Enhanced Adipose Afferent Reflex Contributes to Sympathetic Activation in Diet-Induced Obesity Hypertension

Xiao-Qing Xiong, Wei-Wei Chen, Ying Han, Ye-Bo Zhou, Feng Zhang, Xing-Ya Gao, Guo-Qing Zhu

Abstract—We recently found that adipose afferent reflex (AAR) induced by chemical stimulation of white adipose tissue (WAT) increased sympathetic outflow and blood pressure in normal rats. The study was designed to test the hypothesis that AAR contributes to sympathetic activation in obesity hypertension. Male rats were fed with a control diet (12% kcal as fat) or high-fat diet (42% kcal as fat) for 12 weeks to induce obesity hypertension. Stimulation of WAT with capsaicin increased renal sympathetic nerve activity and mean arterial pressure. Both AAR and WAT afferent activity were enhanced in obesity hypertensive subjects (OH) compared with obesity nonhypertensive (ON) and in ON compared with obesity-resistant or control diet rats. WAT sensory denervation induced by resiniferatoxin caused greater decreases in renal sympathetic nerve activity and mean arterial pressure in OH than ON and in ON than obesity-resistant or control rats. The depressor effect of resiniferatoxin lasted ≥3 weeks in OH. Leptin antagonist in WAT reduced renal sympathetic nerve activity and mean arterial pressure in OH. WAT injection of capsaicin increased plasma renin, angiotensin II, and norepinephrine levels in OH and caused more c-fos expression in paraventricular nucleus in OH than ON and in ON than obesity-resistant or control rats. Inhibiting paraventricular nucleus neurons with lidocaine attenuated renal sympathetic nerve activity in OH and ON, decreased mean arterial pressure in OH, and abolished the capsaicin-induced AAR in all groups. The results indicate that enhanced AAR contributes to sympathetic activation in OH, and paraventricular nucleus plays an important role in the enhanced AAR and sympathetic activation in OH. (Hypertension. 2012;60:1280-1286.) ● Online Data Supplement

Key Words: hypertension ■ obesity ■ adipose afferent reflex ■ sympathetic nerve activity ■ paraventricular nucleus

It is known that sympathetic activity is significantly enhanced in obesity hypertension.1,2 Combined blockade of α- and β-receptors caused a greater decrease in blood pressure in obese hypertensive subjects than that in lean hypertensive subjects.3 Autonomic ganglionic blockade induced a greater depressor response in obese rats than that in lean rats.4 A greater depressor effect of ganglionic blocker was found in obese subjects than normal-weight subjects.3 Renal sympathetic denervation markedly attenuated sodium and water retention and hypertension in diet-induced obesity in dogs.5 Excessive sympathetic activity plays a critical role in the pathogenesis and target organ complication of the obesity hypertension.3-9 Muscle sympathetic nerve activity was more closely associated with the level of abdominal visceral fat than total fat mass or abdominal subcutaneous fat in obese humans.10 Fat in the abdominal viscera has a particularly strong effect on blood pressure.11 Central obesity is characterized by a greater sympathetic activation than that in peripheral obesity.12 Weight loss decreased the sympathetic activity in obese metabolic syndrome subjects.13 These findings indicate that the increased abdominal visceral fat is an important factor contributing to sympathetic activation. The mechanisms involved in the sympathetic activation of obesity hypertension (OH) remain uncertain. However, some encouraging findings about the adipose afferent reflex (AAR) in our laboratory have shed light on revealing the neural mechanisms of sympathetic activation in OH.

Injection of leptin into white adipose tissue (WAT) of rats increased the sympathetic outflow to the WAT,14 brown adipose tissue, pancreas, liver, and kidney.15,16 We recently found that WAT injection of capsaicin, bradykinin, adenosine, or leptin increased renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) in normal rats. WAT injection of capsaicin increased afferent nerve activity of the WAT and efferent nerve activity of both WAT and brown adipose tissue. Chemical stimulation of WAT-induced sympatho-excitatory reflex is called AAR.17 WAT denervation or chemical lesion of the neurons in paraventricular nucleus (PVN) abolished the AAR induced by chemical stimulation. These findings suggest that the AAR is involved in the regulation of sympathetic activity.

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and blood pressure. Neuroanatomical studies have shown sensory and efferent sympathetic innervations of WAT. We hypothesize that the enhanced AAR may play an important role in the sympathetic activation and hypertension in obesity. The present study was designed to determine the contribution of AAR to the sympathetic overdrive and hypertension, as well as the possible mechanisms of enhanced AAR and sympathetic activity in OH induced by a high-fat diet (HFD).

### Methods

Male Sprague-Dawley rats were randomly assigned to receive a control (Ctrl) diet (12% kcal as fat, n=55) or HFD (42% kcal as fat, n=165) for 12 weeks. The rats with weight gains equal to or less than the heaviest Ctrl rats were defined as obesity-resistant (OR) rats, and the rats with greater weight gains were obesity-prone (OP) rats. The OP rats with systolic blood pressure (SBP) ≥150 mm Hg were referred to as OH rats. The OP rats with lower SBP were obesity nonhypertensive (ON) rats, including OH-prone rats (between 140 and 150 mm Hg) and obesity normotensive rats (<140 mm Hg). The rats with Ctrl diet were used as Ctrl.

Acute experiments were carried out at the end of the 12th week under anesthesia with intraperitoneal administration of urethane (800 mg/kg) and α-chloralose (40 mg/kg). RSNA, MAP, and heart rate (HR) were continuously recorded. AAR was evaluated by the RSNA and MAP responses to injections of capsaicin (1.0 nmol/µL) into 4 sites of right inguinal WAT (iWAT) at a rate of 4.0 µL/min for 2 minutes for each site. The c-fos expression in the PVN was determined statistically significant.

### Results

#### Incidence of Obesity and Hypertension

There were 116 OP rats (70.3%) in 165 HFD-fed rats (Table 1). In these OP rats, 70 rats (60.3%) developed hypertension, 15 rats (13.0%) belonged to hypertension-prone rats, and 31 rats (26.7%) remained at normal blood pressure level.

#### Anatomic Data, Blood Pressure, and HR

Body weight (BW) in both ON and OH was higher than Ctrl or OR (Figure 1A), and the SBP in OH was higher than the other 3 groups (Figure 1B). There was no significant difference in BW between ON and OH. A significant linear correlation between the BW and SBP was observed in ON, OH, OR, HFD, or a cohort of rats. There was a tendency of correlation between the BW and SBP in Ctrl or OR, but the correlation did not reach statistical significance (Table 1). MAP and HR in OH were increased compared with the other 3 groups. WAT mass and adiposity index in both ON and OH were increased compared with Ctrl or OR, but no significant difference was found between the ON and OH (Table 2).

#### Basal Sympathetic Activity

Plasma NE level (Figure 1C) and maximal depressor response to hexamethonium (Figure 1D) were used to evaluate the basal sympathetic activity. The basal sympathetic activity was enhanced in OH compared with ON, OR, Ctrl and in ON compared with OR or Ctrl.

#### Adipose Afferent Reflex

Representative recordings of the capsaicin-induced AAR are shown in the online-only Data Supplement. The iWAT

### Table 1. Correlation Analysis of Body Weight to Systolic Blood Pressure After 12 Weeks of Diet

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>% of Total</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (Ctrl+HFD)</td>
<td>220</td>
<td></td>
<td>0.666</td>
<td>0.000*</td>
</tr>
<tr>
<td>Ctrl</td>
<td>55</td>
<td>25.0%</td>
<td>0.115</td>
<td>0.402</td>
</tr>
<tr>
<td>HFD (OR+OP)</td>
<td>165</td>
<td>75.0%</td>
<td>0.633</td>
<td>0.000*</td>
</tr>
<tr>
<td>OR</td>
<td>49</td>
<td>29.7%</td>
<td>0.137</td>
<td>0.347</td>
</tr>
<tr>
<td>OP (ON+OH)</td>
<td>116</td>
<td>70.3%</td>
<td>0.503</td>
<td>0.000*</td>
</tr>
<tr>
<td>ON</td>
<td>46</td>
<td>27.9%</td>
<td>0.475</td>
<td>0.001*</td>
</tr>
<tr>
<td>OH</td>
<td>70</td>
<td>42.4%</td>
<td>0.779</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Ctrl indicates control; HFD, high-fat diet; OR, obesity-resistant; OP, obesity-prone; ON, obesity nonhypertension; OH, obesity hypertension. *P<0.05.

### Figure 1.

Body weight, systolic blood pressure (SBP), plasma norepinephrine (NE), and hexamethonium-induced depressor responses in control (Ctrl), obesity-resistant (OR), obesity-prone nonhypertension (ON), and obesity-prone hypertension (OH) rats. A. Body weight; B. Systolic blood pressure of tail artery in conscious state, n=55, 49, 46, and 70 for Ctrl, OR, ON, and OH, respectively; C. Plasma NE, n=8 for each group; D. Maximal mean arterial pressure (MAP) response to ganglionic blockade with intravenous hexamethonium hydrochloride as an index of sympathetic activity, n=8 for each group. Values are mean±SE. *P<0.05 vs Ctrl; †P<0.05 vs OR; ‡P<0.05 vs ON.
Injection of capsaicin increased RSNA and MAP in all 4 groups. The capsaicin-induced AAR was enhanced in OH compared with ON, OR, or Ctrl and in ON compared with OR or Ctrl. There was no significant difference in the AAR between OR and Ctrl (Figure 2A).

**Plasma Renin, Ang II, and NE Levels**

Simulation of iWAT with capsaicin to induce the AAR increased the plasma renin, Ang II, and NE levels in OH (Figure 2B).

**WAT Sensory Denervation With RTX**

Bilateral iWAT and retroperitoneal WAT (rWAT) injection of RTX caused immediate and short-term increases in RSNA and MAP peaking at 5 minutes in all groups, but a significant increase in HR was found only in OH. Approximately 20 minutes later, RTX caused long-lasting decreases in RSNA and MAP in ON and OH but not in Ctrl and OR. Both excitatory and inhibitory effects of RTX were enhanced in OH compared with ON, OR, or Ctrl and in ON compared with OR or Ctrl (Figure 3A). Effectiveness of WAT sensory denervation was confirmed by the failure to induce the AAR with capsaicin 120 minutes after RTX treatment (Figure 3B).

**Long-Term Effect of RTX on SBP**

Bilateral iWAT and rWAT injection of RTX in OH caused a persistent decrease in SBP lasting ≥3 weeks. Four weeks later, RTX only had a tendency to decrease SBP, and the difference did not reach statistical significance (Figure 4A).

**Effects of WAT Injection of Leptin Antagonist**

Bilateral iWAT and rWAT injection of leptin antagonist in OH decreased the RSNA and MAP peaking at 5 minutes and lasting ≥15 minutes (Figure 4B through 4D).

**Baseline WAT Afferent Nerve Activity**

Baseline WAT afferent nerve activity was evaluated by the percentage change of WAT afferent nerve activity after blocking the WAT nerve at its peripheral end with lidocaine. Lidocaine caused more decrease in OH than ON, OR, or Ctrl and in ON than OR or Ctrl (Figure 5A), suggesting enhanced baseline WAT afferent nerve activity in OH, especially in OH.

**c-fos Expression in PVN**

Injection of capsaicin into WAT increased the c-fos expression in PVN in Ctrl and OH. After injection of capsaicin, the c-fos expression in PVN was increased in OH compared with ON, OR, or Ctrl and in ON compared with Ctrl or OR (Figure 5B). Representative photos of c-fos expression in PVN are shown in the online-only Data Supplement.

**Effects of Lidocaine in PVN**

Bilateral PVN perfusion of lidocaine to inhibit the activity of PVN neurons attenuated baseline RSNA in both OH and ON and decreased baseline MAP and HR in OH. The effect of lidocaine on RSNA was greater in OH than ON (Figure 6A). Lidocaine in the PVN abolished the AAR in all 4 groups (Figure 6B).

**Discussion**

The primary novel findings in the present study are that AAR is enhanced in diet-induced OH, and the enhanced AAR contributes to the sympathetic activation and hypertension. Hypothalamic PVN is an important central site involved in the enhanced AAR and sympathetic activation in OH. Enhanced AAR not only activates the sympathetic nervous system but also increases the circulating renin, Ang II, and NE levels in OH.

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### Table 2. Anatomic Data, Mean Arterial Pressure, and Heart Rate After 12 Weeks of Diet

<table>
<thead>
<tr>
<th>Variables</th>
<th>Ctrl</th>
<th>OR</th>
<th>ON</th>
<th>OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>55</td>
<td>49</td>
<td>46</td>
<td>70</td>
</tr>
<tr>
<td>Initial BW, g</td>
<td>312±2</td>
<td>317±2</td>
<td>313±3</td>
<td>315±2</td>
</tr>
<tr>
<td>Final BW, g</td>
<td>472±6</td>
<td>497±5</td>
<td>627±8†</td>
<td>642±10†</td>
</tr>
<tr>
<td>BW gain, g</td>
<td>160±5</td>
<td>180±4</td>
<td>315±8†</td>
<td>327±10†</td>
</tr>
<tr>
<td>Final BW range, g</td>
<td>365–547</td>
<td>392–546</td>
<td>554–781</td>
<td>552–817</td>
</tr>
<tr>
<td>HW, mg</td>
<td>1571±12</td>
<td>1654±20</td>
<td>2101±28†</td>
<td>2173±27†</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>3.34±0.03</td>
<td>3.33±0.02</td>
<td>3.36±0.03</td>
<td>3.41±0.04</td>
</tr>
<tr>
<td>Inguinal WAT mass, g</td>
<td>8.2±0.2</td>
<td>9.3±0.2</td>
<td>18.0±0.4†</td>
<td>18.2±0.4†</td>
</tr>
<tr>
<td>Retroperitoneal WAT mass, g</td>
<td>7.6±0.2</td>
<td>8.3±0.2</td>
<td>15.8±0.5†</td>
<td>16.4±0.4†</td>
</tr>
<tr>
<td>Epididymal WAT mass, g</td>
<td>5.5±0.1</td>
<td>6.1±0.1</td>
<td>10.1±0.3†</td>
<td>10.3±0.2†</td>
</tr>
<tr>
<td>Mesenteric WAT mass, g</td>
<td>4.6±0.1</td>
<td>5.1±0.1</td>
<td>8.5±0.2†</td>
<td>8.8±0.2†</td>
</tr>
<tr>
<td>Sum of WAT mass, g</td>
<td>25.7±0.4</td>
<td>28.7±0.5</td>
<td>52.4±1.0†</td>
<td>53.7±1.0†</td>
</tr>
<tr>
<td>Adiposity index,%</td>
<td>5.8±0.1</td>
<td>6.1±0.1</td>
<td>9.1±0.1†</td>
<td>9.2±0.1†</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>92.4±1.6</td>
<td>91.8±1.7</td>
<td>98.4±2.1</td>
<td>129.8±2.2†‡</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>334±5</td>
<td>342±6</td>
<td>339±5</td>
<td>387±5†‡</td>
</tr>
</tbody>
</table>

Ctrl indicates control; OR, obesity-resistant; ON, obesity nonhypertension; OH, obesity hypertension; BW, body weight; HW, heart weight; WAT, white adipose tissue. Values are mean±SE. Mean arterial pressure and heart rate were determined under anesthesia.

*P<0.05 vs Ctrl.
†P<0.05 vs OR.
‡P<0.05 vs ON.
It is known that obesity increases the risk of hypertension and cardiovascular diseases, and excessive sympathetic activity contributes to hypertension in obesity.24,25 HFD is widely used to induce obesity and hypertension, and the diet-induced obesity model closely mimics the neurohumoral and hemodynamic changes observed in obese humans.26,27 In the present study, proportions of OR and OP were 29.7% and 70.3% of the total rats. The results confirm the importance of obesity in the development of hypertension. Segregation of OH and ON from OP in the present study is more favorable for the investigation of hypertension in obesity compared with indiscriminate OP. We found that plasma NE level was higher in ON and much higher in OH. Similarly, ganglionic blockade in ON and OH caused greater depressor responses than OR or Ctrl and much greater in OH than ON. These results indicate a sympathetic activation in obesity, especially in hypertension, which is compatible with the findings that SBP decreased more in OH subjects compared with obese normotensive subjects induced by autonomic withdrawal.5

Neuroanatomical studies have shown the sympathetic and sensory innervations of WAT.18,21 The important adipose tissue depot linking obesity with sympathetic activation is abdominal visceral fat rather than subcutaneous fat in humans.30,31 Transient receptor potential vanilloid-1 is a ligand-gated, nonselective, cation channel and a polymodal nocisensor par excellence, being receptive to noxious heat, acidosis, endovanilloids, and a variety of pungent compounds, such as capsaicin, RTX, piperine, gingerol, zingerone, camphor, eugenol, ethanol, and vanillatoxins.32 Some endogenous capsaicin-like substances that activate transient receptor potential vanilloid-I have been identified.33,34 It is noted that the stimulation with capsaicin is not physiological, but the excitatory effects of capsaicin on sensory innervations of WAT.18–21 The important adipose tissue is abdominal visceral fat rather than subcutaneous fat in humans.30,31 Transient receptor potential vanilloid-1 is a ligand-gated, nonselective, cation channel and a polymodal nocisensor par excellence, being receptive to noxious heat, acidosis, endovanilloids, and a variety of pungent compounds, such as capsaicin, RTX, piperine, gingerol, zingerone, camphor, eugenol, ethanol, and vanillatoxins.32 Some endogenous capsaicin-like substances that activate transient receptor potential vanilloid-I have been identified.33,34 It is noted that the stimulation with capsaicin is not physiological, but the excitatory effects of capsaicin on sensory innervations of WAT.18–21 The important adipose tissue is abdominal visceral fat rather than subcutaneous fat in humans.30,31 Transient receptor potential vanilloid-1 is a ligand-gated, nonselective, cation channel and a polymodal nocisensor par excellence, being receptive to noxious heat, acidosis, endovanilloids, and a variety of pungent compounds, such as capsaicin, RTX, piperine, gingerol, zingerone, camphor, eugenol, ethanol, and vanillatoxins.32 Some endogenous capsaicin-like substances that activate transient receptor potential vanilloid-I have been identified.33,34 It is noted that the stimulation with capsaicin is not physiological, but the excitatory effects of capsaicin on sensory innervations of WAT.18–21 The important adipose tissue is abdominal visceral fat rather than subcutaneous fat in humans.30,31 Transient receptor potential vanilloid-1 is a ligand-gated, nonselective, cation channel and a polymodal nocisensor par excellence, being receptive to noxious heat, acidosis, endovanilloids, and a variety of pungent compounds, such as capsaicin, RTX, piperine, gingerol, zingerone, camphor, eugenol, ethanol, and vanillatoxins.32 Some endogenous capsaicin-like substances that activate transient receptor potential vanilloid-I have been identified.33,34 It is noted that the stimulation with capsaicin is not physiological, but the excitatory effects of capsaicin on...
sensory afferents are similar to many exogenous or endogenous chemicals by stimulating transient receptor potential vanilloid-1. Capsaicin is most widely used to investigate the function of afferent fibers as a valuable tool. We have found that stimulation of the WAT afferents with capsaicin, bradykinin, adenosine, or leptin increases the RSNA and MAP in normal rats. The sympato-excitatory reflex, AAR, is involved in the regulation of sympathetic activity and blood pressure. In the present study, capsaicin-induced AAR was enhanced in OP (ON and OH) and the AAR enhancement effect was much greater in OH than ON. Chemical stimulation of the WAT increased plasma NE, renin, and Ang II levels in OH, suggesting that the enhanced AAR not only activates sympathetic nervous system but also increases the activity of renin-Ang system in OH.

RTX is a more potent transient receptor potential vanilloid-1 activator than capsaicin and has been demonstrated to cause degeneration of capsaicin-sensitive afferent neurons in adult rats on systemic administration and is used to deplete capsaicin-sensitive afferent fibers. It is known that inguinal, retroperitoneal, epididymal, and mesenteric fat pads make up the major bulk of total abdominal visceral fat. As shown in Table 2, the iWAT and rWAT mass account for more than half of visceral fat mass. Bilateral injection of RTX into iWAT and rWAT caused immediate and short-term increases in RSNA and MAP, and the responses to RTX were greater in OH than in other groups of rats. The excitatory effects of RTX can be explained as stimulating WAT afferents, which is similar to the capsaicin-induced AAR. It is particularly worth noting that RTX caused long-lasting decreases in RSNA and MAP in OP, especially in OH attributed to the abolishment of enhanced AAR in these rats. Furthermore, WAT afferent activity was
enhanced in OP, especially in OH. These results indicate that the increased WAT afferent activities and enhanced AAR contribute to the excessive sympathetic activation and hypertension in obesity. The depressor effects of RTX in OH lasted ≥3 weeks. The recovery of blood pressure may be involved in a compensatory increase of WAT afferent activity of non-RTX-treated WAT, which is supported by the fact that surgical removal of body fat (partial lipectomy) triggers compensatory increases in nonexcised WAT mass and regrowth of excision sites. Capsaicin failed to induce AAR at the end of the fifth week, suggesting that the recovery of blood pressure is independent of the reinnervation of the denervated WAT. Leptin antagonist resulted in mild decreases in sympathetic activity and blood pressure in OH, suggesting that leptin is one of the chemicals involved in the signal from adipocytes that leads to afferent activation.

PVN is an important integrative site in regulating sympathetic outflow and cardiovascular activity. In the present study, c-fos expression was used as a marker of neuronal activation. Injection of capsaicin into iWAT caused more c-fos expression in the PVN in OH than ON, OR, and Ctrl and in ON than OR or Ctrl. These results suggest that the response of PVN neurons to chemical stimulation of the WAT was enhanced in OP, particularly greatly enhanced in OH. The connection between WAT and PVN was supported by the finding that pseudorabies virus–infected neurons were found in the PVN after injection of trans-synaptic retrograde tracer into the WAT. Lidocaine is commonly used as a tool for reversible functional inactivation in discrete brain areas. We found that PVN perfusion of lidocaine abolished the AAR in all groups and decreased the RSNA in both OH and ON but reduced the MAP and HR only in OH. These data reinforce the conclusion drawn from the RTX that the enhanced AAR contributes to the sympathetic activation and hypertension in obesity. The results also indicate the vital role of the PVN in the enhanced AAR and sympathetic activity in OH. It has been found that Ang II type 1a receptors of Ang II in the PVN are involved in the sympathetic activation in hypertension. Redox signaling in the brain is recognized in neuronal Ctrl of the sympathetic activation in discrete brain areas. In OH, the enhanced AAR and sympathetic activity in obesity hypertension. The discovery shows the fundamental role of the enhanced AAR in sympathetic activation and hypertension in obesity. Furthermore, the results indicate 2 effective targets (the WAT afferents and PVN neurons) at counteracting the enhanced AAR. Intervention of the enhanced AAR may be a strategy for attenuating obesity hypertension.

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**Disclosures**

None.

**References**


Novelty and Significance

**What Is New?**

- AAR, which is caused by stimulation of white adipose tissue and results in sympathetic activation and pressor response, is enhanced in obesity hypertension.
- White adipose tissue sensory denervation or paraventricular nucleus inhibition causes more decreases in sympathetic activity and blood pressure in obesity hypertension.
- Stimulation of white adipose tissue afferents increases plasma renin, angiotensin II, and norepinephrine levels and c-fos expression in paraventricular nucleus.

**What Is Relevant?**

- Better understanding of sympathetic activation in obesity hypertension.
- Inhibiting the enhanced AAR could favorably prevent sympathetic activation and hypertension in obesity.

**Summary**

- White adipose tissue afferents and paraventricular nucleus are targets counteracting the enhanced AAR and sympathetic activity in obesity hypertension.
- Enhanced AAR contributes to sympathetic activation, and paraventricular nucleus is involved in the enhanced AAR and sympathetic activation in obesity hypertension.
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Enhanced Adipose Afferent Reflex Contributes to Sympathetic Activation in Diet-induced Obesity Hypertension

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Supplemental Methods

Animal Models of Obesity Hypertension

Male Sprague-Dawley normotensive rats weighing between 300-350 g were randomly divided into four groups. The rats in one group received a control diet (Ctrl, 12% kcal as fat, n=55), and rats in other three groups were mixed together and received a high fat diet (HFD, 42% kcal as fat, n=165) for 12 weeks. All rats were housed in a temperature and humidity-controlled room with a 12-hour light/dark cycle and were given food and water ad libitum. After 12 weeks, rats consuming the HFD were ranked based on weight gain. Rats with weight gains equal to or less than the heaviest control rats were defined as obesity-resistant rats (OR), and rats with greater weight gains were defined as obesity-prone rats (OP). Then, OP rats were ranked based on systolic blood pressure (SBP). The OP rats with SBP equal to or more than 150 mm Hg were referred to as obesity hypertensive rats (OH). The OP rats with lower SBP were obesity non-hypertensive rats (ON) including obesity hypertension-prone rats (between 140 and 150 mm Hg) and obesity normotensive rats (less than 140 mm Hg). The rats with control diet were used as control (Ctrl). The experimental procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Measurement of Body Weight and SBP

Body weight (BW) and SBP were measured in a conscious state at weekly intervals. SBP of tail artery was measured with a non-invasive computerized tail-cuff system (NIBP, ADInstruments, Sydney, Australia). Before the measurements, the rats were warmed for 10–20 min at 28℃ in order to allow detection of tail artery pulsations and to achieve a steady pulse level. SBP was obtained by averaging 10 measurements as previously reported.

General Procedures of Acute Experiment

Acute experiment was carried out at the end of the 12th week after HFD or control diet. The rats were anesthetized with urethane (800 mg/kg) and α-chloralose (40 mg/kg) intraperitoneally. Supplemental doses of anesthetic agents were administered intravenously to maintain an adequate depth of anesthesia during the experiment. A midline incision was made to expose the trachea and carotid artery. The trachea was cannulated and connected with a rodent ventilator (Model 683, Harvard Apparatus Inc, USA). The right carotid artery was cannulated for recording of mean arterial pressure (MAP) and heart rate (HR). The rats were euthanized at the end of the experiment. The heart and the epididymal, inguinal, mesenteric and retroperitoneal fat pads were harvested and weighed. The adiposity index is calculated as total mass of fat pads × 100 / (body weight - total mass of fat pads).

RSNA recording

Renal sympathetic nerve activity (RSNA) was recorded as previously reported to evaluate the dynamic changes of sympathetic outflow. Simply, the left renal sympathetic nerve was isolated and cut distally to abolish its afferent activity. The nerve was placed on a pair of silver electrodes and immersed in mineral oil. The RSNA was amplified with an AC/DC differential amplifier (Model 3000; A-M System, Washington, DC, USA) and filtered with a band-pass between 60 and
3000 Hz. The amplified and filtered signals were integrated at the time constant of 100 ms. The RSNA, MAP and HR were simultaneously recorded with a PowerLab data acquisition system (8/35, ADInstruments, Sydney, Australia) and stored on a hard disk until analyzed. The baseline noise of the recorded signals was determined after section of the central end of the nerve at the end of experiment and subtracted from the integrated RSNA values. Nerve activity was expressed as the percent change from the baseline value.

**WANA recording**

One of nerves that innervate the right inguinal WAT (iWAT) was isolated, and cut at its central end to abolish its efferent activity to the WAT. The nerve was placed on a pair of silver electrodes and fixed with silicone (Kwik-Sil, WPI Inc., Sarasota, FL, USA). The distal end of the nerve was separated from other tissues with a rubber latex film. WAT afferent nerve activity (WANA) was recorded similarly to the RSNA recording mentioned above.

**Evaluation of AAR**

The AAR was induced as previously reported.\(^{10}\) The right iWAT was exposed through an inguinal area incision. Four thin and sharp stainless steel tubes (0.31 mm outer diameter) were inserted into the fat pad 3 mm below the surface of the fat pad. The tips of these tubes were 4 mm apart from each other and were connected with a 4-channel programmable pressure injector (PM2000B, MicroData Instrument, NJ, USA). The AAR was induced by the injections of capsaicin (1.0 nmol/μl) into four sites of the right iWAT at a rate of 4.0 μl/min for 2 min for each site. AAR was evaluated by the RSNA and MAP responses to injections of capsaicin. At the end of the experiment, the same volume of Evans blue was injected into the iWAT. Histological identification of the WAT was made 30 min later. The dye was localized in the WAT and the diffusion diameter was less than 3 mm in all rats.

**PVN Perfusion**

The rat was fixed in a stereotaxic frame as previously reported\(^{10}\) and two glass micropipettes (50 μm tip diameter) were inserted into the PVN bilaterally. Stereotaxic co-ordinates for the PVN were 1.8mm caudal to bregma, 0.4mm lateral to the mid-line and 7.9mm ventral to the dorsal surface according to the stereotaxis atlas of the rat brain.\(^{11}\) The perfusion of lidocaine into the bilateral PVN was carried out with a dual-channel microdialysis infusion syringe pump (53101V, Stoelting Co., Illinois, USA) at an infusion rate of 10 nl/min for 30 min. The dose of lidocaine was 30 nmol in 300 nl for each side of the PVN. Lidocaine infused at the rate and volume does not cause a diffusion over the range and a discernible tissue damage on histological evaluation based on many studies using higher rates/volumes.\(^{12-16}\) At the end of the experiment, the same volume of Evans Blue dye was perfused into the perfusion site. The histological identification was used to confirm that the dye was localized in the PVN. One rat was excluded for analysis because the perfusion site was outside the PVN.

**c-fos Immunohistochemistry**

The rats were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) and perfused transcardially with 0.01 M phosphate-buffered saline (PBS, 37°C) followed with 4% paraformaldehyde (4°C). Subsequently, the brain was removed and postfixed in
paraformaldehyde overnight at 4°C, then dehydrated in 20% and 30% sucrose at 4°C for 24 h. Coronal sections (30 μm) were cut on a cryostat and placed in PBS (4°C). After washed in 0.3% Triton X-100 and 3% H2O2, the free-floating sections were incubated in 10% normal goat serum at 37°C for 1 h. Sections were incubated with rabbit anti-rat c-fos polyclonal antibody (Santa Cruz) diluted 1:40 in a solution of 1.5% goat serum at 4°C overnight. Sections were washed in PBS prior to incubation in biotinylated secondary antibody (ABC staining system kit, Santa Cruz) in PBS containing 1.5% goat serum for 30min at 37°C. Thereafter, sections were incubated in AB reagents for 30 min at 37°C and peroxidase substrate was used according to the manufacturer’s descriptions of the ABC kit. Following final washes, the sections were mounted on gelatine coated microscope slides.17 Bright-field illumination using a microscope and Image-Pro Plus 6.0 (IPP6.0), were used to assess sections that exhibited c-fos immunoreactivity as detected by black stained nuclei. Bilateral cell counts were averaged for 4 sections of the PVN between 1.8 mm and 2.1 mm behind the bregma.

Measurement of Plasma Renin, Angiotensin II and norepinephrine

Plasma renin, angiotensin II (Ang II) and norepinephrine (NE) were measured with commercial ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s descriptions. The 96-well plates were incubated with antibodies specific for rat renin, Ang II or NE, respectively. Samples and standard diluent buffer were then added, incubated and washed. Horseradish peroxidase-conjugated solution was added and then washed out. The reactions were stopped with stop solution and the final solution was read at 450 nm using a microplate reader (ELX800, BioTek, Vermont, USA).18

Experimental design

All rats received a control diet or HFD for 12 weeks. BW and SBP were measured in a conscious state at weekly intervals. At the end of the 12th week, acute experiments were carried out except the rats in Experiment 5, in which acute experiment was done after another period of 5 weeks. At the beginning of an acute experiment, MAP and HR were recorded in each rat. At the end of the experiment, epididymal, inguinal, mesenteric and retroperitoneal fat pads as well as the heart were harvested and weighed in all rats. The adiposity index is calculated as total WAT mass / (body weight – total WAT mass) and expressed as a percentage. Histological identifications for WAT injection sites and PVN injection sites were carried out in all the rats subjected to corresponding injections.

Experiment 1: Basal sympathetic tone was determined in Ctrl, OR, ON and OH (n=8 for each group). Blood samples were collected from the carotid artery for measuring the plasma NE. Then, maximal depressor response of intravenous injection of ganglionic blockade, hexamethonium hydrochloride (30 mg/kg), was used as another index of sympathetic activity.19

Experiment 2: AAR was evaluated by the RSNA, MAP and HR responses to capsaicin in Ctrl, OR, ON and OH (n=6 for each group). Each rat was randomly subjected to injections of vehicle and capsaicin into the right iWAT. The interval between the vehicle and capsaicin injections was at least 60 min for a complete recovery.

Experiment 3: Changes of plasma renin, Ang II and NE levels caused by WAT injection of capsaicin to induce AAR were determined in OH (n=8). Each rat was subjected to the right iWAT
injections of vehicle, capsaicin and vehicle in turn. The intervals between injections were at least 60 min for complete recovery. The blood samples were collected for measurements 15 min after each injection.

Experiment 4: Effects of WAT sensory denervation with resiniferatoxin (RTX) on the RSNA, MAP and HR were determined in Ctrl, OR, ON and OH (n=12 for each group). The rats in each group were randomly divided into RTX and vehicle (Veh) subgroups (n=6 for each subgroup), which were subjected to injections of RTX (20.0 pmol/μl) or vehicle into bilateral retroperitoneal WAT (rWAT) and iWAT (8.0 μl per site, 4 sites for each WAT pad), respectively. AAR induced by capsaicin in the right iWAT was determined 120 min later to confirm the effectiveness of the WAT sensory denervation.

Experiment 5: Long-term depressor effect of WAT sensory denervation with RTX was determined in a conscious state of OH. Rats were randomly divided into RTX and vehicle (Veh) groups (n=6 for each group), which were subjected to injections of RTX (20.0 pmol/μl) or vehicle into bilateral rWAT and iWAT (8.0 μl per site, 4 sites for each WAT pad). SBP was determined before and after the injections for 5 weeks. Effectiveness of the WAT sensory denervation was confirmed by the failure to induce AAR by injection of capsaicin into the right iWAT at the end of the 5th week after injection of RTX.

Experiment 6: Effects of WAT injection of leptin antagonist on the RSNA, MAP and HR were investigated in OH (n=6). Each rat was randomly subjected to injections of saline and leptin antagonist triple mutant rat recombinant (LA, 1.0 μg/μl) into bilateral rWAT and iWAT (8.0 μl per site, 4 sites for each WAT pad). The interval between saline and LA injections was at least 100 min for a complete recovery. The effectiveness of the LA dose was confirmed by the fact that pretreatment with this dose of LA in the right iWAT abolished the AAR induced by leptin (0.075 pmol/μl, 8.0 μl per site, 4 sites for each WAT pad) in the right iWAT in Ctrl (n=4). The dose of leptin had been used to induce AAR in normal rats as previously reported.\(^{10}\)

Experiment 7: WAT afferent nerve activity (WANA) was recorded for comparison of basal WANA among Ctrl, OR, ON and OH (n=6 for each group). Lidocaine is widely used to block the nerve conduction.\(^{20-24}\) WAT nerve was cut at its central end to abolish its efferent activity to the WAT. The maximal change of WANA caused by the topical application of small pieces of filter paper (4×4 mm) soaked in 2% lidocaine solution on the distal end of the nerve was used to evaluate the basal WANA level. A greater decrease in WANA after application of lidocaine represents a high basal WANA level.

Experiment 8: Change of c-fos immunoreactivity in the PVN caused by WAT injection of capsaicin to induce AAR was investigated in Ctrl, OR, ON and OH (n=3 for each group). The c-fos expression was used as a marker of neural activation. Injection of vehicle or capsaicin into bilateral iWAT was carried out with five replicates. The intervals between the injections were 20 min. The c-fos immunoreactivity in the PVN was determined 10 min after the 5th injection of capsaicin.

Experiment 9: Effects of inhibition of PVN neurons with lidocaine on the baseline RSNA, MAP, HR and AAR were investigated in Ctrl, OR, ON and OH (n=6 for each group). Each rat was subjected to the PVN perfusion of the saline, lidocaine (30 nmol) and saline in turn. Each
perfusion lasted for 30 min. The AAR induced by the iWAT injection of capsaicin was determined 10 min after the beginning of each perfusion. Intervals between the PVN perfusions were at least 60 min for complete recovery.

References


Supplemental Figures

Figure S1. Representative recordings showing the AAR induced by injection of capsaicin into the iWAT in Ctrl, OR, ON and OH. The AAR was enhanced in ON and OH compared with OR or Ctrl, and especially in OH compared with ON. Int. RSNA, integrated RSNA.

Figure S2. Representative photos showing c-fos positive neurons in the PVN after iWAT injection of vehicle (Veh) or capsaicin (Cap) in Ctrl, OR, ON and OH. Injection of capsaicin into iWAT increased the c-fos expression in PVN. The c-fos expression in PVN was increased in OH compared with ON, Ctrl or OR, and in ON compared with Ctrl or OR after injection of capsaicin into iWAT.