

ORIGINAL RESEARCH

Modulation of Sirt1 and FoxO1 on Hypothalamic Leptin-Mediated Sympathetic Activation and Inflammation in Diet-Induced Obese Rats

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BACKGROUND: Hypothalamic leptin-mediated signaling contributes to the exaggerated sympatho-excitation and increased blood pressure in obesity-associated hypertension. The aim of the study was to investigate the roles of energy-sensing enzyme sirtuin1 (Sirt1) and forkhead box protein O1 (FoxO1) on the hypothalamic leptin-mediated high sympathetic nerve activity and inflammation in obesity.

METHODS AND RESULTS: Sprague Dawley rats were fed with high-fat diet (HFD) for 12 weeks. In vivo, the potential of Sirt1 and FoxO1 in the sympathetic effects of leptin was investigated via siRNA injection to knockdown Sirt1 or FoxO1 gene in the arcuate nucleus (ARCN) of hypothalamus in rats. In vitro, the effects of Sirt1 or FoxO1 on leptin-mediated inflammation were observed in proopiomelanocortin (POMC) and microglial cells. Knockdown Sirt1 by siRNA significantly reduced the renal sympathetic nerve activity (RSNA) and blood pressure responses to leptin injection in the ARCN in the HFD rats. Conversely, knockdown FoxO1 significantly enhanced the RSNA and blood pressure responses to leptin injection in the HFD rats. Knockdown Sirt1 reduced the levels of pro-inflammatory cytokines interleukin 6 (IL-6), tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), C1q/TNF-related protein-1 (CTRP1), and immune cell infiltration in the ARCN in the HFD rats. Knockdown FoxO1 significantly increased the level of IL-6 in the ARCN of HFD rats. In cultured hypothalamic POMC and microglial cells, knockdown Sirt1 significantly reduced leptin-induced IL-6 expression, affected the levels of AMP-activated protein kinase (AMPK) and serine/threonine-specific protein kinase (Akt). Knockdown FoxO1 significantly increased leptin-induced IL-6 in both POMC cells and microglial cells.

CONCLUSIONS: These data suggest that both Sirt1 and FoxO1 are the key modulators of leptin signaling in the hypothalamus contributed to the over sympathetic activation and inflammation in obesity.

Key Words: energy-sensing enzyme ■ hypothalamus ■ leptin ■ obesity-associated hypertension ■ sympathetic nerve activity

Accumulated evidence has shown that obesity is associated with over-activation of sympathetic nervous system in humans and in animal models.¹⁻⁴ Chronic sympathetic activation increases cardiovascular risks in obesity.^{5,6} Many studies have demonstrated that central nervous system (CNS) signaling pathways contribute to the etiology and pathogenesis of obesity-associated hypertension.^{2,7} However, the precise mechanisms

linking obesity with sympathetic over-activation are not entirely understood.

In the recent decade, the evidence has indicated the level of circulating adipose-deprived hormone leptin is elevated and this may relate to the onset and maintenance of hyper-sympathetic condition during obesity.^{2,8,9} Leptin action in the brain increases sympathetic nerve activity to the adipose, kidney, and blood vessels.¹⁰ In the CNS, the arcuate nucleus (ARCN) of hypothalamus has

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CLINICAL PERSPECTIVE

What Is New?

- We found the expressions and activity of energy-sensing proteins Sirt1 and FoxO1 were enhanced in the arcuate nucleus of hypothalamus of obese rats.
- Knockdown Sirt1 and FoxO1 in the arcuate nucleus resulted in the changes of sympathetic activation and blood pressure controlled by leptin in diet-induced obese rats.
- Knockdown Sirt1 and FoxO1 affected the levels of pre-inflammatory cytokines in obese rats.

What Are the Clinical Implications?

- This finding provides potential therapeutics targeting leptin and energy-sensing proteins to treat obesity-associated hypertension.

Nonstandard Abbreviations and Acronyms

AgRP	agouti-related peptide
Akt	serine/threonine-specific protein kinase
AMPK	AMP-activated protein kinase
AP	arterial pressure
ARCN	arcuate nucleus
CNS	central nervous system
CTRP1	C1q/TNF-related protein-1
FoxO1	forkhead box protein O1
HFD	high-fat diet
HR	heart rate
POMC	proopiomelanocortin
RSNA	renal sympathetic nerve activity
Sirt1	sirtuin 1
TNF-α	tumor necrosis factor α

emerged as a major site for regulating physiological processes by leptin. Direct injection of leptin into the ARCN increases renal sympathetic nerve activity (RSNA) as well as arterial pressure (AP) demonstrating the action in this nucleus can evoke sympathetic and cardiovascular effects of leptin.¹¹ In the ARCN, various neuronal populations including proopiomelanocortin (POMC) neurons, agouti-related peptide (AgRP) neurons, microglial cells and astrocytes are involved in many of leptin's actions on the cardiovascular and renal sympathetic activity.¹²⁻¹⁴ These sympathetic effects of leptin on cardiovascular system are exaggerated in obese condition and have been shown to act as the key contributing factors for the development of obesity-associated hypertension.^{8,15,16}

The ARCN is located at the mediobasal hypothalamus which can sense the changes of various

nutrients and hormones. The POMC neurons in the ARCN are nutrient and energy responsive and their firing rate changes with the concentration of glucose, amino acid, and lipids.¹⁷ Among the energy-sensing enzymes, sirtuin 1 (Sirt1) is a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone/protein deacetylase and is widely expressed in many tissues, including liver, muscle, adipose tissue, heart, and hypothalamus.¹⁸ Sirt1 is involved in mediating fat and glucose levels related alteration in energy homeostasis both in the periphery and in the brain.¹⁹ In the brain, Sirt1 induces negative energy balance and its activity is directly related to the effects of hypothalamic peptide hormones, such as leptin and insulin.^{18,20} Recent study has shown that over-expression of Sirt1 in the hypothalamus can improve leptin sensitivity to regulate food intake and body weight gain.²¹ However, there is no evidence showing the effects of Sirt1 on the leptin-mediated sympathetic activation and cardiovascular influences from the CNS.

In the hypothalamus, forkhead box protein O1 (FoxO1) is a transcription factor which abundantly expressed in the ARCN POMC and AgRP neurons. FoxO1 is also a metabolic sensor which integrates leptin and insulin signaling.²² The studies have shown that the leptin signaling inhibits food intake by competing with FoxO1. FoxO1 can inhibit POMC promoter to inhibit leptin activity.²² Over-expression of FoxO1 in the hypothalamus of rats by adenoviral microinjection leads to loss of function of leptin on feeding.²³ In the CNS, Sirt1 and FoxO1 play contrary roles in the leptin-signaling. Interestingly, the cross-talks among Sirt1, FoxO1 and leptin signaling have been reported in different experimental systems.²² In the brain, Sirt1-mediated deacetylation of FoxO1 can cause the increasing of FoxO1 activity. In the liver, Sirt1 deacetylates and activates FoxO1 to promote gluconeogenesis. It is quite possible these signaling cross-talks may have functional effects of leptin in the hypothalamus to regulate food intake, body weight as well as sympathetic activation and blood pressure (BP) controlling.

Therefore, the present study was conducted to investigate the effects of Sirt1 and FoxO1 on hypothalamic leptin-mediated sympathetic activation and potential leptin-related inflammatory mechanism in rats with high-fat diet-induced obesity.

METHODS

All supporting data are available within the article.

Animals

This animal protocol was approved by the Institutional Animal Care and Use Committee of the University of South Dakota and was in accordance with the

American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male Sprague-Dawley rats (130–150 g, age 6–7 weeks) were obtained from Envigo (Indianapolis, IN) and maintained in a temperature and humidity-controlled environment with a 12-/12-hour light/dark cycle. Rats were fed with the high-fat diet (HFD) (TD.88137, 42% of calories are from fat, Envigo) for 12 weeks (n=40). The regular chow fed rats were used as non-HFD controls (n=40). Body weight and food consumption were monitored weekly. The experiments were performed after 12 weeks exposure to the HFD or normal diet.

Measurement of Visceral Fat, Plasma Glucose, and Leptin Levels

Rats were euthanized at 19 to 20 weeks of age. The visceral fat including retroperitoneal fat and epididymal fat from each rat was collected and immediately weighed. Plasma glucose levels were monitored from tail blood and measured by Accu-check. The levels of plasma leptin were measured by commercial enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, Salem, NH) according to the manufacturer's instructions. The blood was collected from a tail incision and centrifuged at 2000g. The supernatant (plasma) was used for ELISA.

In Vivo Physiological Experiments

Knockdown *Sirt1* and *FoxO1* by Small Interference RNA Microinjection in the ARC/N

After 12 weeks of HFD or non-HFD feed, pre-designed small interference RNA (siRNA) targeting rat *Sirt1* or *FoxO1* or scramble (a negative control siRNA) (Thermo Fisher Scientific) were microinjected into the ARC/N bilaterally. siRNA transfection was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA). 100 pmol siRNA was diluted with lipofectamine and incubated for 45 minutes at room temperature. Rats were anesthetized with 2% to 3% isoflurane with 100% oxygen. An incision was made on the midline of the scalp. The coordinates of the ARC/N were 2.3 mm posterior to the bregma, 0.5 mm lateral to the midline, and 9.6 to 9.9 mm ventral to the dura.^{24,25} A microsyringe needle (0.2 mm OD) was inserted into the ARC/N for siRNA or scramble injection (200 nL). After 10 minutes, the needle was removed. The scalp skin was sutured. Rats were returned to their cages. Five days after siRNA or scramble injections, rats were used for physiological or molecular biological experiments.

Verification the Gene Knockdown of *Sirt1* and *FoxO1* in the ARC/N

Five days after siRNA or scramble injections, rats were euthanized. Then brains were removed and frozen on

dry ice. The serial coronal sections of the ARC/N (100 μ m/section, total 15 sections, from –2.0 to –3.5 mm to bregma) were cut with a cryostat according to a stereotaxic atlas. The sections were bilaterally punched using the Palkovits and Brownstein technique.²⁶ The punched ARC/N tissue was put in TRI Reagent (Molecular Research Center Inc, Cincinnati, OH). The total RNA in the homogenate was extracted and subjected to reverse transcription. The cDNA was amplified by real-time quantitative RT-PCR with the StepOne Real-Time PCR system (Thermo Fisher Scientific). The primer pairs used were *Sirt1* (the sense primer 5'-ACCGATGGACTCCTCACTAA-3', the antisense primer 5'-ATCTGCCACAGCGTCATATC-3'), *FoxO1* (the sense primer 5'-GTCCTCGAACAGCTCAAAT-3', the antisense primer 5'-CTGTCCTGAAGTGTCTGCATAG-3') and β -actin (the sense primer 5'-GAGGTATCCTGACCCTGAAGTA-3', the antisense primer 5'-GCTCGAAGTCTAGAGCAACATAG-3'). Relative mRNA expressions of *Sirt1* and *FoxO1* were calculated using the Pfaffl equation which relates expression of the target gene to expression of a reference gene (β -actin).

Recording of Arterial Pressure, Heart Rate, and Renal Sympathetic Nerve Activity

Five days after siRNA injection, rats were anesthetized with urethane (0.75–1.5 g/kg, i.p) and α -chloralose (140 mg/kg, i.p). The femoral vein was cannulated for administration of extra anesthesia and 0.9% saline. The femoral artery was cannulated and connected to the MacLab (ADInstruments, Colorado Spring, CO) for computer-based recording to record AP and heart rate (HR).

The left kidney was exposed through a retroperitoneal flank incision. A bunch of renal nerve was isolated and placed on a bipolar platinum electrode and fixed with Wacker Silgel. The electrical signal was amplified with a Grass amplifier. The rectified output signal was displayed and recorded with the MacLab system as described previously.^{27,28}

ARC/N Microinjection Experimental Protocol

Leptin-induced sympathetic responses and BP changes were recorded in six groups of rats (control+scramble, control+siRNA *Sirt1*, control+siRNA *FoxO1*, HFD+scramble, HFD+siRNA *Sirt1*, HFD+siRNA *FoxO1*, n=5–6/group). Recombinant leptin (R&D Systems, Minneapolis, MN) (50, 100, 200 ng in 50 nL artificial cerebrospinal fluid) was microinjected into the ARC/N. The responses of RSNA, mean AP (MAP) and HR over the following 30 minutes were recorded. At the end of the experiment, blue dye (2% Chicago blue, 30 nL) was injected into the brain for histological verification.

Western Blot Analysis

In the separate groups of rats with or without siRNA injection (n=5–6/group), the punched ARC/N tissue was homogenized in 100 μ L of radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail (Promega, Madison, WI) and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The total protein concentrations were measured with a bicinchoninic acid assay kit (Pierce, Rockford, IL). Samples were adjusted to have the same total protein concentration. 4 \times loading buffer was added and samples were loaded onto a sodium dodecyl sulfate polyacrylamide electrophoresis gel and subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane. Then, the membrane was incubated with primary antibody overnight. After the incubation with secondary antibody conjugated with fluorescent dye (1:10 000, Thermo Fisher Scientific), the bands were detected using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE). The intensity of the band was quantified using NIH ImageJ software. The protein expression was calculated as the ratio of the intensity of the protein to the intensity of β -actin.

The following primary antibodies were used: Sirt1 (9475S, 1:500), pSirt1 (2314S, 1:500), FoxO1 (2880S, 1:500), pFoxO1 (9461S, 1:500), tumor necrosis factor α (TNF- α , 11948S, 1:1000) (Cell Signaling Technology, Danvers, MA), interleukin 6 (IL-6, ab9324, 1:500), interleukin 1 β (IL-1 β , ab9722, 1:500) (Abcam, Cambridge, MA), C1q/TNF-related protein-1 (CTRP1, sc-81943, 1:500), AMP-activated protein kinase (AMPK, sc-398861, 1:1000) and serine/threonine-specific protein kinase (Akt, sc-5298, 1:1000), and β -actin (sc-47778, 1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA).

Analysis of Immune Cell Infiltration in the ARC/N

In another separate group of rats with or without siRNA injection (n=3–4/group), under deep anesthesia with isoflurane, rats were perfused through left ventricle with heparinized saline followed by 4% paraformaldehyde. Brain was removed, post fixed, and then placed in 30% sucrose. Brain sections with the ARC/N (each section 15 μ m thick) were cut with a cryostat according to a stereotaxic atlas and preserved in the cryoprotectant. The floating brain sections containing ARC/N were blocked with 10% normal donkey serum and incubated with macrophage/microglia cell marker CD11b/c antibody conjugated with DyLight 488 (1:500, NB110-40766, Novus Biologicals, Centennial, CO) overnight at 4°C. After washing, the sections were mounted on the glass slides and coverslipped with Vectashield mounting medium (Vector Laboratory, Burlingame, CA). The fluorescent images of CD11b/c

within the ARC/N were visualized and imaged using a Leica SP8 lightning confocal microscope (Leica, Germany).

In Vitro Studies

POMC and Microglial Cell Culture

Adult mouse hypothalamic POMC cell line (mHypoA-POMC/GFP) was purchased from CEDARLANE Cellutions Biosystems (CLU500, Burlington, Ontario, Canada). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum and 1% penicillin/streptomycin. Mouse microglial cell line (SIM-A9) were purchased from American Type Culture Collection (ATCC CRL-3265, Manassas, VA). Cells were grown in DMEM:F12 medium with 10% fetal bovine serum and 5% horse serum. Both cells were maintained at 37°C and 5% CO₂ until 60% to 70% confluent.

Knockdown Sirt1 and FoxO1 Gene Expression in the Cultured Cells

Cells were then maintained in the medium without serum for differentiation purpose. Control or siRNA Sirt1 or siRNA FoxO1 were premixed with lipofectamine 2000 reagent and then added into the cell media with final siRNA concentration (5–10 nmol/L). After 24-hour incubation, media were changed with fresh medium. Cells were treated with recombinant leptin at the concentrations of 50 to 400 ng/mL or vehicle for 24 hours. Each treatment was repeated 3 times. Cultured cells growing on the glass coverslips were prepared for immunofluorescent staining of Sirt1, FoxO1 (Cell Signaling Technology) and leptin receptor (Abcam). Cultured cells growing in the 60 mm dishes were subject to protein extraction procedure and Western blot analysis for IL-6, TNF- α , IL-1 β , AMPK, and Akt protein.

ELISA for Anti-inflammatory Bioactive Lipid Lipoxin A4 Measurement

After knockdown Sirt1 and FoxO1 by siRNA in the cultured POMC and microglial cells, POMC and microglial cells were treated with leptin at the concentrations of 50 to 400 ng/mL or vehicle for 24 hours. Cultured cells were collected and subjected to measure the level of lipoxin A4 by ELISA kit (LifeSpan BioSciences, Seattle, WA) as per the manufacturer instructions.

Statistical Analysis

The baseline values of MAP, HR and RSNA were obtained by averaging variables over a period of 5 minutes before each injection. The background noise was determined by the RSNA recorded at the end of the

experiment after ganglionic blocker hexamethonium (30 mg/kg, iv) injection. The value of RSNA during experiment was calculated by subtracting the background noise from the actual recorded value. The changes of RSNA were expressed as a percentage from basal value of RSNA. All data are presented as means \pm SE. Statistical significance was determined by one-way ANOVA followed by comparison for individual group differences with the Tukey's test using GraphPad Prism 7 Software. For comparison of values in smaller sample size groups ($n < 4$), t test with non-parametric analysis was performed. A $P < 0.05$ is considered statistically significant.

RESULTS

General Characteristics of Control and HFD Rats

General characteristics of control and HFD rats used in the experiments are summarized in the Table. Twelve weeks of HFD increased rat body weight, retroperitoneal fat pad weight, and epididymal fat pad weight. The plasma glucose and leptin levels were significantly different between the groups. The levels of fast plasma glucose and plasma leptin was significant higher in the HFD rats than those in control rats. These data confirmed 12 weeks HFD-induced hyperlipidemia, hyperglycemia, and hyperleptinemia in the rats.

The basal RSNA were significantly increased in the HFD rats, suggesting that there was an elevated overall sympathetic activity in the HFD rats. The basal MAP was also significantly increased in the HFD rats compared to control rats. There was no significant difference of HR in the two groups of rats. Knockdown Sirt1 and FoxO1 by siRNA had no significant effects on the body weight, basal sympathetic activity, and BP in both control and HFD rats (data not shown in the Table).

Altered Sirt1 and FoxO1 Expressions and Activity in the ARCNe in the HFD Rats

Western blot analysis showed the HFD rats had higher protein levels of Sirt1 (ratio of intensity: 0.61 ± 0.06

versus 0.31 ± 0.02 , $P = 0.0013$, $n = 4$), pSirt1 (ratio of intensity: 0.82 ± 0.11 versus 0.29 ± 0.03 , $P = 0.0006$, $n = 4$), FoxO1 (ratio of intensity: 0.79 ± 0.10 versus 0.47 ± 0.06 , $P = 0.0214$, $n = 4$), and pFoxO1 (ratio of intensity: 0.35 ± 0.09 versus 0.17 ± 0.02 , $P = 0.0480$, $n = 4$) in the ARCNe compared to the controls (Figure 1). The HFD rats showed significantly increased expressions and activity for Sirt1 and FoxO1 in the ARCNe.

Knockdown Sirt1 and FoxO1 Gene in the ARCNe

To verify the efficacy of Sirt1 or FoxO1 gene knockdown by siRNA injection, Sirt1 and FoxO1 mRNA levels of the ARCNe were compared in control and HFD rats. There were significant increases of Sirt1 and FoxO1 mRNA levels in the ARCNe of HFD group (relative expression of Sirt1 mRNA increased 2.5-folds, $P = 0.0035$, $n = 4$; FoxO1 mRNA increased 1.84-folds, $P = 0.0069$, $n = 4$) (Figure 2). siRNA Sirt1 injection significantly reduced the higher Sirt1 mRNA level in both control and HFD rats (relative expression of Sirt1 mRNA reduced 50% in HFD rats, $P = 0.0189$, $n = 4$) (Figure 2A). siRNA FoxO1 injection significantly reduced the higher level of FoxO1 mRNA in control and HFD rats (relative expression of FoxO1 mRNA reduced 54% in HFD rats, $P = 0.0028$, $n = 4$) (Figure 2B).

Effects of Knockdown Sirt1 or FoxO1 in the ARCNe on Leptin-Mediated Sympathetic Responses and Blood Pressure Changes

In anaesthetized rats, microinjections of leptin (50, 100, and 200 ng) into the ARCNe induced dose dependent increases of RSNA, MAP, and HR in both control and HFD rats. Leptin administration (200 ng) elicited significant higher increases in RSNA, MAP, and HR (reaching Δ RSNA: $24 \pm 2\%$, Δ MAP: 15 ± 1 mm Hg, Δ HR: 20 ± 2 bpm) in the ARCNe in the HFD rats compared to control rats (Δ RSNA: $11 \pm 1\%$, $P = 0.0001$; Δ MAP: 6 ± 1 mm Hg, $P = 0.0001$; Δ HR 11 ± 1 bpm, $P = 0.0022$, $n = 6$) (Figure 3).

Knockdown Sirt1 with siRNA in the ARCNe significantly attenuated leptin-induced increases of RSNA,

Table 1. General Characteristics of Control and HFD Rats

	Control (n=15)	HFD (n=16)		Control (n=15)	HFD (n=16)
Body weight, g	425 \pm 18	488 \pm 20*	Fast plasma glucose, mmol/L	5.2 \pm 0.6	7.5 \pm 1.0*
Retroperitoneal pad, g	4.4 \pm 0.4	10.7 \pm 2.7*	Plasma leptin, ng/mL	377 \pm 28	2443 \pm 274*
Epididymal fat pad, g	4.8 \pm 0.2	11.0 \pm 1.8*	Basal Int. RSNA, μ V·s	2.1 \pm 0.3	4.8 \pm 0.5*
Basal MAP, mm Hg	92 \pm 5	104 \pm 5*	Basal heart rate, beat/min	349 \pm 22	367 \pm 35

Values are mean \pm SE. Int.RSNA indicates integrated renal sympathetic nerve activity; HFD indicates high-fat diet; and MAP, mean arterial pressure.

* $P < 0.05$ vs control group.

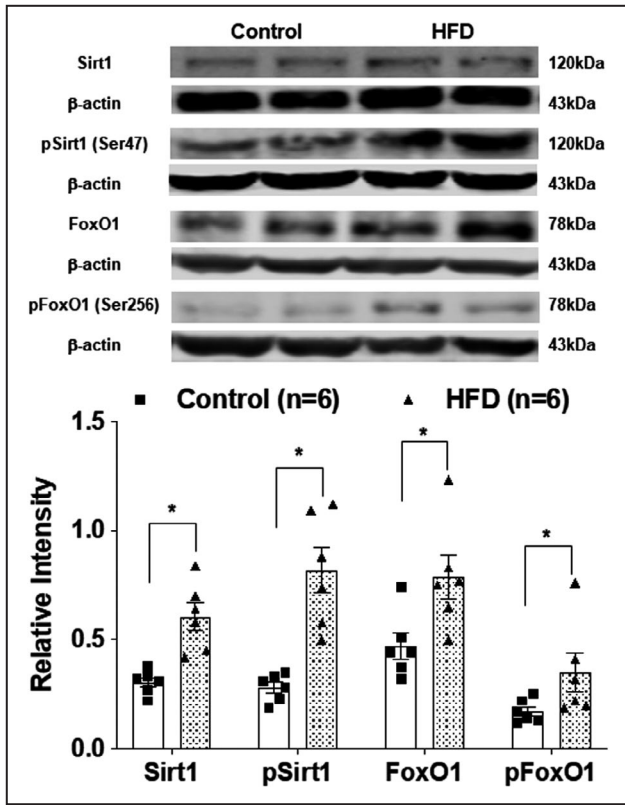


Figure 1. Representative gel and mean protein expressions of sirtuin 1 (Sirt1), pSirt1 (Ser47), forkhead box protein (FoxO1), and pFoxO1 (Ser256) in the arcuate nucleus (ARCNC) in control and high fat diet (HFD) rats. * $P < 0.05$ vs control group.

MAP, and HR in the ARCNC in the HFD rats compared to the HFD scramble group (Δ RSNA: $16 \pm 1\%$ versus $24 \pm 2\%$, $P = 0.0037$; Δ MAP: 9 ± 1 mm Hg versus 15 ± 1 mm Hg, $P = 0.0006$; Δ HR: 14 ± 2 bpm versus 20 ± 2 bpm, $P = 0.0447$, $n = 6$). On the contrary,

knockdown FoxO1 with siRNA in the ARCNC significantly enhanced leptin-induced increases of RSNA and MAP (Δ RSNA: $31 \pm 2\%$ versus $24 \pm 2\%$, $P = 0.0300$; Δ MAP: 18 ± 2 mm Hg versus 15 ± 1 mm Hg, $P = 0.0480$, $n = 6$), and had no enhanced effect on HR response in the ARCNC in the HFD rats (Δ HR: 20 ± 2 bpm versus 20 ± 2 bpm, $P = 0.7858$, $n = 6$) (Figure 3). Both siRNA Sirt1 and siRNA FoxO1 had no significant effects on the leptin-induced RSNA, MAP, and HR increases in the control rats.

Knockdown Sirt1 or FoxO1 in the ARCNC Affects Pro-inflammatory Cytokine Expressions and Immune Cell Infiltration in the ARCNC in the HFD rats

Western blot analysis showed the HFD rats had significant higher protein levels of IL-6 (ratio of intensity: 1.02 ± 0.09 versus 0.67 ± 0.09 , $P = 0.0314$, $n = 4$), TNF- α (ratio of intensity: 1.06 ± 0.09 versus 0.79 ± 0.08 , $P = 0.0440$, $n = 4$), IL-1 β (ratio of intensity: 1.16 ± 0.03 versus 0.53 ± 0.11 , $P = 0.0015$, $n = 4$), and CTRP1 (ratio of intensity: 1.61 ± 0.14 versus 0.94 ± 0.03 , $P = 0.0029$, $n = 4$) in the ARCNC (Figure 4A). Knockdown Sirt1 significantly reduced the levels of these pro-inflammatory cytokines in the HFD rats (ratio of intensity IL-6: 0.72 ± 0.07 versus 1.02 ± 0.09 , $P = 0.0345$, $n = 4$; TNF- α : 0.71 ± 0.10 versus 1.06 ± 0.09 , $P = 0.0438$, $n = 4$; IL-1 β : 0.86 ± 0.07 versus 1.16 ± 0.03 , $P = 0.0094$, $n = 4$) and CTRP1 (0.97 ± 0.06 versus 1.61 ± 0.14 , $P = 0.0052$, $n = 4$) compared to the HFD scramble group. Conversely, knockdown FoxO1 significantly increased the level of IL-6 (1.44 ± 0.09 versus 1.02 ± 0.09 , $P = 0.0015$, $n = 4$), but had no significant effects on the TNF- α (1.11 ± 0.10 versus 1.06 ± 0.09 , $P = 0.6843$, $n = 4$), IL-1 β (1.01 ± 0.09 versus 1.16 ± 0.03 , $P = 0.1823$, $n = 4$), and CTRP1 (1.39 ± 0.14 versus

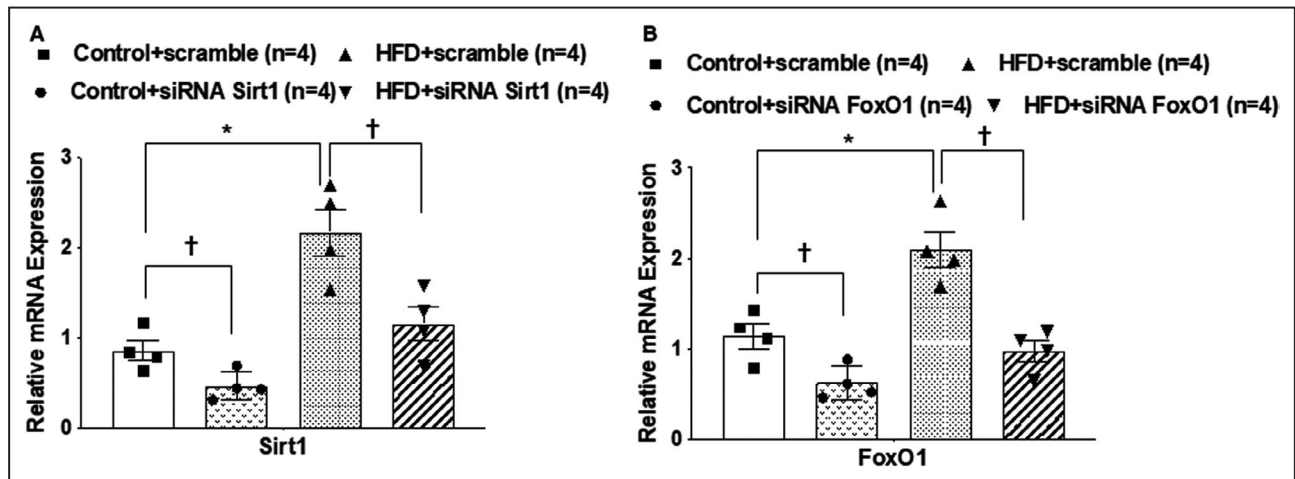


Figure 2. Mean data of relative mRNA expression of Sirt1 (A) (in the control+scramble, control+siRNA Sirt1, HFD+scramble and HFD+siRNA Sirt1 rats) and FoxO1 (B) (in the control+scramble, control+siRNA FoxO1, HFD+scramble and HFD+siRNA FoxO1 rats) in the punched ARCNC tissues measured by real-time RT-PCR. * $P < 0.05$ vs control+scramble group. † $P < 0.05$ vs scramble group.

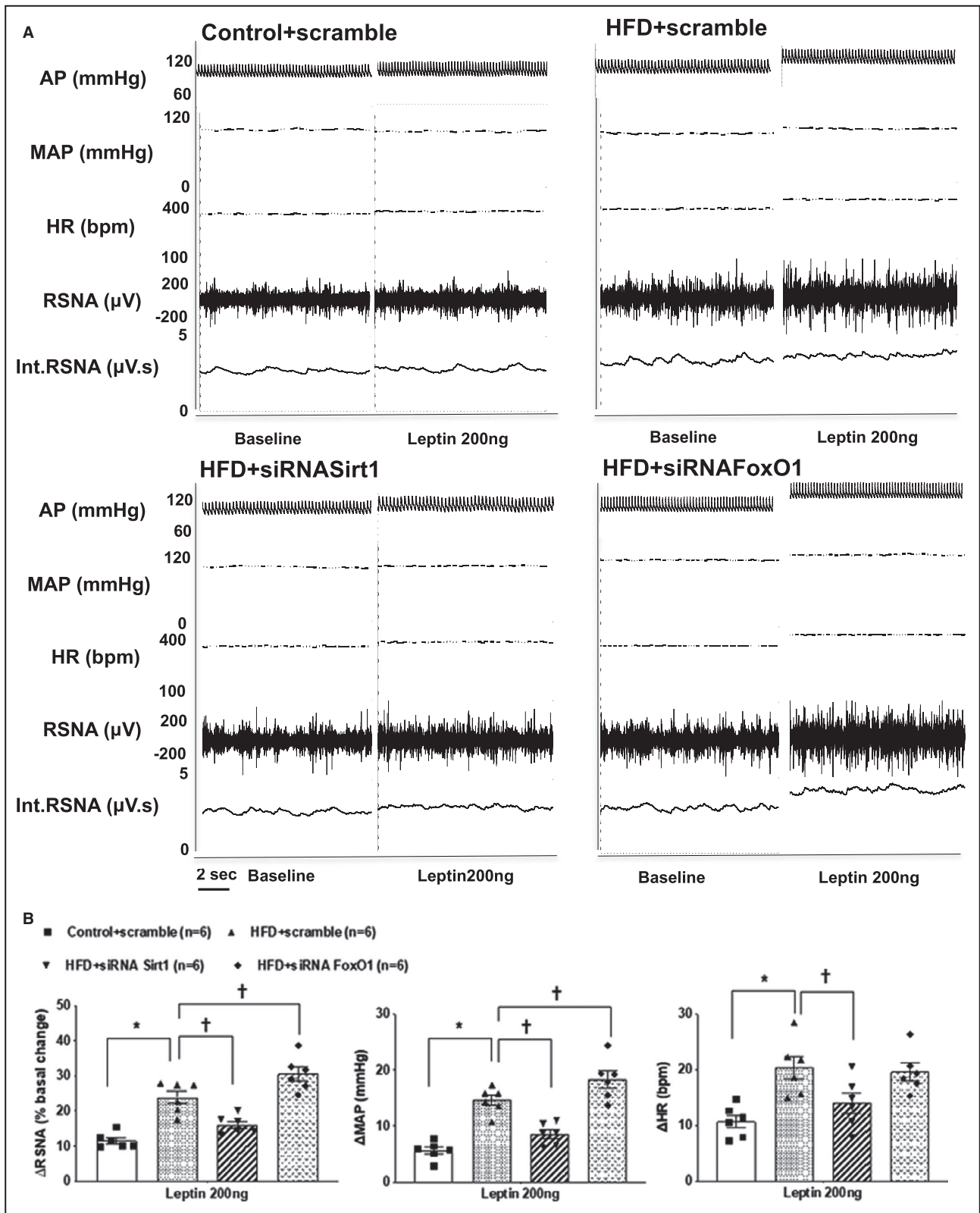


Figure 3. Effects of knockdown Sirt1 or FoxO1 in the ARC on leptin-mediated sympathetic responses, blood pressure (BP), and heart rate (HR) changes.

A, Representative tracer of arterial pressure (AP), mean arterial pressure (MAP), HR, renal sympathetic nerve activity (RSNA), integrative RSNA (int.RSNA) responses to microinjection of leptin (200 ng) in the ARC in the control+scramble, HFD+scramble, HFD+siRNA Sirt1, and HFD+siRNA FoxO1 rats. **B**, Mean changes in RSNA, MAP, and HR to microinjection of leptin (200 ng) in the ARC. **P*<0.05 vs control+scramble group. †*P*<0.05 vs HFD+scramble group.

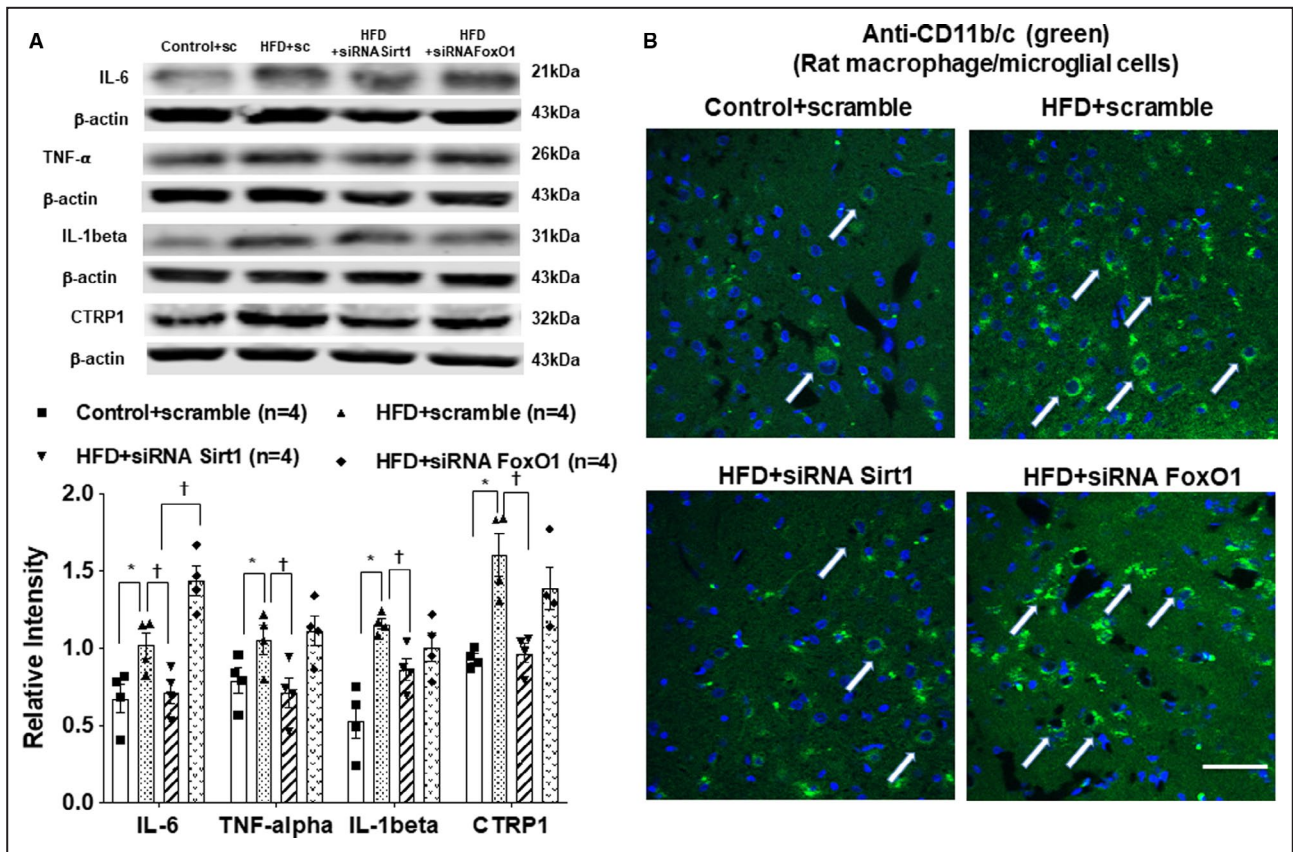


Figure 4. Knockdown Sirt1 or FoxO1 in the ARCn affects pro-inflammatory cytokine expressions and immune cell infiltration in the ARCn.

A, Representative gel and mean protein expressions of interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and C1q/TNF-related protein-1 (CTRP1) in the ARCn in four groups of rats (control+scramble, HFD+scramble, HFD+siRNA Sirt1 and HFD+siRNA FoxO1). **P*<0.05 vs control+scramble group. †*P*<0.05 vs HFD+scramble group; sc indicates scramble. **B**, Immunofluorescent photomicrographs from the sections of the ARCn region stained for macrophage/microglial cell marker CD11b/c (in green) in the control+scramble, HFD+scramble, HFD+siRNA Sirt1, and HFD+siRNA FoxO1 rats. Blue: 4',6-diamidino-2-phenylindole (DAPI). Scale bar=50 μm.

1.61±0.14, *P*=0.2940, *n*=4) expressions in the HFD rats compared to the HFD scramble group (Figure 4A).

Furthermore, immunofluorescent staining showed increased fluorescent signal of macrophage/microglial cell marker CD11b/c in the ARCn of HFD rats which suggesting there was higher immune cell infiltration in the HFD rats. In addition, siRNA Sirt1 attenuated the enhanced immune cell infiltration within the ARCn while siRNA FoxO1 had no significant effect on the immune cell infiltration in the HFD rats (Figure 4B). Both siRNA Sirt1 and siRNA FoxO1 had no significant effects on the pro-inflammatory cytokine expressions and CD11b/c fluorescent signal in the control rats.

Knockdown Sirt1 or FoxO1 in Vitro Affects Leptin-induced Pro-inflammatory Cytokine Expressions

Immunofluorescent staining confirmed the expressions of Sirt1 and FoxO1 in the hypothalamic POMC

cells and microglial cells (Figure 5A). Leptin receptor immunofluorescent signal co-localized with Sirt1 and FoxO1 in both cells. Western blot analysis showed 24-hour leptin treatment significantly increased the protein levels of IL-6 (ratio increased to 2.19±0.06 at dose 400 ng/mL, *P*=0.0001, *n*=3), TNF-α (ratio increased to 2.21±0.10, *P*=0.0003, *n*=3), and IL-1β (ratio increased to 1.74±0.04, *P*=0.0001, *n*=3) in the POMC cells as dose dependent manner compared to the vehicle treated group (Figure 5B). Twenty-four hour leptin treatment also significantly increased the protein levels of IL-6 (ratio increased to 1.58±0.10 at dose 400 ng/mL, *P*=0.0040, *n*=3), TNF-α (ratio increased to 1.82±0.06, *P*=0.0001, *n*=3), and IL-1β (ratio increased to 2.15±0.20, *P*=0.0042, *n*=3) in the microglial cells (Figure 5B). Leptin treatment significantly reduced the levels of anti-inflammatory bioactive lipid lipoxin A4 in both POMC (ratio reduced to 0.25±0.06 at dose 400 ng/mL, *P*=0.0002, *n*=3) and microglial cells (ratio reduced to 0.26±0.05, *P*=0.0002, *n*=3) as does dependent manner (Figure 5C).

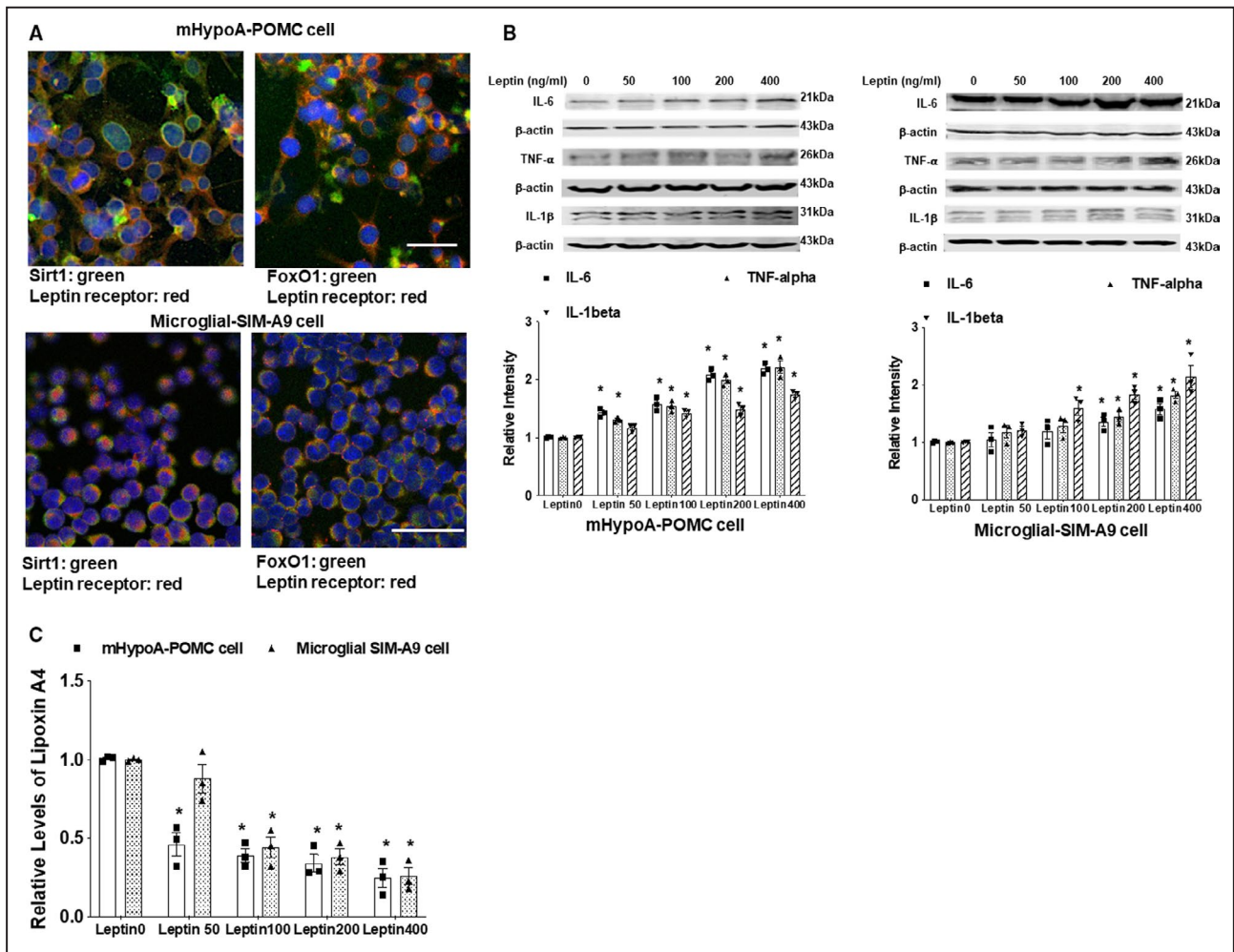


Figure 5. Leptin-induced pro-inflammatory cytokine and anti-inflammatory bioactive lipid lipoxin A4 (lipoxin A4) changes in vitro.

A, Immunofluorescent photomicrographs stained for Sirt1 (in green), FoxO1 (in green) with leptin receptor (in red) in mHypoA-POMC cells (top panel), and microglial-SIM-A9 cells (bottom panel). Blue: DAPI. Scale bar=20 μm. **B**, Representative gel and mean protein expressions of IL-6, TNF-α, IL-1β in mHypoA-POMC cells (left panel), and microglial-SIM-A9 cells (right panel) treated with leptin (50–400 ng/mL). **P*<0.05 vs control without leptin treatment. **C**, Mean level of lipoxin A4 in mHypoA-POMC cells and microglial-SIM-A9 cells treated with leptin (50–400 ng/mL). **P*<0.05 vs control without leptin treatment.

Furthermore, knockdown Sirt1 significantly reduced leptin treatment-induced (200 ng/mL) pro-inflammatory cytokine IL-6 in both POMC (ratio reduced to 0.53±0.06, *P*=0.0014, *n*=3) and microglial cells (ratio reduced to 0.66±0.06, *P*=0.0041, *n*=3) compared to the scramble group (Figure 6A). Knockdown FoxO1 significantly increased leptin-induced IL-6 in both POMC cells (ratio increased to 2.30±0.32, *P*=0.0155, *n*=3) and microglial cells (ratio increased to 1.48±0.11, *P*=0.0107, *n*=3) (Figure 6B).

Knockdown Sirt1 in Vitro Affects AMPK and Akt in the POMC and Microglial Cells

Knockdown Sirt1 directly affected the levels of AMPK and Akt protein in cultured POMC and microglia cells.

Twenty-four hours of siRNA Sirt1 significantly reduced the level of AMPK in both POMC cells (ratio reduced to 0.27±0.09, *P*=0.0011, *n*=3) and microglial cells (ratio reduced to 0.35±0.06, *P*=0.0004, *n*=3) compared to the scramble group (Figure 7A). siRNA Sirt1 treatment also significantly reduce the level of Akt in the POMC cells (ratio reduced to 0.50±0.11, *P*=0.0083, *n*=3) (Figure 7B).

DISCUSSION

For the recent decade, studies have demonstrated that the adipose tissue generated adipokine leptin is involved in the central control of sympathetic nerve activity and cardiovascular function.^{2,8} Hyperleptinaemia is common in obesity and causing leptin resistance

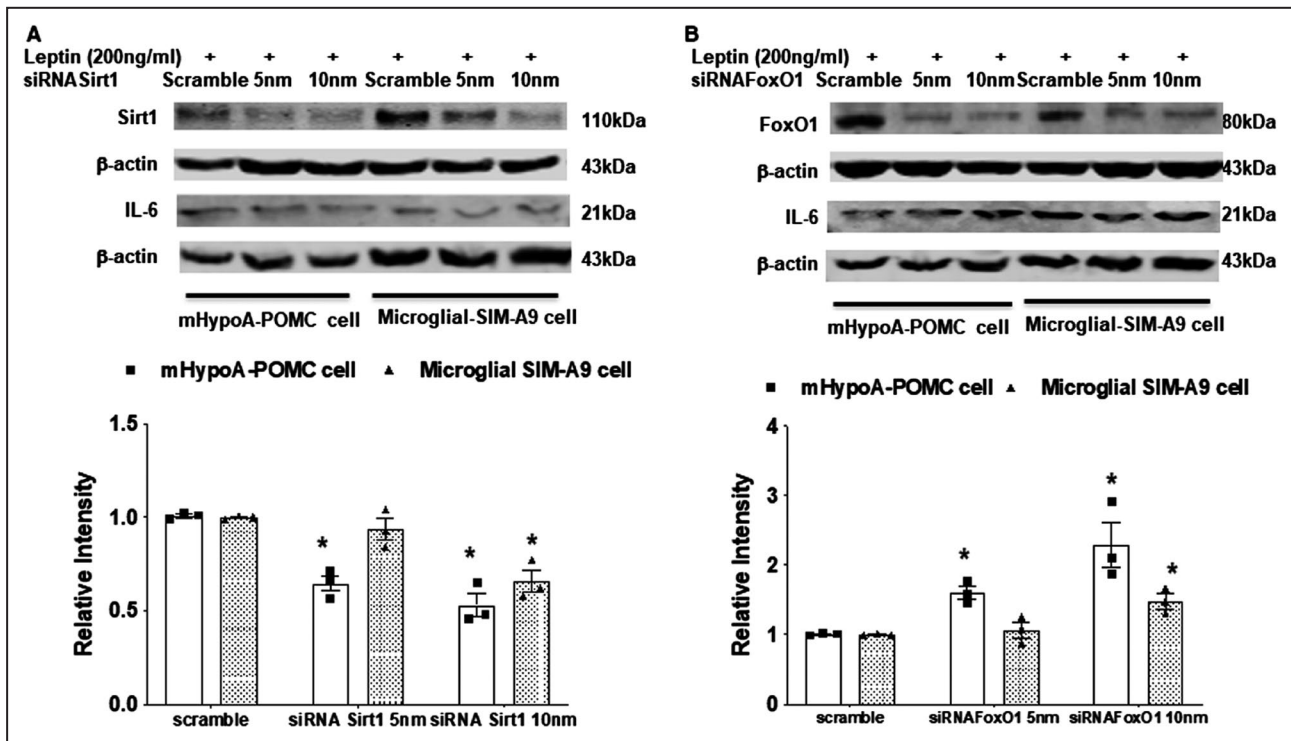


Figure 6. Knockdown Sirt1 or FoxO1 in vitro affects leptin-induced pro-inflammatory cytokine expressions.

A, Representative gel and mean protein expressions of Sirt1 and IL-6 after knockdown Sirt1 by siRNA in microglial-SIM-A9 cells and microglial cells. The cells were incubated with leptin (200 ng/mL) for 24 hours. **B**, Representative gel and mean protein expressions of FoxO1 and IL-6 after knockdown FoxO1 by siRNA in microglial-SIM-A9 cells and microglial cells. The cells were incubated with leptin (200 ng/mL) for 24 hours. * $P < 0.05$ vs scramble group.

to food intake and body weight control. However, the evidence has supported that several other actions of leptin such as cardiovascular sympathetic activation are still preserved and even augmented in obese subjects.^{29,30} The enhanced action on renal sympathetic nerve of leptin may play an important role in the pathological development of obesity-associated hypertension. However, the signaling mediating leptin system dysfunction in the CNS is not fully understood. The present study was designed to investigate the contribution of energy-sensing enzyme Sirt1 and FoxO1 a key transcriptional factor of leptin signaling involved in the hypothalamic leptin-mediated high sympathetic nerve activity and inflammation in obesity. Our major findings in the study included (1) the expressions and activity for Sirt1 and FoxO1 in the ARC of hypothalamus were enhanced in obese rats; (2) knockdown Sirt1 and FoxO1 in the ARC resulted in the changes of sympathetic activation and BP responses controlled by leptin; (3) knockdown Sirt1 and FoxO1 affected the levels of pre-inflammatory cytokines in obese rats and in vitro as well; (4) knockdown Sirt1 directly affected the levels of other leptin signaling effectors including AMPK and Akt.

The role of leptin in activating sympathetic drive through CNS has been highlighted in many reviews.^{1,4,30}

In the HFD-induced obese rat model used in the study, we observed that 12 weeks of HFD produced hyperleptinemia, hyperglycemia, and hyperlipidemia in the rats. Hyperleptinemic condition might be implicated in generating the elevation of sympathetic activation in obese conditions.^{2,31,32} We also observed higher basal renal sympathetic activity and elevated BP in the HFD rats. This suggests there is an increased overall sympathetic tone and hypertension in this HFD rat model.

Central nervous system plays a critical role in integrating peripheral afferent signals to regulate sympathetic outflow and cardiovascular function.^{33,34} Adipose-deprived leptin is able to cross the blood brain barrier to interact with leptin receptor in the hypothalamic nuclei such as ARC, paraventricular nucleus (PVN), dorsal medial hypothalamus, anterior hypothalamus and ventromedial hypothalamus to result in sympathetic activation and exert pressor effect.^{11,35,36} The ARC of hypothalamus has been shown as an important gateway for the actions of leptin signaling on controlling sympathetic activity.³⁷ In our animal studies, we observed that direct leptin administration into the ARC increased RSNA, MAP, and HR. The electrophysiological study functionally confirmed the higher sympathetic activation by central leptin stimulation in obese rats.

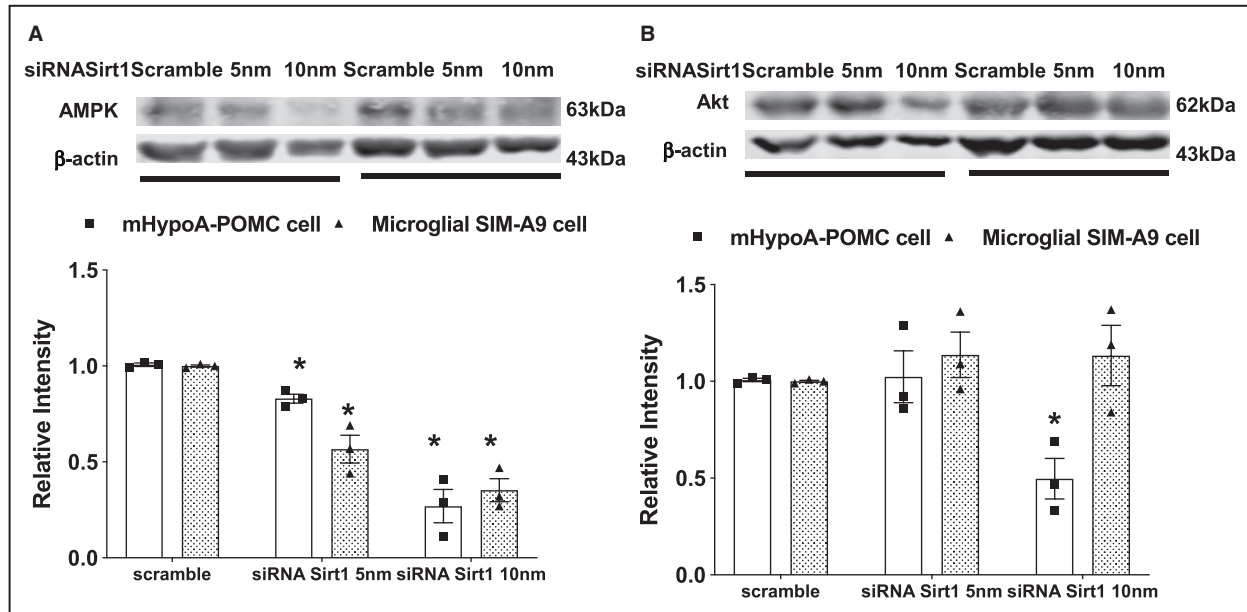


Figure 7. Representative gel and mean protein expressions of AMP-activated protein kinase (AMPK) (A) and serine/threonine-specific protein kinase (Akt) (B) after knockdown by siRNA Sirt1 in mHypoA-POMC cells and microglial-SIM-A9 cells.

* $P < 0.05$ vs scramble group.

In the CNS, leptin has shown to exert its effects on sympathetic activity and BP through a number of downstream mediators. Among the contributing factors regulating energy balance are nutrient sensors such as Sirt1 which plays a critical role in metabolic health and functions in various tissues and organs including the hypothalamus.^{38,39} In the hypothalamus, Sirt1 induces negative energy balance and is directly related to the effects of hypothalamic peptide hormones such as leptin and insulin.²¹ Sirt1 can modulate protein activity through its removal of acetyl functional groups. Our study showed the HFD rats with hyperleptinaemia exhibited elevated Sirt1 level and enhanced Sirt1 activity in the ARC. This was consistent with other reports showing diet-induced obese mice and leptin db/db mice had elevated Sirt1 levels in their ARC.⁴⁰ FoxO1, a downstream transcription factor for the Sirt1 signaling is also elevated in the ARC in our HFD animal model. We believe that the augmented Sirt1 and FoxO1 signaling in obese condition may impact the hyperactivity of leptin on the sympathetic and cardiovascular controlling. Therefore, we tested if genetic knockdown Sirt1 or FoxO1 in the ARC could affect leptin-mediated sympathetic nerve activity and BP increases in HFD rats. Our results showed that knockdown Sirt1 by siRNA significantly reduced the renal sympathetic nerve activation and BP elevation by leptin in the HFD rats. Conversely, knockdown FoxO1 by siRNA significantly increased the renal sympathetic nerve activation and BP elevation by leptin in the HFD rats. This is the first evidence showing both Sirt1 and

FoxO1 regulate the action of leptin on sympathetic cardiovascular function. The cross-talks among leptin, Sirt1 and FoxO1 may be functional in the hypothalamus to regulate sympathetic cardiovascular effects (Figure 8). There is study showing both genetic knockdown and pharmacological inhibition of Sirt1 resulted in long-term decreased food intake and body weight gain in animal models.⁴¹ Our data showed that knockdown Sirt1 and FoxO1 had no significant effects on the body weight and basal sympathetic parameters such as RSNA and BP in both control and HFD rats. These results might be due to the relative short-term of experimental time period. Using virus containing shRNA or genetic knockout animal may better explore the long-term action of Sirt1 and FoxO1 in the brain. Our study did not show Sirt1 or FoxO1 knockdown affecting on leptin-induced sympathetic activity and BP in the normal diet fed animal. This might be due to the elevated leptin level and enhanced leptin actions which is seen only in the HFD rats but not in the normal diet fed rats.

Over the years, hypothalamic inflammation has been linked to the development of progression of obesity and its sequelae, including obesity-associated hypertension. The high inflammatory condition has been found in several hypothalamus nuclei, such as ARC, PVN and lateral hypothalamus in obese animal.⁴² The neuroinflammation in the hypothalamus is related to the HFD-induced obesity and hyperleptinemia. Inflammatory mediators released from neuronal or non-neuronal cell types give rise to long-lasting impaired metabolic control of the hypothalamus.⁴²

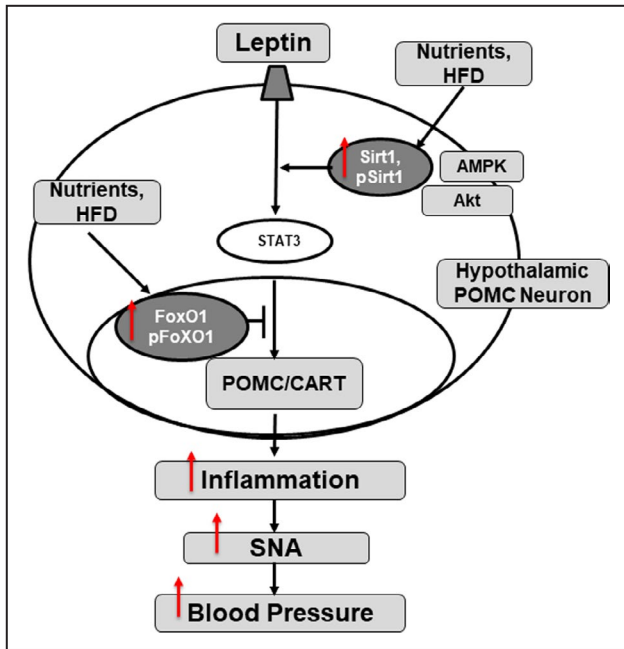


Figure 8. Proposed the mechanism within the hypothalamic nuclei proopiomelanocortin (POMC) neurons that leptin signaling may contribute to the high sympathetic nerve activity (SNA) and increased blood pressure observed in the HFD obese rats.

Sirt1 and FoxO1 in hypothalamus are the key modulators of leptin signaling pathway in the hypothalamus contributed to over sympathetic activation and inflammation in obesity. CART indicates cocaine- and amphetamine-regulated transcript; and STAT3, signal transducer and activator of transcription 3.

Prolonged inflammation leads to apoptosis of hypothalamic neurons and results in an imbalance of the hypothalamic control of energy homeostasis.⁴³ In the ARC/N, leptin can modulate several inflammatory mediators. Leptin can increase the expression of monocyte chemoattractant protein-1 and macrophage lipoprotein lipase, a potent atherogenic cytokine in type 2 diabetes.⁴⁴ However, the knowledge about potential associations between leptin and central inflammatory responses are not fully elucidated. Our results showed that the HFD rats had higher levels of pro-inflammatory cytokines, CTRP1, and immune cell infiltration in the ARC/N. Knockdown Sirt1 influenced the levels of pro-inflammatory cytokines, CTRP1 and immune cell infiltration in the HFD rats. To further test if knockdown Sirt1 or FoxO1 affected the levels of pro-inflammatory cytokines induced by leptin, we performed *in vitro* studies in both neuronal POMC and microglial cells. We found that leptin incubation increased the levels of pro-inflammatory cytokines and reduced the anti-inflammatory bioactive lipid lipoxin A4 in both cultured POMC and microglial cells. Furthermore, knockdown Sirt1 or FoxO1 significantly altered the level of leptin-induced pro-inflammatory cytokine IL-6 in both cells. The results suggest the

potential associations between leptin and hypothalamic inflammation, this may contribute to the augmented leptin signaling to sympatho-excitation in obese rats. Both Sirt1 and FoxO1 functionally work together as downstream effectors in the leptin signaling pathway.

In the study, we also tested some effectors on the leptin signaling pathway by directly knockdown of Sirt1. AMPK is a nutrient/energy sensor involving in the regulation of numerous biochemical pathways to turn off anabolism. Activation of Sirt1 is associated with the stimulation of catabolic pathways and inhibition of anabolic pathways.^{45,46} Since Sirt1 and AMPK are closely related to energy expenditure, they may be potential biomarkers to evaluate cellular energy balance.⁴⁷ In our *in vitro* study, decreased AMPK expression was observed by knockdown Sirt1 in the neuronal POMC and microglial cells. The close relationship of Sirt1 and AMPK may play a critical role in maintaining cellular function and integrity regulated by leptin. Akt is another important regulator in the insulin and leptin signaling pathway which can be regulated by Sirt1.^{22,48} Deacetylation by Sirt1 is necessary for the binding of Akt to phosphatidylinositol-trisphosphate (PIP3) and for its membrane localization and activation. The Sirt1 and Akt signaling pathway serves in many biological processes including cardiac hypertrophy, angiogenesis, and aging.⁴⁹ Our cell culture study indicated the interaction of Sirt1 and Akt in the hypothalamic POMC cells and microglial cells, suggesting this close interplay between Sirt1 and Akt may play important roles to regulate leptin actions in the hypothalamus.

In conclusion, these studies provide evidence that within the hypothalamic nuclei, leptin signaling in the ARC/N may contribute to the exaggerated sympatho-excitation observed in the HFD obese rats. Sirt1 and FoxO1 in the hypothalamus are the key modulators of leptin signaling pathway contributed to over sympathetic activation and inflammation in obesity. This finding provides a rationale for development of novel targeting hypothalamic leptin and energy-sensing proteins to treat obesity-associated hypertension.

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Disclosures

None.

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