Sympathetic activation by chemical stimulation of white adipose tissues in rats

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The sympathetic nerve innervation of the WAT may involve contact with adipocytes and/or their associated vasculature (3, 4). After injection of pseudorabies virus (PRV), a trans-synaptic retrograde tracer tract into the inguinal WAT (iWAT) of rats or eWAT or iWAT of Siberian hamsters, PRV-infected cells were found throughout the neural axis including the intermediolateral nucleus (ILM), nucleus of the solitary tract (NTS), rostral ventrolateral medulla (RVLM), hypothalamic paraventricular nucleus (PVN), and several other areas (2). Similar distribution of PRV-infected cells in the brain was observed after injection of PRV into the retroperitoneal WAT (rWAT), iWAT, or eWAT of Siberian hamsters (8). However, no neuroanatomical evidence for parasympathetic nerve innervation of WAT was found (6). On the other hand, a sensory WAT pathway to the brain has been well documented. After implantation of an anterograde neuroanatomical tracer, true blue, into the iWAT or dorsal subcutaneous fat depots, fluorescent cell bodies were observed in dorsal root ganglia of rats (16). By using the herpes simplex virus-1 (HSV-1), an anterograde trans-neuronal viral tract tracer, the afferent circuits projecting from iWAT or eWAT to the brain related to modulating sympathetic nerve activity were defined, including the PVN, RVLM, and NTS in Siberian hamster (33).

Although much neuroanatomical evidence supports a close bidirectional connection between the WAT and the brain, little is known about the AAR. The present study was designed to determine whether other chemicals besides leptin can stimulate the WAT afferents to cause the AAR and whether stimulation of other WAT such as iWAT or rWAT besides pWAT can reflexly increase the RSNA, WAT efferent nerve activity (WENA), and BAT efferent nerve activity (BENA). Furthermore, the role of the WAT afferent fibers and the PVN in the AAR was determined.

MATERIALS AND METHODS

Experiments were carried out in male Sprague-Dawley rats weighing between 300 and 400 g. The 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). The rat was anesthetized by intraperitoneal injection of urethane (800 mg/kg) and α-chloralose (40 mg/kg). Supplemental doses of anesthetic agents were administered intravenously to maintain an adequate depth of anesthesia during the experiment. The rat was ventilated with room air using a rodent ventilator (683, Harvard Apparatus). The right carotid artery was cannulated for recording of arterial blood pressure and heart rate (HR).

Chemical stimulation of WAT to induce AAR. The right iWAT or rWAT was exposed through an inguinal area incision or an extraperitoneal longitudinal 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 four tubes were 4 mm apart from each other and were connected with four microinjectors by PE50 polyethylene cannulas, respectively. The total injection volume was 8.0 µl (2.0 µl for each injection site). The injections were completed within 2 min at a rate of 1.0 µl/min using a syringe infusion pump. The histological identification of the WAT was made 30 min after injection of same volume of Evans blue into the WAT. The dye was localized in the WAT, and the diffusion diameter was less than 3 mm.

**Recording of nerve activities.** The nerve activity recordings were reported previously in our lab (21, 40). The nerves innervating the left kidney, iWAT, and interscapular BAT were isolated and cut at their distal end for RSNA, WENA, and BENA recordings, respectively. The nerve innervating the right iWAT was isolated and cut at its central end for the recording of WAT afferent nerve activity (WANA). The nerves were placed on silver recording electrodes and then covered with a fast-setting silicone (Kwik-Sil, World Precision Instruments, Sarasota, FL). The signals were amplified with an AC/DC differential amplifier (model 3000, A-M System) with low-frequency cut-off at 60 Hz and high-frequency cut-off at 3,000 Hz. The amplified and filtered signals were integrated at time constant of 0.1 s. The background noise was determined after section of the other end of these nerves at the end of the experiment. The raw nerve activities, integrated nerve activities, mean arterial pressure (MAP), and HR were recorded on a PowerLab data-acquisition system (8SP; ADInstruments). All nerve activities were expressed as the percent change from the baseline value.

**Denervation of iWAT.** The right iWAT was isolated without damaging the vessels. A drop of 1% toluidine blue was applied to the fat pad to facilitate visualization of the nerves. All nerve fibers that were visible in these areas were cut (17). The vessels connected with the iWAT were painted with 10% phenol solution to destroy any remaining nerve fibers in this area.

**Measurement of substance P and norepinephrine in iWAT.** The iWAT was homogenized in the lysis buffer and centrifuged at 13,000 rpm at 4°C for 30 min. The protein concentrations in the supernatants were separately determined with the SP and NE ELISA kits (R&D systems, Minneapolis, MN). The absorbance (450 nm) was monitored, and the SP and NE levels in the supernatants were measured using protein assay kit (BCA, Pierce, Rockford, IL). The substance P (SP) and norepinephrine (NE) levels in the supernatants were measured using protein assay kit (BCA, Pierce, Rockford, IL). The absorbance (450 nm) was measured with a microplate reader (Eix 800, Bio-Tek Instrument, Horsham, PA).

**PVN microinjection.** The coordinates of the PVN are 1.8 mm caudal from bregma, 0.4 mm lateral to the midline, and 7.9 mm ventral to the dorsal surface. Microinjection into the PVN was performed with a glass micropipette (50 µm tip diameter). The bilateral PVN microinjections were completed within 1 min with 50 nl of volume for each side of the PVN (9).

**Experimental protocols.** First, to determine the RSNA and MAP changes induced by different doses of capsaicin in the iWAT, the rats were subjected to the right iWAT injection of saline, 0.25, 1.0, or 4.0 nmol/µl of capsaicin (n = 6 for each group).

Second, to determine the RSNA and MAP changes induced by different chemicals in the iWAT, the rats were subjected to the right iWAT injection of saline, capsaicin (4.0 nmol/µl), bradykinin (0.15 nmol/µl), adenosine (3.5 nmol/µl), adenosine-triphosphate (ATP; 3.5 nmol/µl), or leptin (0.3 pmol/µl) (n = 6 for each group).

Third, to compare the RSNA and MAP changes induced by capsaicin in iWAT with that in rWAT and to exclude the possible diffusion effects of capsaicin, the rats were subjected to injection of capsaicin (4.0 nmol/µl) into the right iWAT, right rWAT, jugular vein, skeletal muscle, or skin adjacent to the right iWAT (n = 6 for each group).

Fourth, to determine whether the iWAT stimulation-induced AAR was mediated by the iWAT afferents, the RSNA and MAP responses to capsaicin (4.0 nmol/µl) in the denervated iWAT or sham-denervated iWAT were determined (n = 6). To confirm the effectiveness of the denervation, the SP and NE levels of the iWAT were determined 1 wk after the iWAT denervation or sham denervation (n = 6).

Fifth, to determine the effect of PVN lesions on the AAR, the right iWAT injection of capsaicin (4.0 nmol/µl) was carried out 120 min after the PVN microinjection of saline or kainic acid (KA; 2 nmol) in rats (n = 6 for each group). To identify the microinjection sites and diffusion, 50 nl of 2% Evans blue was injected into each microinjection site at the end of the experiment for histological identification. One rat was excluded from the data analysis because the microinjection site was close to the boundary of the left PVN and some dye was out of the range of the PVN. To exclude the possible diffusion effect of KA, the effect of microinjection of KA into the anterior hypothalamic area, which is adjacent to the PVN, on the AAR was determined (n = 3). To confirm the PVN lesion, toluidine blue staining of brain sections (27) was carried out 2 days after the PVN microinjection of saline or KA (n = 5 for each group).

Last, to determine the changes of the WAT afferent activity and the changes of efferent activity to the WAT and BAT during the AAR, the effects of saline or capsaicin (4.0 nmol/µl) in the right iWAT on the right WANA, left WENA, and left BANA were determined, respectively (n = 6 for each group).

**Drugs.** Capsaicin, bradykinin, leptin, adenosine, ATP, and KA were obtained from Sigma Chemical.

**Statistics.** The RSNA and MAP changes were determined by averaging 2 min of their maximal responses. Comparisons between two observations in the same animal were assessed by Student’s paired t-test. One-way ANOVA followed by the Bonferroni’s test for post hoc analysis was used when multiple comparisons were made. A linear regression analysis was made to determine the correlation between the doses of capsaicin and the RSNA or MAP changes. All data were expressed as means ± SE. A value of P < 0.05 was considered statistically significant.

## RESULTS

**Time-effect relationship of capsaicin in iWAT.** Injection of capsaicin (4.0 nmol/µl) into the iWAT significantly increased the RSNA in the period of 5–25 min and MAP in the period of 5–20 min but had no significant effect on the HR. The RSNA change reached its maximum of −18% at 10–15 min, whereas the MAP change reached its maximum of −4 mmHg at 5–10 min. Injection of the same volume of saline into the iWAT had no significant effect on the RSNA, MAP, and HR (Fig. 1A). The representative recordings showed that the iWAT injection of capsaicin increased the RSNA and MAP (Fig. 2A).

**Dose-effect relationship of capsaicin in iWAT.** Injection of capsaicin into the iWAT increased RSNA and MAP in a dose-dependent manner. Moderate dose and high dose of capsaicin significantly increased the RSNA and MAP (Fig. 1B).

**Effects of other chemicals in iWAT.** Injection of bradykinin, adenosine, and leptin into the iWAT caused similar increases in RSNA and MAP as the injection of capsaicin, but injection of ATP into the iWAT had no significant effect on the RSNA and MAP (Fig. 3).

**Effects of capsaicin in rWAT, vein, adjacent muscle, and skin.** The rWAT injection of capsaicin caused similar increases in the RSNA and MAP to the iWAT injection of capsaicin. Intravenous injection or intramuscular injection of the same dose of capsaicin into the adjacent skeletal muscle had no significant effect on the RSNA and MAP. Intradermal injection of capsaicin into the near or remote skins only caused a tendency to increase the RSNA slightly, but these changes did not reach statistical significance (Fig. 4).
Effects of iWAT denervation on AAR. Unilateral iWAT denervation had no significant effect on the baseline RSNA and MAP, but completely abolished the RSNA and MAP responses to the injection of capsaicin into the denervated iWAT (Fig. 5A). The surgical denervation of the iWAT decreased the levels of SP, a sensory nerve marker, and NE, a sympathetic nerve marker, in the iWAT (Fig. 5B).

Effects of PVN lesion with KA on AAR. Our previous study found that the bilateral PVN microinjection of KA caused immediate and great increases in the baseline RSNA and MAP, which recovered in 40–60 min and then remained at the baseline levels (40). Similar baseline RSNA and MAP changes were observed in the present study. The PVN microinjection of KA abolished the RSNA and MAP responses to the injection of capsaicin into the iWAT at the 120th min after KA (Fig. 6A). At the end of the experiment, microinjection of Evans blue into each microinjection site was carried out. The visible extent of dye spread was less than 0.30 mm in diameter. Similar dye spread in the PVN and histological observation in our lab have been reported previously (9, 21). Microinjection of KA into the anterior hypothalamic area that is adjacent to the PVN caused the similar baseline RSNA and MAP responses to KA in the PVN, but failed to abolish the AAR. The toluidine blue staining of the brain sections showed the number of neurons in the PVN decreased 2 days after KA microinjection (Fig. 6B). These findings confirmed the effectiveness of the PVN lesion.

Effects of capsaicin in iWAT on WANA, WENA, and BENA. Injection of capsaicin into the iWAT significantly increased the WANA, WENA, BENA, and RSNA. However, capsaicin caused greater increases in the WENA and BENA than in the RSNA (Figs. 2 and 7).

DISCUSSION

Sympathetic activation is common in overweight subjects, obese, and obesity-related hypertensive patients (13). The plasma NE and muscle sympathetic nerve activity (MSNA) are increased in obese compared with lean individuals (20). Weight loss decreases the sympathetic activity (35). The increased fat is a very important factor contributing to the
sympathetic activation in obese and obesity-related hypertension (12, 22, 29, 35). Neuroanatomical studies have shown bidirectional pathways between the WAT and brain (4, 6). It has been found that leptin in the eWAT reflexly increases the efferent activity of sympathetic nerve innervating bilateral eWAT (26) and leptin in the pWAT increases the RSNA (37). The reflexly sympathetic activation may be beneficial to increase energy expenditure, accelerate lipolysis, and reduce body weight (26). There is a possibility that the abnormal reflex is partially involved in the pathogenesis of sympathetic activation in obesity, metabolic syndrome, and obesity-related hypertension. It is important to realize the sympathoexcitatory reflex induced by chemical stimulation of the WAT [adipose afferent reflex (AAR)].

It is known that capsaicin causes neuronal excitation, although high concentration of capsaicin may cause damage of the sensory neurons (7, 31). Low concentration of capsaicin is usually used to determine the function of sensory afferents (19, 30, 38). The capsaicin in the present study was used to stimulate sensory afferents, and its concentration was much lower than the concentration that causes damage of the sensory neurons. The iWAT injection of capsaicin resulted in dose-related increases in the RSNA and MAP. Capsaicin, bradykinin, leptin, or adenosine in the iWAT caused similar RSNA and MAP effects as capsaicin. These results indicate that several chemicals in the iWAT, not merely leptin, can induce the AAR. Although ATP can be converted to adenosine in the tissue, ATP in the iWAT failed to induce the AAR. The results show that ATP had no effects on stimulating the iWAT afferents. A possible explanation is that little ATP was actually converted to adenosine. On the other hand, injection of capsaicin into the rWAT also caused similar increases in RSNA and MAP. We consider that the AAR can be induced by stimulating the WAT in many places of the body, which was supported by previous reports that leptin in pWAT increased the RSNA (37) and leptin in eWAT increased the efferent activity of sympathetic nerve innervating BAT, adrenal medulla, pancreas, and liver (RSNA was not observed) (25). Intravenous, intramuscular, or intradermal injection of capsaicin had no significant effects on the RSNA and MAP, indicating that the reflex was induced by chemical stimulation of the WAT, and the possible diffusion effects of capsaicin could be excluded.

The iWAT injection of capsaicin increased the activity of iWAT afferent nerve fibers, whereas unilateral iWAT denervation abolished the effects of capsaicin in the denervated iWAT. These results indicate that the AAR induced by capsaicin in the iWAT was mediated by the iWAT afferent nerve fibers rather than some chemicals released from WAT to the circulation, which is supported by the findings that the pWAT injection of leptin increases the RSNA without significant effect on circulating leptin, insulin, glucose, and lactate (37). The effectiveness of the iWAT denervation in the present study was confirmed by the reduced SP and NE levels in the iWAT. A small quantity of SP and NE left in the denervated iWAT may have originated from circulating SP and NE.

Hypothalamic PVN is an important integrative site in the control of sympathetic outflow and cardiovascular activity via sympathetic afferents in obese and obesity-related hypertension (12, 22, 29, 35). Neuroanatomical studies have shown bidirectional pathways between the WAT and brain (4, 6). It has been found that leptin in the eWAT reflexly increases the efferent activity of sympathetic nerve innervating bilateral eWAT (26) and leptin in the pWAT increases the RSNA (37). The reflexly sympathetic activation may be beneficial to increase energy expenditure, accelerate lipolysis, and reduce body weight (26). There is a possibility that the abnormal reflex is partially involved in the pathogenesis of sympathetic activation in obesity, metabolic syndrome, and obesity-related hypertension. It is important to realize the sympathoexcitatory reflex induced by chemical stimulation of the WAT [adipose afferent reflex (AAR)].

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KA can be used as a tool to destroy neuronal perikarya selectively without damage of axons of passage and terminals (11, 23). At the KA injection site, the neurons start firing immediately at a very high rate after injection of KA but no neuronal firing can be observed 1 h later (23). Previous study in our lab showed that the bilateral PVN microinjection of KA caused immediate and great increases in the baseline RSNA and MAP, which recovered in 40–60 min and then remained at the baseline levels. The KA-induced PVN lesions were confirmed by the prominent vacuolation and dilatation of the mitochondria in the neuronal perikarya of the PVN at the 100th min after KA (40). An important finding in the present study was that microinjection of KA into the PVN abolished the AAR induced by the iWAT injection of capsaicin when the RSNA and MAP had returned to the baseline level 120 min after microinjection of KA. The results indicate that the PVN neurons mediate the AAR. The results are supported by the findings that the PRV-infected cells were found in the PVN after injection of the PRV, a retrograde trans-neuronal viral tract tracer, into the WAT (2) and that the HSV-1-infected cells were found in the PVN after the WAT injection of HSV-1, an anterograde trans-neuronal viral tract tracer (33). The bidirectional pathways between the PVN and the WAT support the important role of the PVN in regulating the AAR. However, these virus-infected cells were also observed in IML, NTS, and RVLM. The relationship of the PVN with other nuclei or regions involved in the control of AAR-related sympathetic activation needs to be further investigated.

It is interesting that an intact PVN is not necessary for food deprivation-induced lipid mobilization (18). The result is not a conflict with present findings because the food deprivation-induced lipid mobilization is only a special condition. It has been reported that the PVN electrolytic lesions increase body and WAT pad masses compared with sham-treated Siberian

**Fig. 5.** Effects of the iWAT denervation on adipose afferent reflex (AAR), substance P (SP) level, and norepinephrine (NE) level in the iWAT. A: right iWAT denervation abolished the RSNA and MAP responses to capsaicin in the denervated iWAT; B: right iWAT denervation decreased the SP and NE levels in the denervated iWAT. Values are means ± SE. *P < 0.05 compared with sham-treated rats. n = 6 for each group.

**Fig. 6.** Bilateral paraventricular nucleus (PVN) lesions with kainic acid (KA). A: effects of PVN microinjection of saline and KA on the RSNA and MAP responses to capsaicin in the right iWAT. Values are means ± SE. *P < 0.05 compared with saline. n = 6 for each group. B: representative sections showing the PVN lesion. Compared with saline, KA treatment decreased the number of neurons in the PVN.

**Fig. 7.** Effects of capsaicin in the iWAT on the WANA, WENA, brown adipose tissue efferent nerve activity (BENA), and RSNA. Right iWAT injection of capsaicin increased the homolateral WANA, contralateral WENA, BENA, and RSNA. Values are means ± SE. *P < 0.05 compared with saline; †P < 0.05 compared with WENA or BENA. n = 6 for each group.
hamsters and bilateral PVN lesions cause more increases in food intake, body, and WAT pad masses than unilateral WAT lesion. The sympathetic innervation of WAT, rather than adrenomedullary catecholamines, is important for lipid mobilization from WAT (18). These results suggest the importance of the PVN in control of WAT mass, which support our findings that the PVN is involved in the AAR-related sympathetic activation.

A general outline of the neural pathway of the AAR is postulated as follows. Capsaicin in the iWAT stimulates its sensory nerve endings to increase the activities of the jWAT. The PVN is involved in the AAR via its projections to the RVLM and IML to increase the RSNA and blood pressure. However, the speculated pathway of AAR needs further experimental evidence. The iWAT injection of capsaicin increased the efferent activity of the sympathetic nerve innervating the contratral iWAT, BAT, and kidney, which is similar to previous reports that unilateral eWAT injection of leptin increases the efferent activity of the sympathetic nerve innervating the bilateral eWAT (26), BAT, adrenal medulla, pancreas, and liver (25). It appears that the AAR induced by the chemical stimulation of the WAT widely activates the sympathetic nervous system. It is well documented that the sympathetic innervation of the WAT is a principal initiator of lipolysis (5, 28, 34). We expect that an important role of the AAR is to accelerate lipolysis, increase energy expenditure, and thereby reduce body weight.

Chronic activation of the sympathetic nervous system is the dominant contributor to systemic hypertension (14, 15). The excess weight gain, especially when associated with increased visceral adiposity, is associated with increased sympathetic activation, and the overweight category is almost invariably associated with increased risk for hypertension (12, 22, 29, 36). Combined administration of α- and β-adrenergic receptor blockers caused a greater decrease in blood pressure in obese hypertensive subjects than that in lean hypertensive subjects (39). Application of the ganglionic blocker caused a greater reduction in blood pressure in obese subjects than that in normal-weight subjects (32). These results indicate that the enhanced sympathetic nerve activity plays an important role in obesity-related hypertension. An ongoing study in our lab has shown that the AAR is involved in the sympathetic activation and hypertension in obese rats. On the other hand, activation of the intrarenal renin-angiotensin system may be an important contributor to systemic hypertension (24). It is interesting to investigate whether the AAR is involved in the activation of the intrarenal renin-angiotensin system for further study.

In summary, stimulation of iWAT afferents with capsaicin, bradykinin, adenosine, or leptin reflexly increases the RSNA and blood pressure. The WAT afferents and the PVN are involved in the AAR induced by capsaicin in the WAT.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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