Carotid baroreceptor stimulation in obese rats affects white and brown adipose tissues differently in metabolic protection

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Running Title: Effects of CBS on Obesity.

Abbreviations: CBS, carotid baroreceptor stimulation; WAT, white adipose tissue; BAT, brown adipose tissue; PWAT, perirenal white adipose tissue; EWAT, epididymal white adipose tissue; IBAT, interscapular brown adipose tissue; SNS, sympathetic nerve system; AUCs, areas under the curves; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BMI, body mass index; NE, norepinephrine; ACH, norepinephrine; Ang-II, angiotensin-II; CD36, fatty acid translocase CD36; FATP, fat acid transport protein; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; UCP-1, uncoupling protein-1; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); IL-1\(\beta\), interleukin-1 beta; CLS, crown-like structure; PKA, protein kinase A; AMPK, AMP-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; CPT1a, carnitine palmitoyl transferase 1a; ACOX, acyl-CoA oxidase; LPL, lipoprotein lipase; aP2, adipocyte fatty acid-binding protein.
Abstract

Sympathetic nervous system (SNS) regulates the functions of white adipose tissue (WAT) and brown adipose tissue (BAT) tightly. Carotid baroreceptor stimulation (CBS) efficiently inhibits SNS activation. We hypothesized that CBS would protect against obesity. We administered CBS to obese rats and measured sympathetic and AMP-activated protein kinase (AMPK)/peroxisome proliferator activated receptor (PPAR) pathway responses as well as changes in perirenal white adipose tissue (PWAT), epididymal white adipose tissue (EWAT), and interscapular brown adipose tissue (IBAT). CBS alleviated obesity related metabolic changes, improving insulin resistance; reducing adipocyte hypertrophy, body weight, and adipose tissue weights; and decreasing norepinephrine (NE) but increasing acetylcholine (ACH) in plasma, PWAT, EWAT, and IBAT. CBS also downregulated fatty acid translocase (CD36), fat acid transport protein (FATP), phosphorylated and total hormone sensitive lipase, phosphorylated and total protein kinase A, and PPARγ in obese rats. Simultaneously, CBS upregulated phosphorylated adipose triglyceride lipase, phosphorylated and total AMPK, and PPARα in PWAT, EWAT, and IBAT. However, BAT and WAT responses differed; although many responses were more sensitive in IBAT, responses of CD36, FATP, and PPARγ were more sensitive in PWAT and EWAT. Overall, CBS decreased chronically activated SNS and ameliorated obesity related metabolic disorders by regulating the AMPK/PPARα/γ pathway.

Keywords: obesity, carotid baroreceptor stimulation, sympathetic nerve system, white adipose tissue, brown adipose tissue
Introduction

The prevalence of obesity is escalating worldwide. Obesity is characterized by excessive fat storage in white adipose tissue (WAT) and impaired thermogenesis in brown adipose tissue (BAT) (1-3). Obese subjects generally show chronic activation of the sympathetic nerve system (SNS) (4-6), which may be the common pathophysiological mechanism for various obesity-related illnesses, such as hyperlipidemia, insulin resistance, diabetes mellitus, and hypertension (4, 7).

WAT and BAT are both innervated by sympathetic nerve endings, and the SNS regulates the functions of WAT and BAT tightly (8-10). In the normal physiological state, acute SNS activation increases lipid mobilization and decreases body weight (11). However, in obese subjects, the SNS is chronically activated and the responses of the SNS to a variety of metabolism-relevant stimuli, including glucose consumption, circulating insulin, and adipokines, are obviously blunted (4, 12-15). Elevated SNS outflow and blunted SNS in established obesity play promotive roles in insulin resistance and leptin resistance, ultimately resulting in a state of hyperinsulinemia and hyperleptinemia. A recent study demonstrated that an increase in adipokines in obesity may contribute to the state of sympathetic overdrive (16). This leads to a positive feedback loop in which hyperinsulinemia and hyperleptinemia contribute to more pronounced sympathetic tone and the development of metabolic disorders (5, 17).

Therapies targeted at reducing sympathetic activity improve insulin resistance and dyslipidemia in obese subjects (18). The ganglionic blocker trimethaphan improves insulin sensitivity and glucolipid metabolism by reducing the SNS efferent to peripheral organs in obese individuals (17). Several recent studies suggested that device-based renal denervation and transcutaneous auricular vagus nerve stimulation exert therapeutic effects on metabolic disorders (19, 20).

Carotid baroreceptor stimulation (CBS) has proved efficient in inhibiting SNS activation and has been clinically applied for the treatment of heart failure and resistant hypertension (21, 22). Our previous studies showed that CBS exerts anti-arrhythmia effects by decreasing the
discharging of the left stellate ganglion (sympathetic ganglion of the heart) in a canine model (23, 24). In the current study, we hypothesized that CBS would exert protective effects against obesity and we aimed to reveal the underlying mechanisms using a high-fat diet (HFD)-induced rat model of obesity.
Materials and methods

Animal treatments and study design

Thirty Sprague Dawley rats (male, body weight 210–230 g, 5 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China). The rats were randomized into three groups: control rats receiving sham operation (CBS device implantation without delivery of stimulation; C-sham, N = 10), obese rats receiving sham operation (O-sham, N = 10), obese rats receiving CBS device implantation and stimulation (O-CBS, N = 10). All rats were housed in a breeding facility under a 12-h light/dark cycle and had unrestricted access to food and water.

All rats were adaptively fed a control normal diet (CND) for one week prior to surgery. The CND contained 10% fat, 20% protein, and 70% carbohydrate (D12450B; Beijing HFK Bioscience, China). One week after the implantation surgery, the C-sham group continued to receive the CND, whereas the O-sham and O-CBS groups were changed to a high HFD that consisted of 60% fat, 20% protein, and 20% carbohydrate (D12492, Beijing HFK Bioscience, China). Food intake, body weight and core body temperatures (by a rectal digital thermometer, MC-347, OMRON, Japan) were measured weekly between 19:00 and 19:30.

All the rats were housed at constant temperature (22 ± 2°C) and the body temperature was maintained between 36°C and 37°C by a heating pad during tissue harvest. All procedures were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). The study was approved by the Institutional Animal Care and Use Committee at Renmin Hospital, Wuhan University, China (IACUC Issue No. WDRM20151210).

Implantation of CBS device

The CBS device (custom-designed and -made by Ensense Biomedical Technologies Co., Ltd.,
Shanghai, China) consists of a battery-powered impulse generator and a bipolar platinum electrode with conductive leads. All the surfaces are coated by biocompatible materials. The device is programmable by a telemeter system and allows the operator to non-invasively modulate the stimulation parameters delivered to the carotid sinus wall. The rats were anaesthetized by gaseous anesthetic system. The pulse generator was implanted on the back subcutaneously, and the conductive leads went across the back and neck subcutaneously. The bipolar platinum electrode was planted around the exterior surface of the right carotid sinus wall adjacent to the right common carotid artery, and the location of the electrode was slightly adjusted to achieve a clear blood pressure response. The stimulation protocol was previously described (20, 21). The stimulating frequency was 10 Hz, the pulse duration was 1 ms, and the stimulating cycle was set with an interval of 1 min in each cycle of 5 min. The output of the stimulator was set at a working voltage that was 80% of the threshold voltage. The threshold voltage was defined as the voltage that induces a 10% decrease in blood pressure. Sham surgery was the same as that of CBS device implantation, without delivery of stimulation.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

Four days before the sacrifice, GTT was conducted after an overnight fast by intraperitoneally injecting 50% D-glucose (2.0 g/kg). Two days before the sacrifice, ITT was performed by intraperitoneally injecting insulin (0.5 units/kg) after a 4-h fast. For GTT and ITT, small tail cuts were made and blood glucose was measured at 0, 15, 30, 60, 120 min after the injection, with a hand-held glucometer (HEA-230; OMRON Corp., Kyoto, Japan). Areas under the curves (AUCs) of glucose and insulin were calculated by the trapezoidal rule based on the data obtained during the 2-h measurements.

Blood pressure measurement and tissue sample analysis
At the end of the study, body length (from the nose tip to the end of the scrotum) and body weight were measured under isoflurane anesthesia. Thereafter, a catheter was punctured into the left carotid artery and was directly connected to a pressure transducer to obtain carotid artery blood waveforms. LabChart (ADInstruments, Colorado Springs, USA) was used to analyze the waveform data of systolic and diastolic blood pressure. Then, blood samples were taken from the inferior caval vein following an overnight fast. Plasma biochemical parameters were analyzed at the clinical laboratory of Renmin Hospital of Wuhan University, using an Advia 2400 automatic biochemical analyzer (Siemens, Germany) and kits for free fatty acids (FFAs; A042-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), triglyceride (TG; 74023; Siemens, USA), total cholesterol (TCH; Siemens, 74018), low-density lipoprotein (LDL; Siemens, 74028), and high-density lipoprotein (HDL; Siemens, 74027). Finally, following decapitation, perirenal WAT (PWAT), epididymal WAT (EWAT), and interscapular BAT (IBAT) were resected and were weighed separately.

**Enzyme-linked immunosorbent assay (ELISA)**

Plasma levels of insulin, renin, and angiotensin-II (Ang-II) were determined using commercial sandwich ELISA kits (insulin: CEA448Ra; Cloud-Clone Corp., Houston, USA; renin: CSB-E08702r; CUSABIO, Wuhan, China; Ang-II: CSB-E04494r; CUSABIO). The levels of norepinephrine (NE), acetylcholine (ACH), leptin, adiponectin, and tumor necrosis factor-α (TNF-α) in plasma, PWAT, EWAT, and IBAT were detected using commercial sandwich ELISA kits (NE: CSB-E07022r; CUSABIO; ACH: CEA912Ge, Cloud-Clone Corp; TNF-α: CSB-E11987r; CUSABIO; leptin: CSB-E07433r; CUSABIO; adiponectin: CSB-E07271r; CUSABIO). All assays were conducted according to the manufacturer’s protocol.

**Immunohistochemistry analysis**
PWAT, EWAT and IBAT was fixed, embedded in paraffin blocks. Tissue sections were stained with anti-CD68 antibody (GB11067, Servicebio, Wuhan, China) following standard procedures. Both macrophages and Crown-like structure (CLS) were counted using a light microscope. CLS were identified as single adipocyte surrounded by three or more macrophages.

**Oil red O staining**

Liver sections were stained using the Oil red O work solutions containing 6ml Oil red O stock solution (G1015, Servicebio) and 4 mL ddH2O for 15 min as previously described (25). Integrated optical density (IOD; IOD = area × average intensity) of Oil Red O staining was quantified by using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

**Real-time qPCR analysis**

Total RNA was extracted from PWAT, EWAT, and IBAT using Trizol Reagent (15596-026, Invitrogen Life Technologies, USA). The concentration and purity of total RNA were measured. Then 5 μg of the total RNA was reversely transcribed. The 2^ΔΔCT method was used to analyze the relative mRNA levels of C-reaction protein (CRP), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), carnitine palmitoyl transferase 1 (CPT1a), acyl-CoA oxidase (ACOX), lipoprotein lipase (LPL), and adipocyte fatty acid-binding protein (aP2). The PWAT in C-sham group acted as internal control.

**Western blotting**

Western blotting was conducted using extracts from PWAT, EWAT, and IBAT. The adipose tissues were grinded thoroughly and then total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (G2002, Servicebio). Protein concentrations of the adipose tissue homogenates were measured by using a Pierce® BCA Protein Assay Kit.
Proteins (50 μg) were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then were transferred to a polyvinylidene difluoride membrane. The blots were incubated with primary antibodies against fatty acid translocase CD36 (CD36; bs-8873R, Bioss, Beijing, China), fatty acid transport protein (FATP; bs-10556R, Bioss), adipose triglyceride lipase (ATGL; ab109251, Abcam, USA), pS460-ATGL (ab135093, Abcam), hormone sensitive lipase (HSL; ab45422, Abcam), pSer660-HSL (bs-3358R, Bioss), uncoupling protein-1 (UCP-1; bs-1925R, Bioss), protein kinase A (PKA, bs-0520R, Bioss), pThr198-PKA (ab75991, Abcam), AMP-activated protein kinase (AMPK; 5831, CST, USA), pThr172-AMPK (50081, CST), peroxisome proliferator-activated receptor α (PPARα; bs-23398R, Bioss), and peroxisome proliferator-activated receptor γ (PPARγ; bs-0530R, Bioss) overnight at 4°C. The membrane was then incubated with horseradish peroxidase-labeled secondary antibody IgM at room temperature for 1 h. Immunocomplexes were detected with a chemiluminescent substrate (G2014; Servicebio) and the membrane was exposed to X-ray film for semi-quantitative analysis of the target proteins. Protein expression was normalized to that of GAPDH and was expressed relative to that in the C-sham group.

**Statistical analysis**

Quantitative data were expressed as the mean ± standard error of the mean (SEM). Homogeneity of variance was analyzed by Levene’s test. Means were compared by analysis of variance (ANOVA), followed by Tukey’s post-hoc test. Statistical significance was considered at P < 0.05. SPSS 22.0 was used for analysis.
Results

Obesity induced by 8 weeks of HFD feeding is characterized by glucose intolerance, insulin resistance, and hyperlipidemia

Food intake of O-sham and O-CBS group began to be significantly higher than C-sham group since the fifth week. CBS made no difference on the food intake between O-CBS and O-sham group (Figure 1A). O-sham and O-CBS group had significantly elevated body weights than C-sham group since the third week. Body weights of O-CBS group began to be apparently lower than O-sham group since the sixth week (Figure 1B). After 8 weeks of HFD feeding, O-sham group showed higher body weight gain and BMI than C-sham group, which were significantly suppressed in the O-CBS group (Figure 1C). Core body temperature of O-sham and O-CBS group began to be obviously lower than C-sham group since the seventh week. Core body temperature of O-CBS group began to be apparently higher than O-sham group since the ninth week (Figure 1D). GTT, ITT, and plasma insulin indicated distinct glucose and insulin intolerance in O-sham group compared to C-sham group. These abnormalities were all partially alleviated in O-CBS group (Figure 1E-I).

Plasma FFAs, TG, TCH, and LDL were all evidently higher, whereas HDL was obviously lower in O-sham group compared to C-sham group (Figure 1J). Plasma FFAs were significantly higher and TG was lower in the O-CBS than O-sham group (Figure 1J). However, CBS had no significant effect on plasma TCH, LDL, and HDL (Figure 1J).

CBS suppresses the activation of the SNS and the renin-angiotensin-aldosterone system in obese rats

Levels of NE in plasma, PWAT, EWAT, and IBAT were distinctly higher in O-sham group than C-sham group (Figure 2A, B). Inversely, levels of ACH in plasma, PWAT, EWAT, and IBAT were lower in O-sham group than C-sham group (Figure 2C, D). Compared with C-sham group, O-sham group had higher plasma levels of renin and Ang-II (Figure 2E, F). All above abnormalities
were partially ameliorated by CBS (Figure 2A-F). Systolic and diastolic blood pressure were significantly elevated in O-sham group compared to C-sham group. CBS obviously alleviated the increase in systolic blood pressure, but did not affect the diastolic blood pressure (Figure 2G, H).

**CBS inhibits adipocyte hypertrophy, fat deposition, and hepatic steatosis in obese rats**

As shown in Figure 3A, lipids were deposited in a single unilocular droplet in adipocytes of PWAT and EWAT, and in multiple droplets in adipocytes of IBAT. Compared with C-sham group, the mean areas of adipocytes in PWAT and EWAT, fat accumulation in adipocytes of IBAT, weights and fat mass ratios (vs. body weight) of three adipose tissues, the amounts of neutral lipids in liver were all significantly higher in O-sham group. CBS partly ameliorated all above abnormalities (Figure 3A-F).

**CBS differentially affects lipid metabolism-related enzymes in adipose tissues**

Compared with C-sham group, protein levels of CD36 and FATP were significantly upregulated in O-sham group, which was partially suppressed in O-CBS group compared to O-sham group (Figure 4A-C). In O-sham group, levels of UCP-1, pSer660-HSL, total HSL, and pSer660-HSL/total HSL were significantly upregulated, whereas pS460-ATGL, total ATGL, pS460-ATGL/total ATGL decreased distinctly when compared to C-sham group in PWAT, EWAT, and IBAT (Figure 4A-I). CBS made no difference on UCP-1 protein levels in PWAT and EWAT, but exerted a prominent downregulation of UCP-1 in IBAT (Figure 4A, D). pS460-ATGL and pS460-ATGL/total ATGL were partly upregulated in O-CBS group relative to O-sham group in PWAT, EWAT, and IBAT (Figure 4E, F, H). O-CBS group showed slight, albeit not statistically significant, upregulation of total ATGL in PWAT, EWAT, and IBAT relative to O-sham group (Figure 4E, G). The levels of pSer660-HSL, total HSL, and pSer660-HSL/total HSL were completely reversed in PWAT, EWAT, and IBAT of O-CBS group (Figure 4E, I-J).
Effects of CBS on adipocytokines and inflammation in adipose tissues of obese rats

Leptin levels in plasma, PWAT, EWAT, and IBAT were evidently higher in O-sham group than C-sham group (Figure 5A, B). In contrast, adiponectin levels in plasma, PWAT, EWAT, and IBAT were obviously lower in O-sham group than C-sham group (Figure 5C, D). Compared with O-sham group, all above abnormalities were partially alleviated in the O-CBS group (Figure 5A-D). Expression levels of inflammatory factors, including TNF-α, CRP, IL-1β, IL-6, and MCP-1 in PWAT, EWAT, and IBAT increased significantly in obese rats. CBS made no difference on TNF-α, CRP, and IL-1β, but partially downregulated IL-6 and MCP-1 in PWAT, EWAT, and IBAT of O-CBS group (Figure 5E, G, H). Macrophages and CLS were obviously more in PWAT and EWAT of O-sham group compared to C-sham group (Figure 3A, 5I, J). Macrophages were not significantly different in IBAT among C-sham, O-sham, and O-CBS group, but CLS in IBAT of O-sham group was significantly higher than C-sham group (Figure 3A, 5K, L). Macrophages were obviously decreased in PWAT and EWAT of O-CBS group compared to O-sham group (Figure 3A, 5I).

Effects of CBS on AMPK-PPARα/γ signaling in PWAT, EWAT, and IBAT

Compared to C-sham group, levels of pThr172-AMPK, total AMPK, pThr172-AMPK/total AMPK, and PPARα were significantly downregulated in PWAT, EWAT, and IBAT of O-sham group, which were partly rectified by CBS in O-CBS group (Figure 6A, E-G, H, I). Levels of pThr198-PKA, total PKA, pThr198-PKA/PKA, and PPARγ in PWAT, EWAT, and IBAT of O-sham group were distinctly higher than C-sham group (Figure 6A-D, H, J). In PWAT and EWAT, O-CBS group had prominently lower levels of pThr198-PKA, total PKA, pThr198-PKA/total PKA, and PPARγ than that of O-sham group (Figure 6A-D, H, J). In IBAT, CBS significantly decreased the levels of pThr198-PKA, total PKA, and pThr198-PKA/total PKA, but did not affect the expression of PPARγ (Figure 6A-D, H, J). Relative mRNA levels of PPARα target genes CPT1a and ACOX were apparently lower, while relative mRNA levels of PPARγ target genes
LPL and ap2 were significantly higher in PWAT, EWAT, and IBAT of O-sham group than C-sham group. CBS partly upregulated CPT1a and ACOX in PWAT, EWAT, and IBAT of O-CBS group. In O-CBS group, LPL and ap2 were partially downregulated in PWAT and EWAT, but not in IBAT.

**PWAT, EWAT, and IBAT show different responses to HFD and CBS**

In C-sham group, NE, ACH, UCP-1, pS460-ATGL, total ATGL, total HSL, pThr172-AMPK, total AMPK, and PPARα levels were relatively lower in PWAT and EWAT than IBAT (Figure 2A, C; Figure 4D-G, J; Figure 6A, E, F, I; Table 1). Responses of NE, ACH, UCP-1, pS460-ATGL, pSer660-HSL, total HSL, pThr198-PKA, total PKA, pThr172-AMPK, and PPARα to both HFD and CBS were less sensitive in PWAT and EWAT than IBAT (Figure 7A-D, F-H; Table 1). In C-sham group, CD36, FATP, pSer660-HSL, TNF-α, pThr198-PKA, total PKA, and PPARγ levels were not significantly different among PWAT, EWAT, and IBAT (Figure 4A-C; Figure 5E; Figure 6A-C, J), while leptin and adiponectin levels in PWAT and EWAT were significantly higher than that of IBAT (Figure 5A, C). CD36, FATP, and PPARγ expression responded more sensitively to HFD and CBS in PWAT and EWAT than IBAT (Figure 7B, H; Table 1). Responses of total ATGL, leptin, adiponectin, TNF-α, and total AMPK to HFD and CBS were not significantly different among PWAT, EWAT, and IBAT (Figure 7C, E, G; Table 1).
Discussion

**Chronic SNS activation is common in obesity and plays a key role in disease progression**

Obesity, especially when accompanied by excessive accumulated visceral fat and high adipokine secretion, critically contributes to sympathetic activation and metabolic disorders (16, 26, 27). WAT and BAT are both innervated by sympathetic nerves, and the functions of WAT and BAT are tightly regulated by the SNS (8-11). Physiologically, acute SNS activation is intended to increase lipolysis in WAT and promote thermogenesis in BAT in response to cold (11). However, chronically activated SNS in obesity responses to a variety of metabolism relevant stimuli are blunted, which contributes to a state of hyperinsulinemia and hyperleptinemia. Hyperinsulinemia and hyperleptinemia promote fat synthesis, thus establishing a vicious circle between sympathetic activation and obesity (11). In short, chronic SNS activation promotes obesity. In this study, obesity is successfully induced by HFD in rats as indicated by high BMI, dyslipidemia, glucose intolerance, insulin resistance, and adipocyte hypertrophy. CBS exerts significant alleviating effects on these abnormalities. While CBS improves main metabolic disorders, it does not affect plasma TCH, LDL, and HDL in the current study. It worth a further clarification.

**CBS inhibits sympathetic tone and improves main metabolic disorders in obese rats**

In our previous study, CBS was demonstrated to significantly lower the discharging of the left stellate ganglion (sympathetic ganglion of heart) in a canine model (23). As the primary transmitter released by sympathetic nerves, NE is commonly used as the index for sympathetic outflow (28). In this study, increased NE levels in PWAT, EWAT, IBAT, and plasma, as well as increased plasma renin, Ang-II, and systolic blood pressure, are partly reversed by CBS. These findings, along with results in previous studies above, provide evidences that CBS is able to inhibit SNS activation acutely and chronically. It is widely accepted that adipose tissues are innervated by sympathetic endings, and parasympathetic innervation of adipose pads is also
supported by recent findings (29). In our study, decreased ACH levels in plasma and adipose tissues of obese rats are partly rectified by CBS, it indicates that CBS may also play an active role in the parasympathetic nervous system.

Interestingly, β-blockers reportedly increase the propensity of obesity by decreasing insulin sensibility and energy expenditure (30). Our data suggest that CBS exerts anti-obesity effects through an unclear mechanism that is quite different from that of β-blockers. Acute increase in SNS outflow is intended to prevent further storage of excess energy as fat via increasing β-adrenergic stimulation of thermogenesis. However, the chronic SNS activation is demonstrated to actually impair β-adrenergic signaling, further reducing β-adrenergic responsiveness in peripheral tissue and decreasing energy expenditure (31). Thus, elevated SNS tone initiated to prevent additional storage of body fat ultimately evolves into a mechanism contributing to the development of HFD-induced obesity. Owing to the inhibitive effects on SNS, CBS may increase β-adrenergic stimulation of thermogenesis by ameliorating the desensitization of the β-adrenergic signaling pathway in obesity. Recent study demonstrates that a 1°C increase in body temperature is associated with a 10–13% increase in metabolic rate (32). In our study, O-CBS group shows no significant difference on food intake, but shows significant increase in core body temperature compared to O-sham group. It indicates that CBS may increase the energy expenditure of O-CBS group by sensitizing β-adrenergic signaling pathway. This complex process remains to be elucidated in future studies.

**FFA uptake and lipolysis might be the key targets of CBS**

Anatomically, WAT comprises two major depots, subcutaneous WAT and visceral WAT. Visceral WAT is closely related to metabolic disorders and various chronic diseases (33, 34). After food intake, FFAs derived from dietary lipids are taken up by white adipocytes and are esterified into TGs in lipid droplets. CD36 is a high affinity receptor which has been shown to facilitate FFAs uptake into adipose tissues and muscle in rodents and humans (35). FATP is an
integral membrane protein implicated in the trans-membrane transport of FFAs (36). In obese individuals, insulin resistance induces upregulation of CD36 and FATP, which results in excessive fat synthesis in white adipocytes by promoting FFA uptake (37). In our study, the upregulation of CD36 and FATP in obese rats is partly inhibited by CBS.

When required, TGs are hydrolyzed by lipolysis and released into the circulation as FFAs. ATGL and HSL are responsible for most of the TG hydrolysis in adipose tissues (38). In our study, increased levels of phosphorylated HSL, total HSL and phosphorylated HSL/total HSL in PWAT, EWAT, and IBAT of obese rats are completely reversed by CBS in O-CBS group. CBS does not restore total ATGL downregulation, but levels of phosphorylated ATGL and phosphorylated ATGL/ATGL were partly restored in PWAT, EWAT, and IBAT of obese rats. The mechanism involved in the regulation of total ATGL may be different from that of phosphorylated ATGL and HSL in adipocytes (39, 40). Total ATGL is mainly upregulated by glucocorticoid (PPAR) agonists (41). During fasting, phosphorylated ATGL and HSL mainly respond to adrenocorticotrophic hormone, adrenaline, or some kinases (such as AMPK), and they are all in high relation to the SNS activity (40, 42, 43). This explains why CBS differently regulates ATGL and HSL in adipose tissues.

Quite different from WAT, BAT is specialized in UCP1-mediated non-shivering thermogenesis through lipid oxidation when exposed to cold (44). UCP-1 is expressed uniquely in brown adipocytes, therefore regarded a specific biomarker of BAT (45-47). Clusters of “brown-like” adipocytes with low UCP-1 expression occur in WAT (47, 48). Brown and brown-like adipocytes generate heat by β-oxidation of fatty acids when exposed to cold or upon SNS activation (45, 49). Our data support the idea that UCP-1 expression is increased in brown and inducible brown-like adipocytes owing to hyperactivity of the SNS in obesity (50). CBS is demonstrated to decrease UCP-1 abundance in IBAT, without affecting UCP-1 levels in PWAT and EWAT. Even though decreasing non-shivering thermogenesis in BAT adipocytes promotes obesity, this effect might be too weak to alter adipocyte hypertrophy.
Different effects of CBS on adipocytokines and inflammation in adipose tissues of obese rats

In our study, obese rats have significant fat accumulation and high levels of TNF-α in plasma and adipose tissues, as well as increased other inflammatory markers, including CRP, IL-1β, IL-6 and MCP-1 in adipose tissues. In addition, obese rats show more macrophages and CLS in adipose tissues. These data correspond to the recent points that obesity is always accompanied by chronic low-intensity inflammation and macrophages accumulation in expanding adipose tissues (51, 52). CBS partially alleviates the abnormal secretion of leptin and adiponectin. It is interesting that CBS significantly decreases the content of IL-6, and MCP-1 in PWAT, EWAT, and IBAT, but not the level of TNF-α, CRP, IL-1β. Meanwhile, CBS obviously decreases macrophage infiltration in PWAT and EWAT, but not in IBAT. CBS also made no difference on the number of CLS in PWAT, EWAT and IBAT. These results indicate that the effects of CBS on inflammatory reaction and macrophages accumulation in adipose tissues vary with the type of inflammatory markers and adipose tissue.

CBS attenuates metabolic disorders of adipose tissue by regulating the AMPK-PPARα/γ pathway

PPARα and PPARγ are both ligand-activated transcription factors that are involved in the differentiation of adipocytes and lipid metabolism. In adipose tissues, PPARα increases FFA β-oxidation and insulin sensitivity, whereas PPARγ promotes lipid synthesis and endocrine function (53, 54). AMPK, a serine/threonine protein kinase, acts as an efficient sensor of the cellular energy state and is a critical modulator for PPARα and PPARγ (55-57). AMPK is regarded as a master switch in the regulation of lipid metabolism and involves in mediating beneficial effects of autonomic nervous system (57-59). In addition, circulating adipokines, such as adiponectin, increase insulin sensitivity and enhance fatty acid oxidation by upregulating AMPK in the peripheral tissues (57, 58) In our study, CBS upregulates phosphorylated AMPK, total AMPK,
phosphorylated AMPK/total AMPK and may consequently upregulate PPARα and downregulate PPARγ in adipose tissues, which in turn increase FFA β-oxidation and decrease FFA uptake. This is supported by alterations in the enzymes mediating FFA β-oxidation (ATGL, HSL, and UCP-1) and uptake (CD36 and FATP) in adipocytes. Herein, we furthermore explore the effects of CBS on the upstream and downstream of AMPK-PPARα/γ pathway. Our data correspond to the recent study that PKA negatively modulates AMPK (60). The modulations of CBS on PPARα target genes (CPT1α and ACOX) and PPARγ target genes (LPL and aP2) are accorded with the effects of CBS on PPARα and PPARγ respectively. Taken together, these novel findings demonstrate that CBS exerts beneficial effects against obesity via the AMPK-PPARα/γ pathway in adipose tissues.

**BAT and WAT differentially respond to HFD and CBS**

In our study, responses of the NE, ACH UCP-1, phosphorylated ATGL, phosphorylated HSL, total HSL, phosphorylated PKA, total PKA, phosphorylated AMPK, and PPARα to both HFD and CBS are less sensitive in PWAT and EWAT than those in IBAT. This may be explained by the fact that IBAT is innervated by more nerve endings than WAT, and the regulating proteins above are highly regulated by SNS (40, 61-66). CD36, FATP, and PPARγ expression respond more sensitively to both HFD and CBS in PWAT and EWAT than IBAT. This may be because CD36, FATP, and PPARγ are largely involved in FFA uptake in PWAT and EWAT, whereas HSL, UCP-1, and PPARα are mainly involved in FFA β-oxidation in IBAT (53, 54). The differences of leptin and UCP-1 expression among different adipose tissues and the contribution of HFD to their expression have ever been reported. Van Harmelen et al. showed that leptin secretion is positively correlated with adipocyte size and the subcutaneous adipose tissue has a higher leptin secretion than that of visceral adipose (67). This point is further confirmed by our study in which PWAT and EWAT are similar in cell size and leptin levels in plasma and adipose tissues are respectively approximate. In recent study, HFD is indicated to increase UCP-1 expression in BAT generally, but exerts inconsistent effects on UCP-1 expression in WAT (68). Our data match with the points
that PWAT and EWAT may play more critical roles in secretes several adipokines whereas IBAT play more important roles in UCP-1 mediated thermogenesis (44, 69).

Conclusion

Based on the results of this study, we draw three conclusions: 1) CBS exerts protective effects against obesity by suppressing the chronic activation of SNS in obese rats; 2) CBS exerts anti-obesity effects likely by regulating the AMPK-PPARα/γ pathway; 3) WAT and BAT differentially respond to HFD and CBS.

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Table 1. Differential responses of PWAT, EWAT, and IBAT to carotid baroreceptor stimulation

| Tissue      | PWAT | EWAT | IBAT |
|-------------|------|------|------|
| NE          | ↓    | ↓    | ↓↓   |
| ACH         | ↑    | ↑    | ↑↑   |
| CD36        | ↓↓   | ↓↓   | ↓    |
| FATP        | ↓↓   | ↓    | ↓    |
| pS460-ATGL  | ↑↑   | ↑↑   | ↑↑↑  |
| total ATGL  | --   | --   | --   |
| pSer660-HSL | ↓↓   | ↓↓   | ↓    |
| total HSL   | --   | --   | --   |
| UCP-1       | ↓    | ↓    | ↓    |
| Leptin      | ↑    | ↑    | --   |
| Adiponectin | ↑    | ↑    | --   |
| TNF-α       | --   | --   | --   |
| pThr198-PKA | ↓↓   | ↓↓   | ↓    |
| total PKA   | ↓↓   | ↓↓   | ↓    |
| pThr172-AMPK| ↑↑   | ↑↑   | ↑↑↑  |
| total AMPK  | ↑↑   | ↑↑↑  | ↑↑   |
| PPARα       | ↑↑   | ↑↑   | ↑↑   |
| PPARγ       | ↓↓   | ↓    | --   |

Notes: ↓, ↓↓, and ↓↓↓ indicate a decrease of 0–50.0%, 51.1–150.0%, and more than 151.1% respectively in the mean value of O-CBS group compared to the O-sham group; ↑, ↑↑, and ↑↑↑ indicate an increase of 0–50.0%, 51.1–150.0%, and more than 151.1% respectively in O-CBS group compared to the O-sham group; --, the mean value is not significantly different between the O-CBS and O-sham groups.
Figure 1. Effects of CBS on BMI, plasma biochemical parameters, glucose tolerance, and insulin tolerance in obese rats

Effects of CBS on weekly food intake (A), weekly body weight (B), body weight gain and BMI (kg/m²) (C), and core body temperature (D). Curves for GTT (E) and ITT (F). AUCs for GTT (G) and ITT (H). Effects of CBS on fast plasma levels of insulin (I), FFAs, TG, TCH, LDL, and HDL (J). All data are expressed as the mean ± SEM, N = 10 for each group. #: O-sham group vs C-sham group; #: O-sham group vs O-CBS group; *: O-CBS group vs C-sham group; +,#,*,P < 0.05; ##,++,**,P < 0.01.
Figure 2. Effects of CBS on the activation of sympathetic nervous system and the renin-angiotensin-aldosterone system in obese rats

Effects of CBS on fast norepinephrine levels in PWAT, EWAT, IBAT (A), and plasma (B). Effects of CBS on fast acetylcholine levels in PWAT, EWAT, IBAT (C) and plasma (D). Effects of CBS on fast plasma levels of renin (E) and angiotensin-II (F). Effects of CBS on systolic blood pressure (G) and diastolic blood pressure (H). All data are expressed as the mean ± SEM, N = 10 for each group. #: O-sham group vs C-sham group; +: O-sham group vs O-CBS group; *: O-CBS group vs C-sham group; §: EWAT vs IBAT; $: IBAT vs PWAT; §§,$,$,##,++,**,P < 0.05; §§§,$$$,##,,(++,**P < 0.01.
Figure 3. Effects of CBS on adipocyte hypertrophy and fat deposition in obese rats

Immunohistochemical staining for macrophages (with light blue nucleus) and CLS (arrowheads) in PWAT, EWAT, and IBAT (the first-third panel) (A). Oil red O staining for liver (the fourth panel). Magnification: 400×. Scale bar = 20 μm. Effects of CBS on weights (B) and fat weight ratios (C) of PWAT, EWAT, and IBAT. Effects of CBS on adipocyte areas in PWAT, EWAT (D), fat storage in IBAT (E), and neutral lipids accumulation in liver (F). All data are expressed as the mean ± SEM, N = 10 for each group. #: O-sham group vs C-sham group; +: O-sham group vs O-CBS group; *: O-CBS group vs C-sham group; +, #, *P < 0.05; ##, ++, **P < 0.01.
Figure 4. Effects of CBS on lipometabolism-related enzymes in adipose tissues of obese rats

Representative blots for protein abundances of CD36, FATP, and UCP-1 in PWAT, EWAT, and IBAT (A). Effects of CBS on protein abundances of CD36 (B), FATP (C), and UCP-1 (D) in PWAT, EWAT, and IBAT. Representative blots for protein abundances of pS460-ATGL, total ATGL, pSer660-HSL, and total HSL in PWAT, EWAT, and IBAT (E). Effects of CBS on levels of pS460-ATGL (F), total ATGL (G), pS460-ATGL/total ATGL (H), pSer660-HSL (I), total HSL (J), and pSer660-HSL/total HSL (K) in PWAT, EWAT, and IBAT. All data are expressed as the mean ± SEM, N = 10 for each group. #: O-sham group vs C-sham group; +: O-sham group vs O-CBS group; *: O-CBS group vs C-sham group; §: EWAT vs IBAT; $: IBAT vs PWAT; §§,$$,#,#,++,*P < 0.05; §§, $$,#,#,++,**P < 0.01.
Figure 5. Different effects of CBS on adipocytokines and inflammation in adipose tissues of obese rats

Effects of CBS on fast leptin levels in PWAT, EWAT, IBAT (A), and in plasma (B). Effects of
CBS on fast adiponectin levels in PWAT, EWAT, IBAT (C) and plasma (D). Effects of CBS on TNF-α levels in PWAT, EWAT, IBAT (E) and plasma (F). Effects of CBS on relative mRNA levels of CRP (G), IL-1β (G), IL-6 (H), and MCP-1 (H) in PWAT, EWAT, and IBAT. Effects of CBS on the number of macrophages (I) and CLS (J) in PWAT and EWAT. Effects of CBS on the number of macrophages (K) and CLS (L) in IBAT. All data are expressed as the mean ± SEM, N = 10 for each group. #: O-sham group vs. C-sham group; +: O-sham group vs O-CBS group; *: O-CBS group vs C-sham group; §: EWAT vs IBAT; $: IBAT vs PWAT; §, $, +, #, * P < 0.05; §§, $$, ##, ++, ** P < 0.01.
Figure 6. Effects of CBS on the AMPK-PPARα/γ signaling pathway in adipose tissues of obese rats

Representative blots for protein abundances of pThr198-PKA, total PKA, pThr172-AMPK, and total AMPK in PWAT, EWAT, and IBAT (A). Effects of CBS on levels of pThr198-PKA (B), total PKA (C), pThr198-PKA/total PKA (D), pThr172-AMPK (E), total AMPK (F), and pThr172-AMPK/total AMPK (G) in PWAT, EWAT, and IBAT. Representative blots for protein abundances of PPARα and PPARγ in PWAT, EWAT, and IBAT (H). Effects of CBS on protein abundances of PPARα (I) and PPARγ (J) in PWAT, EWAT, and IBAT. Effects of CBS on relative mRNA levels of PPARα target genes (CPT1a and ACOX) (K) and PPARγ target genes (LPL and aP2) (L) in PWAT, EWAT, and IBAT. All data are expressed as the mean ± SEM, N = 10 for each group. #: O-sham group vs C-sham group; +: O-sham group vs O-CBS group; *: O-CBS group vs C-sham group; §: EWAT vs IBAT; $: IBAT vs PWAT; §§, $§, #§, ++, **P < 0.01.
Figure 7. Differential responses to HFD and CBS among PWAT, EWAT, and IBAT

Differential responses to HFD and CBS among PWAT, EWAT, and IBAT: norepinephrine and acetylcholine (A), CD36 and FATP and UCP-1 (B), pS460-ATGL and total ATGL (C), pSer660-HSL and total HSL (D), leptin and adiponectin (Adp), and TNF-α (E), pThr198-PKA and total PKA (F), pThr172-AMPK and total AMPK (G), PPARα and PPARγ (H). All data are expressed as the mean ± SEM, N = 10 for each group. §: EWAT vs IBAT; $: IBAT vs PWAT; §§, $$P < 0.05; §§§, $$$P < 0.01.