et al.*, 2000).

Feeding rats with a moderately high-fat diet for an extended period of time can lead to the segregation of obesity-resistant (OR) and obesity-prone (OP) rats, whereby the latter group exhibits several characteristics of obesity-induced hypertension in humans, such as elevated levels of circulating leptin, reduced growth hormone concentration, and activation of the renin-angiotensin system (Dobrian *et al.*,

2000; Carroll *et al.*, 2006; Stocker *et al.*, 2007). Experimental, clinical, and population studies have demonstrated that excess weight gain raised blood pressure, thus representing a good predictor for the development of hypertension (Hall, 2003). According to Stocker *et al.* (2007), hypertension in rats with diet-induced obesity is attributed to the involvement of RVLM neurons. The use of muscimol instead of EAA blockers in the latter study could not provide direct evidence on the nature of the input to the RVLM. Rather, the results merely indicated that the tonic activity of RVLM neurons is necessary for the elevated blood pressure in rats with obesity-induced hypertension (Stocker *et al.*, 2007). Thus, this present study aimed to investigate the neural pathway that underlies the activation of RVLM neurons by determining whether EAA input, particularly L-glutamate within the RVLM neurons, contributes to elevated blood pressure in obesity. We further examined whether microinjections of EAA receptor antagonist into the RVLM vicinity reduce arterial pressure in rats with obesity-induced hypertension.

Methods and materials

Animals

All procedures involving animals had been approved by the Animal Ethics Committee of our university. Male Sprague-Dawley rats (280-300 g) were housed individually in a temperature-controlled room (24°C to 25°C) with a 12-hour/12-hour light-dark cycle. Rats were fed with a low-fat (LF) diet (10% kcal from fat; Research Diets, Inc. D12489B) or a moderately high-fat diet (MHF) (32% kcal from fat; Research Diets, Inc. D12266B) for 16 weeks. Rats on the MHF diet were segregated into OR and OP rats based on the body weight gain distribution at the end of 16 weeks, as described by Lauterio *et al.* (1994). Rats with the greatest body weight gain were grouped as OP while those with the lowest body weight gain were grouped as OR, and the remainder was excluded from this study (Boustany *et al.*, 2005; Carroll *et al.*, 2006). Food intake was measured daily throughout the 16-week period.

Systolic blood pressure

The development of hypertension was assessed using the tail cuff method (Powerlab; ADInstruments, Australia). Tail systolic blood pressure (SBP) was measured at weeks 0, 8, 12 and 16. Rats were acclimatised to the process two to three days prior to actual measurements. Three consecutive measurements with a difference of less than 5 mmHg were recorded (Dobrian *et al.*, 2000).

Microinjection into RVLM

Rats were anaesthetised with urethane (Sigma, China) (1.3 g/kg) intraperitoneally and tracheotomy was performed to facilitate respiration. Right femoral vein and artery were catheterised with PE50 polyethylene tubing for the administration of anaesthetic and the measurement of invasive MAP and heart rate (HR), respectively (Yusof *et al.*, 2009). The animals were kept on a heating pad to maintain a constant body temperature (36-37°C) and evaluated with a rectal thermometer (Harvard Apparatus). Room temperature was maintained at 26°C. Adequacy of the level of anaesthesia was verified by the absence of a withdrawal response to nociceptive stimulation of the hindpaw. Supplemental doses of urethane were administered whenever necessary.

Anaesthetised rats were then positioned onto a stereotaxic frame (Narishige, Japan). Craniotomy was performed and RVLM was microinjected using reference coordinates from the Paxinos and Watson atlas (Paxinos and Watson, 1986). The pipette tip was positioned 12 mm caudally to the bregma, 1.9 mm lateral to the midline and 8 mm ventral to the surface of the cerebellar vermis (Yang *et al.*, 2001). Microinjections were performed using modified stainless steel micropipettes (30G) sealed with PE10 polyethylene tubing. A 10-μL Hamilton syringe was then inserted at the end of the polyethylene tubing. All injections were made bilaterally with a volume of 100 nL of physiological saline administered for 3-7 seconds. Upon completion of the surgical procedures, rats were stabilised for an hour before commencement of the experiment. Initially 1 nmol of L-glutamate in 100 nL was injected bilaterally into the RVLM to confirm the functional pressor site (Tagawa and Dampney, 1999). MAP and HR were recorded using a computerised data acquisition system (Biopac Systems Inc, California). Then, the micropipette was removed and rinsed thoroughly with physiological saline. Different concentrations of KYN (4 nM, 40 nM) were injected into the RVLM with at least five minutes between each injection. At the end of the experiment, hexamethonium (20 mg/kg) was injected intravenously to determine the total autonomic blockade effect on MAP in all groups.

Histological study

At the end of the experiment, Chicago sky blue (1%) dye (Sigma, US) was injected into the RVLM

to verify the centre of the microinjection site. Rats were euthanised with an overdose of urethane. The brain stem was removed and fixed in 10% neutral buffered formalin. Three days later, the frozen brain stem was sliced into 45 μm coronal sections on a freezing microtome and stained with neutral red (Fluka, India) (Yusof and Coote, 1988). Brain stem sections were examined using light microscopy and the microinjection site was identified according to standard anatomical structures of the brain stem (Paxinos and Watson, 1986). The RVLM area was identified as medial to the inferior olive or pyramidal tracts, ventral to the compact formation of the nucleus ambiguus and lateral to the spinal trigeminal nucleus (Stocker *et al.*, 2007; Kashihara *et al.*, 2008).

Calculation of the adiposity index

After removal of the brain stem, a midline incision was made in each rat to expose the abdominal region. Epididymal and retroperitoneal fat pads were isolated and weighed for calculation of the adiposity index using the following equation (Boustany *et al.*, 2005):

$$\text{Adiposity index} = \frac{(\text{epididymal fat} + \text{retroperitoneal fat})}{\text{body weight} - (\text{epididymal fat} + \text{retroperitoneal fat})} \times 100$$

Drugs

All drugs were freshly prepared prior to commencement of the experiment. L-glutamate (Sigma, US) and hexamethonium (Sigma, Switzerland) were dissolved in physiological saline. KYN (Sigma, UK) was dissolved in a minimum volume of sodium hydroxide (NaOH, 0.1 M), then topped up with distilled water. pH was adjusted to pH 7.0 with hydrochloric acid (HCl, 0.1 M). Then, KYN was diluted in physiological saline.

Data analysis

All the variables were expressed as mean ± SEM and then analysed using one-way ANOVA followed by Bonferroni's post-hoc test. Data for different KYN concentrations in the same strain were analysed with one-way ANOVA followed by Dunnett's post-hoc test.

Results

Body Weight, Food intake, and Measurement of Systolic Blood Pressure

At the onset of the study (week 0) body weight did not differ significantly among LF (n = 6), OR (n = 6) and OP rats (n = 6) (Fig. 1). However, by week 4, OP rats showed significant increases ($p < 0.05$) in body weight as compared with LF and OR rats. This increase remained present throughout the study period. Moreover, no significant difference in body weight between LF and OR rats was observed. Food intake for OP rats was significantly greater ($p < 0.05$) compared to LF and OR rats throughout the 16-week period, while there was no significant difference in food intake between LF and OR rats (Fig. 2). There was greater fat deposition in OP rats as shown by the adiposity index, which was significantly different ($p < 0.001$) as compared to both LF and OR rats (Table 1).

In this study, SBP was measured by tail cuff method. Figure 3 displays the measured SBP as a function of time. We found that starting at week 12, SBP was significantly elevated ($p < 0.001$) in OP rats as compared to both LF and OR rats. The SBP at week 16 was also significantly increased in all groups when compared to baseline SBP at week 0 (Table 1).

Invasive blood pressure measurement demonstrated that OP rats had significantly elevated MAP at week 16 as compared with LF and OR rats (LF: 99 ± 3; OR: 101 ± 4; OP: 130 ± 1 mmHg, $p < 0.05$). HR values displayed a similar pattern, which was significantly higher in OP rats as compared with LF and OR rats (LF: 356 ± 10; OR: 376 ± 5; OP: 411 ± 6 bpm, $p < 0.05$).

Effects of L-glutamate injection into RVLM

The functional pressor site in the RVLM was identified by an increase in MAP after the microinjection of L-glutamate (1 nmol). The L-glutamate-evoked pressor response was significantly higher in OP rats as compared to both LF and OR rats (LF: 9.6 ± 0.3; OR: 7.4 ± 1.2; OP: 13.8 ± 0.9 mmHg, $p < 0.05$).

Effects of KYN injection into RVLM

A KYN injection of 4 nM into RVLM produced no change in the baseline MAP of all groups, while at 40 nM, KYN injection significantly reduced the

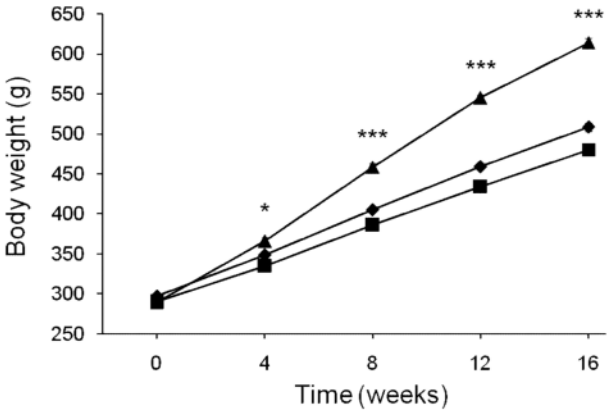

FIG. 1. — Initial body weight did not differ in low-fat diet-treated (LF) rats (◆), obesity-resistant (OR) rats (■) and obesity-prone (OP) rats (▲). However, beginning at week 4, body weight for OP rats was significantly higher as compared to LF and OR rats. Data are presented as the mean ± SEM (n = 6). *Significant difference between OP rats versus LF and OR rats ($p < 0.05$). ***Significant difference between OP rats versus LF and OR rats ($p < 0.001$).

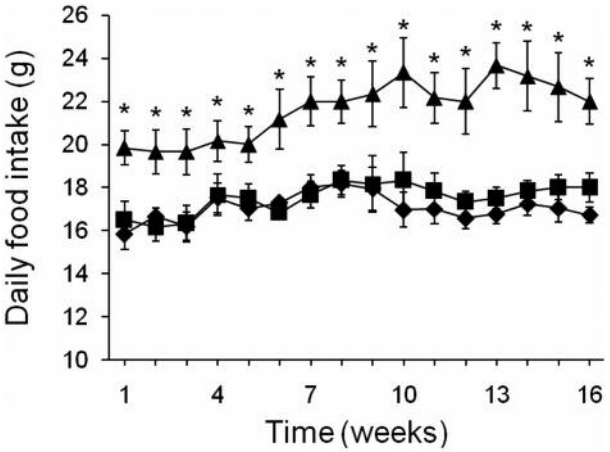

FIG. 2. — Average daily food intake for obesity-prone (OP) rats (▲) was significantly higher as compared to both low-fat diet-treated (LF) rats (◆) and obesity-resistant (OR) rats (■). Data are presented as the mean ± SEM (n = 6). *Significant difference between OP versus LF and OR rats ($p < 0.05$).

Table 1

Variables of LF, OR and OP rats after 16 weeks of diet

Group	LF rats	OR rats	OP rats
Body weight			
Baseline (g)	298 ± 2	291 ± 2	290 ± 3
Week 16 (g)	509 ± 5	481 ± 4	614 ± 5***
Epididymal fat pad (g)	5.0 ± 0.2	5.1 ± 0.2	9.3 ± 0.2***
Retroperitoneal fat pad (g)	9.1 ± 0.2	9.2 ± 0.2	15.1 ± 0.4***
Adiposity index (%)	2.9 ± 0.1	3.1 ± 0.1	4.1 ± 0.1***
Systolic blood pressure			
Baseline (mmHg)	108 ± 2	110 ± 2	111 ± 2
Week 16 (mmHg)	127 ± 2#	135 ± 3#	156 ± 2*#

Values are represented as mean ± SEM (n = 6).

*Significant difference between obesity-prone (OP) rats versus low-fat diet-treated (LF) and obesity-resistant (OR) rats ($p < 0.05$).

***Significant difference between OP rats versus LF and OR rats ($p < 0.001$).

#Significant difference between systolic blood pressure (SBP) measured by tail cuff at week 16 versus baseline SBP in the same strain ($p < 0.05$).

MAP in OP rats by 7 ± 0.8 mmHg ($p < 0.05$), as compared to LF and OR rats which did not exhibit any change in the MAP (Fig. 4). Hexamethonium was administered intravenously at the end of the experiment to determine the total autonomic blockade. In OP rats, administration of hexamethonium decreased MAP by 51 ± 3 mmHg while in LF and OR rats, the decrease was about -34 ± 2 and -32 ± 5 mmHg, respectively (Fig. 5).

Histology

Histological analysis confirmed that all microinjections reached the RVLM site. This was shown with the presence of blue spots on the coronal sections through the medulla at a level ~12 mm caudal from the bregma, using the Paxinos and Watson atlas as reference. Schematic representation of the coronal section is shown in Figure 6.

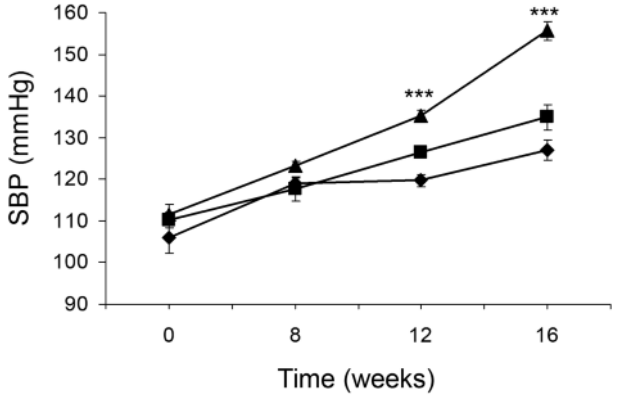

FIG. 3. — In obesity-prone (OP) rats (▲), development of hypertension was seen at week 12 (as shown by systolic blood pressure (SBP)) and became more evident at week 16. Data are presented as the mean ± SEM (n = 6). *Significant difference between OP rats versus low-fat diet-treated (LF) rats (◆) and obesity-resistant (OR) rats (■) ($p < 0.05$). ***Significant difference between OP rats versus LF and OR rats ($p < 0.001$).

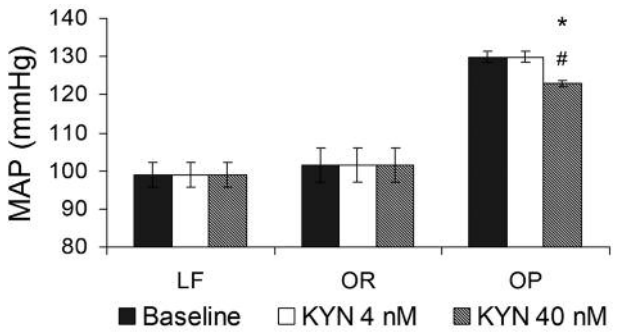

FIG. 4. — Effects of different concentrations of KYN microinjected into the RVLM of low-fat diet-treated (LF) rats, obesity-resistant (OR) and obesity-prone (OP) rats on MAP. Data are presented as the mean ± SEM (n = 6). *Significant difference between OP rats versus LF and OR rats ($p < 0.05$). #Significant difference between KYN concentration versus baseline MAP in the same strain ($p < 0.001$).

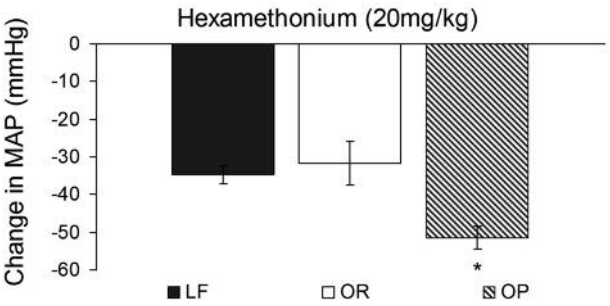

FIG. 5. — Effects of administration of intravenous hexamethonium on MAP of low-fat diet-treated (LF), obesity-resistant (OR) and obesity-prone (OP) rats. Data are presented as the mean ± SEM (n = 6). *Significant difference between OP rats versus LF and OR rats ($p < 0.05$).

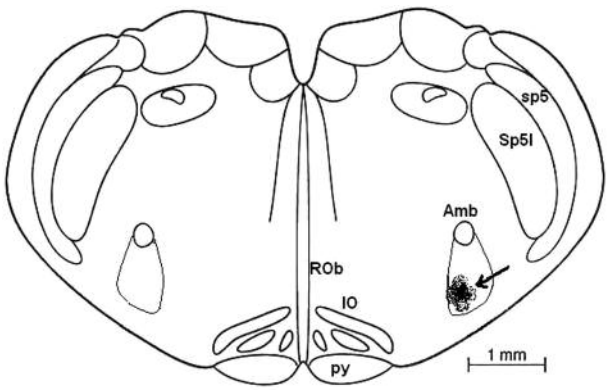

FIG. 6. — Schematic representation of coronal section through the medulla at a level ~12 mm caudal from bregma with reference to the Paxinos and Watson atlas. Arrow indicates the injection site in the RVLM as indicated by Chicago sky blue dye (shaded area). Amb indicates nucleus ambiguus; IO, inferior olive; py, pyramidal tract; SP5I, spinal trigeminal nucleus; sp5, spinal trigeminal tract; ROb, raphe obscurus.

Discussion

The key finding of this present study is that injection of 40 nM KYN into the RVLM of OP rats decreased the MAP. Although the magnitude of the response is small, the difference between the OP rats in comparison to both LF and OR rats is significant since KYN injection into the RVLM at the same concentration did not produce any change in baseline MAP. These data are consistent with the hypothesis that injection of EAA antagonist into the RVLM vicinity will result in a reduction of arterial blood pressure in rats with obesity-induced hypertension.

A previous study proposed that resting arterial pressure in normotensive rats is determined by the balance between tonic EAA-mediated excitation of RVLM vasomotor neurons and indirect inhibition of RVLM vasomotor neurons. Hence, chronic elevation of sympathetic vasomotor activity may result from either increased EAA-mediated excitation of RVLM neurons or decreased inhibition of RVLM neurons (Ito *et al.*, 2000). The decrease in MAP after KYN injection at 40 nM in this present study suggests that EAA input to the RVLM is imbalanced such that the balance is shifted towards excitation in OP rats. However, this disruption in the balance of EAA is not easily discerned since the magnitude of the decrease in MAP is small following injection of EAA antagonist into the RVLM. Moreover, the decrease in MAP after KYN injection reveals that tonically

active EAA input to the RVLM modulates MAP in this rat model of obesity-induced hypertension.

In the present study, KYN injection into the RVLM at a lower concentration (4 nM) did not produce any change in MAP in any of the groups. This showed that low concentrations of KYN are unable to reverse the shift in the balance of excitatory and inhibitory input to the RVLM in rats with obesity-induced hypertension. Meanwhile, a study by Ito and his colleagues (2001) showed that KYN injection at a concentration less than 4 nM was enough to block the EAA receptor in the RVLM of Dahl salt-sensitive rats.

Another possible explanation for the lack of KYN's effect is that our study is limited to the role of NMDA receptors. It has been reported that cardiovascular responses in RVLM of anesthetised rats are partly mediated by different EAA receptor subtypes, namely, metabotropic EAA receptors (Tsuchihashi *et al.*, 1994) and non-EAA receptors (Horiuchi *et al.*, 2004). This is supported by the finding in spontaneously hypertensive rats that stimulation of metabotropic glutamate receptors in the RVLM enhanced pressor and sympathoexcitatory responses. These results suggest the role of metabotropic receptors in the maintenance of blood pressure in hypertension (Tsuchihashi *et al.*, 1994).

Local administration of L-glutamate in brain will excite the cell bodies of neurons but not the axons (Ross *et al.*, 1984). In this present study, the L-glutamate-evoked pressor response was greater in OP rats as compared to LF and OR rats. This pressor response to L-glutamate cannot be attributed to mechanical distortion since injection of vehicle with equal volume did not elicit any response. Greater L-glutamate pressor response in OP rats might be due to increased sensitivity of the RVLM towards EAA, especially glutamate, and might also be associated with an increased role of the excitatory input to the vasomotor RVLM (Brooks *et al.*, 2004). In other models of hypertension, magnitude of the glutamate-evoked pressor response was greater (≈ 40 mmHg). This is plausibly due to higher baseline MAP values (≈ 165 mmHg) in those models (Ito *et al.*, 2000; 2001) as compared to OP rats. Moreover, it should be noted that anaesthesia can also affect the responsiveness of the RVLM neurons to EAA, as studies have shown that pressor responses to local injection of glutamate are reduced in anaesthetised rats (Bachelard *et al.*, 1990; Mayorov and Head, 2003).

In this present study, intravenous administration of hexamethonium further decreased the MAP in all groups. The depressor response was stronger in OP rats as compared to both LF and OR rats, suggesting the presence of elevated sympathetic vasomotor tone in OP rats (Schreihofer *et al.*, 2007). Additionally, it seems that in our model of obese-hypertensive rats, the RVLM is not the dominant centre in the regulation of blood pressure (Araujo *et al.*, 1999). In recent years, other brain regions have been recognised to have a role in the regulation of vasomotor tone. Among these, the paraventricular nucleus (PVN) of the hypothalamus is the region most studied by investigators. PVN neurons consist of three classes: neurons that project to RVLM neurons, neurons that project to spinal cord and branch to innervate the RVLM, and neurons that project directly to spinal cord (Badoer, 2001; Yang *et al.*, 2001; Yusof, 2007). Elevated levels of circulating angiotensin II and adipocyte-derived leptin have been observed in rats with obesity-induced hypertension. These factors can act centrally to activate PVN neurons. Activation of these neurons, which is mediated by other neurotransmitters, can lead to a rise in sympathetic outflow to the kidneys (Lohmeier *et al.*, 2003). Therefore, it is possible that elevation of blood pressure in rats with diet-induced obesity is not primarily due to imbalanced EAA input to the RVLM, but rather involves other pathways in addition to those that synapse on the RVLM.

In addition, a study in the Dahl salt-sensitive rat model also reported that KYN injection into the RVLM of this strain resulted in a significant drop in MAP as compared to Dahl salt-resistant rats. The response to KYN injection was exaggerated in association with salt-induced hypertension in this strain. These observations reflect the imbalance between tonically active EAA-mediated excitation of RVLM vasomotor neurons and the tonic inhibition of these neurons through the action of EAA input to the RVLM (Ito *et al.*, 2001). However, it should be noted again that anesthetic agents could produce differences in the results obtained (Ito *et al.*, 2000).

Apart from excitatory amino acid input to RVLM, brain angiotensin II also has an effect on the regulation of blood pressure. Studies have reported that blockade of angiotensin type 1 (AT_1) receptors in RVLM decreased blood pressure in spontaneously hypertensive rats (Ito *et al.*, 2002). Moreover, it appears that the amount of angiotensin and its receptor was altered in discrete brain regions of this hypertensive model (Ito *et al.*, 2002). In Dahl salt-sensitive rats, injection of angiotensin II into RVLM increased the MAP as compared to MAP in normotensive Dahl salt-resistant rats. In addition, injection of valsartan, an antagonist for AT_1 receptors, into RVLM reduced the elevated MAP in Dahl salt-sensitive rats (Ito *et al.*, 2003). Therefore, further investigation is needed to identify whether angiotensin

II input to RVLM also contributes to the elevated MAP in diet-induced obesity.

Feeding rats a moderately high-fat diet resulted in a clear segregation of OR and OP rats with the latter group showing significantly greater weight gain and also elevated blood pressure. Greater body weight in OP rats reflects an increase in adipose mass as shown by higher adiposity index, while elevation of blood pressure suggests that hypertension develops in response to the obesity and that diet is not the major determinant of elevated blood pressure in this model (Dobrian *et al.*, 2000; Stocker *et al.*, 2007). The increase in SBP at week 16 in LF and OR rats, although significant, cannot be considered as hypertension since it did not exceed the upper normal limit (SBP > 140 mmHg) (Dobrian *et al.*, 2000). Additionally, **t**he greater increase in invasive MAP and HR in OP rats at week 16 as compared to other groups may reflect a certain level of sympathetic activation in the rat model of obesity-induced hypertension (Dobrian *et al.*, 2001).

In conclusion, the present study demonstrates that EAA input to RVLM contributes to the development of hypertension in rats with diet-induced obesity. However, the role of EAA input to RVLM is not dominant in determining the basal MAP since injection of KYN produces slight decreases in MAP in OP rats. Furthermore, it is possible that the RVLM acts in association with other brain regions to cause elevated blood pressure in response to obesity.

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