Cell Reports, Volume *20*

Supplemental Information

Heparin Increases Food Intake through AgRP Neurons

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Supplemental Figures

Figure S1

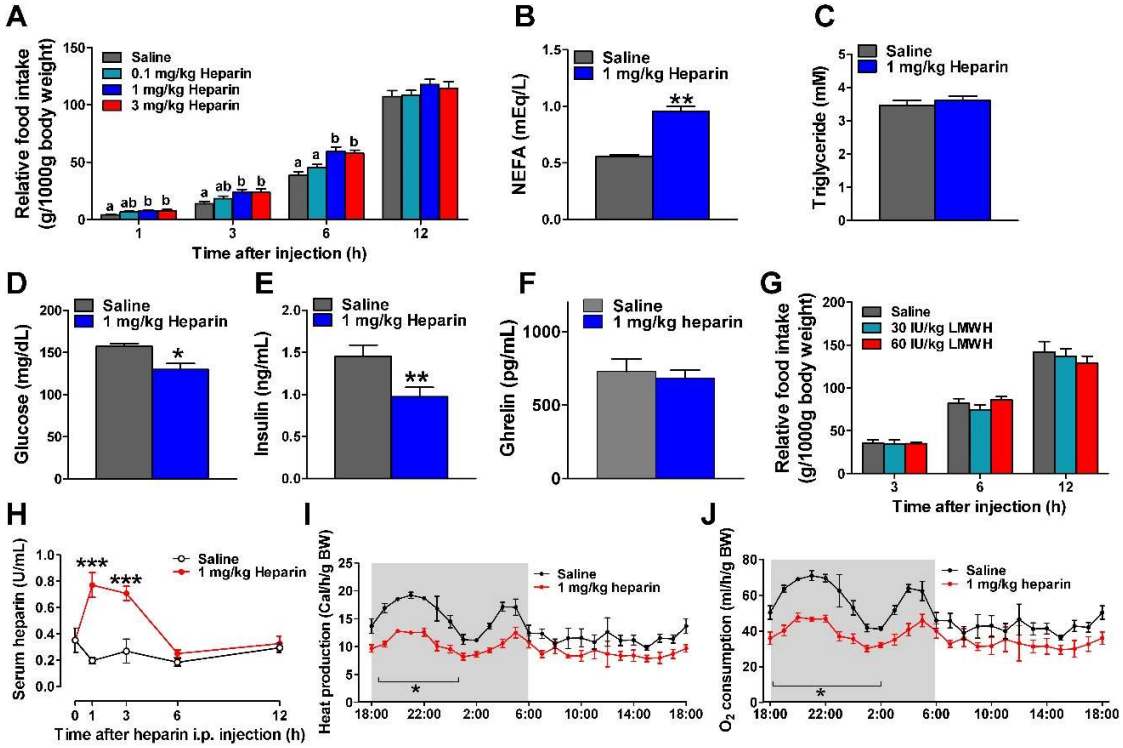

Figure S1. Acute metabolic effects of heparin. Related to Figure 1. (A) Dark cycle food intake of male Qunmin mice after i.v. injection of saline, 0.1, 1 or 3 mg/kg heparin. N=8-10/group. (B-F) Serum NEFA (B), triglyceride (C), glucose (D), insulin (E) and ghrelin (F) concentrations in male C57BL6/J mice 1 hr after i.p. injection of saline or 1 mg/kg heparin. N=5/group. (G) Dark cycle food intake of male Qunmin mice after i.v. injection of saline, 30 or 60 IU/kg LMWH. N=8, 9 or 10/group. (H) Serum heparin concentration-time profile obtained after i.p. administration of single dose of heparin in male C57BL6/J. N=6/group. (I-J) Heat production (I) and O2 consumption (J) of male C57BL6/J mice after i.p. injection of saline or 1 mg/kg heparin. N=4/group. Results are presented as mean ± SEM. (A and G) Different letters between bars mean P≤0.05 in two way ANOVA analyses followed by post hoc Bonferroni tests. (B-F) *, P≤0.05; **, P≤0.01 in non-paired student's t test. (H-J)*, P≤0.05; ***, P≤0.001 in two way ANOVA analyses followed by post hoc Bonferroni tests.

Figure S2

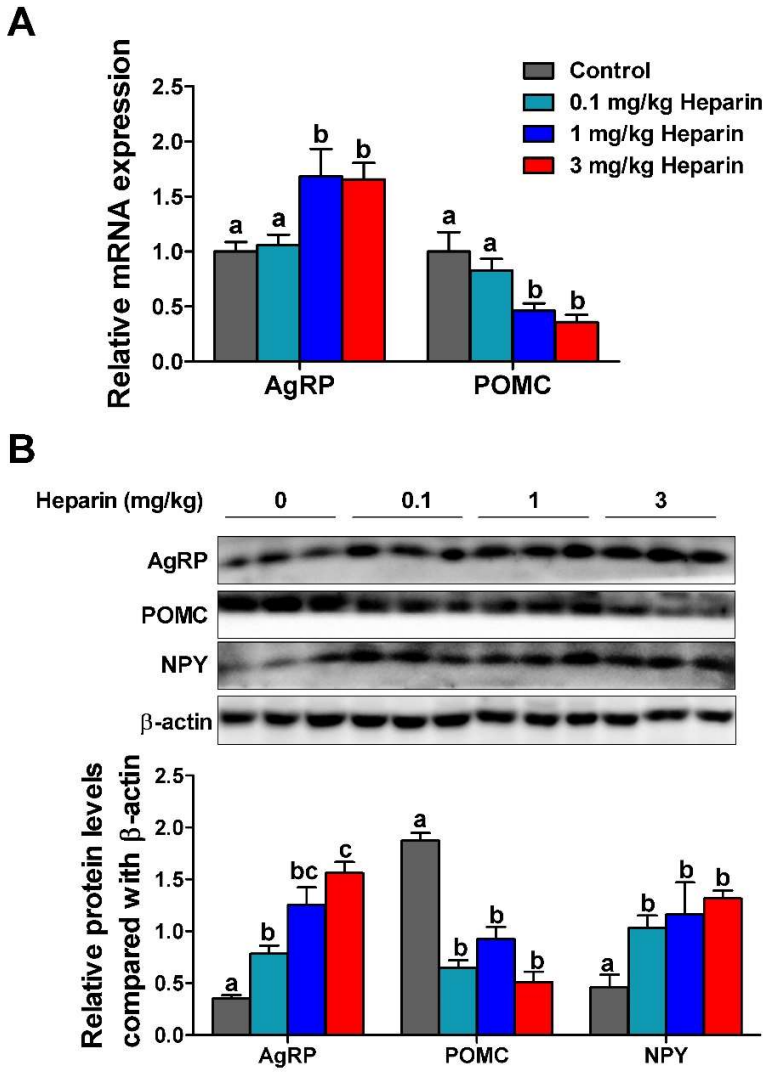

Figure S2. Dose response of heparin on hypothalamic expression of AgRP, NPY and POMC. Related to Figure 2. (A) mRNA expression of AgRP and POMC in the hypothalamus of male Qunmin mice after i.v. injection of saline, 0.1, 1 or 3 mg/kg heparin. N=6/group. (B) Immunoblots and quantification of AgRP, POMC and NPY protein expression in the hypothalamus of male Qunmin mice after i.v. injection of saline, 0.1, 1 or 3 mg/kg heparin. N=6/group. Results are presented as mean ± SEM. Different letters between bars mean $P \leq 0.05$ in one way ANOVA analyses followed by post hoc Tukey's tests.

Figure S3

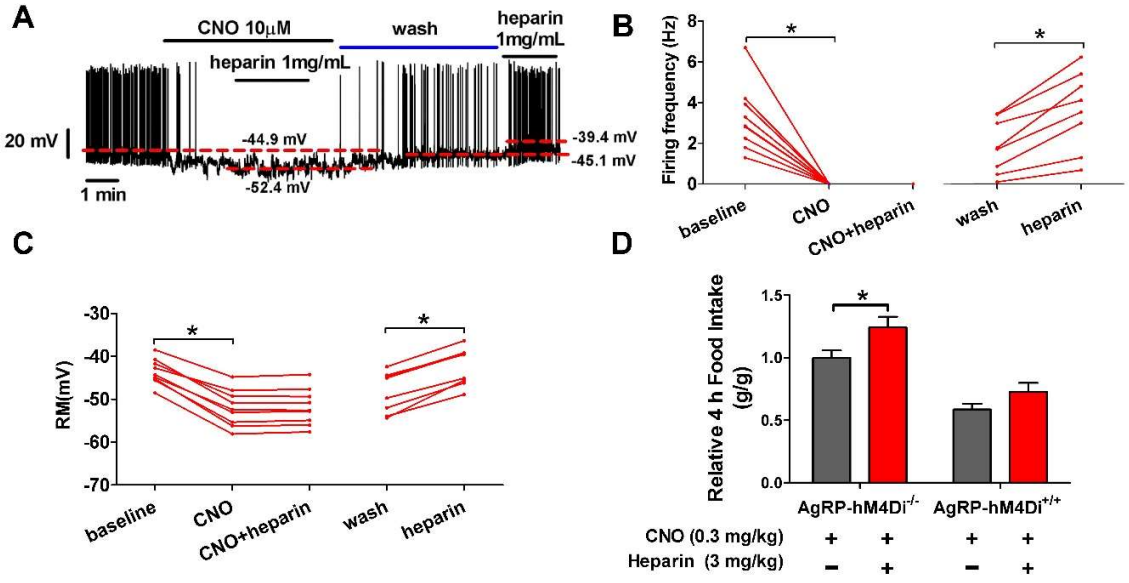

Figure S3. Effects of high dose of heparin. Related to Figure 5. (A) Representative electrophysiological response to CNO (10 μM) and high dose of heparin (1 mg/mL) in ARC AgRP neurons infected with inbhibitory AAV-hM4Di-mCherry. (B-C) Statistic of firing frequency (B) and resting membrane potential (C). (D) Effects of CNO (0.3 mg/kg) co-injected with saline or high dose of heparin (3 mg/kg) on dark cycle food intake measure in male AgRP-Cre mice receiving inhibitory AAV-hM4Di-mCherry infection in the ARC or injected with same virus but with missed target. N=7 or 4/group. Results are presented as mean ± SEM. *, P≤0.05 in non-paired student's t test.

Figure S4

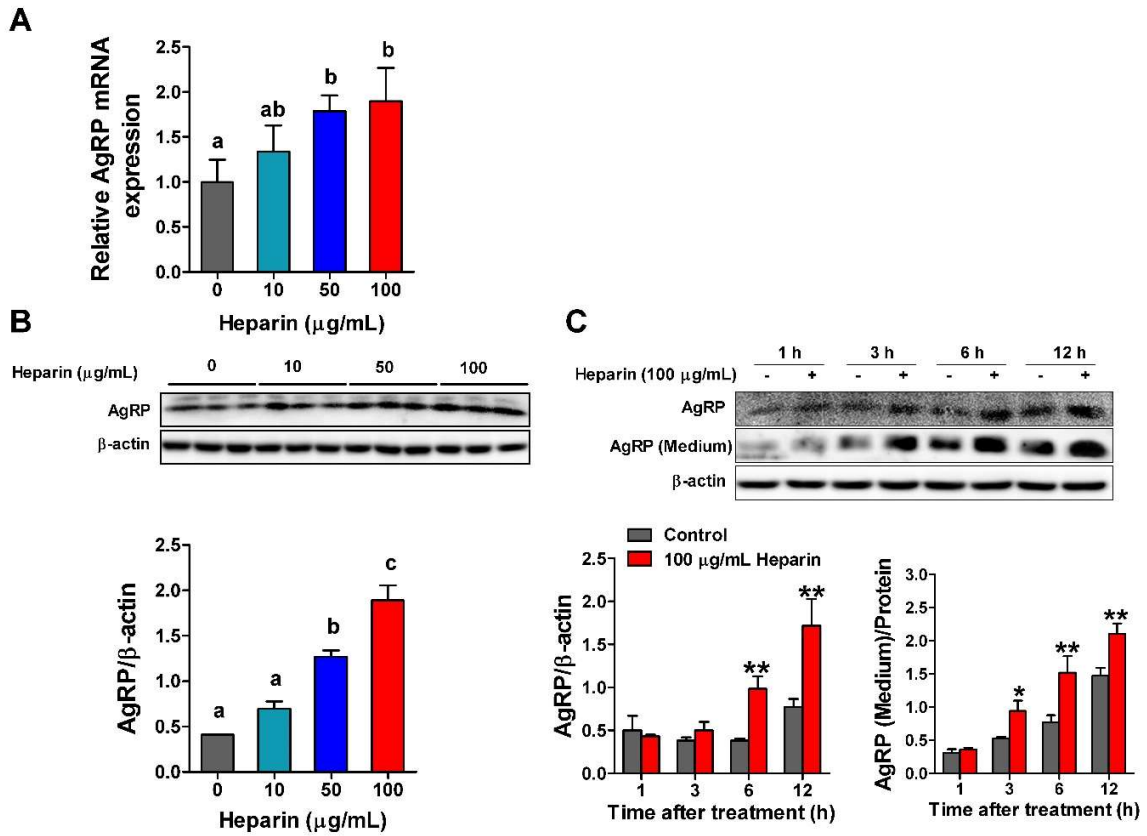

Figure S4. Heparin increases AgRP expression and secretion in N38 cells *in vitro*. Related to Figure 6. (A) mRNA expression of AgRP in N38 cells cultured with vehicle, 10, 50 or 100 μg/mL heparin for 12 hrs. N=6/group. (B) Immunoblots and quantification of AgRP protein expression in N38 cells cultured with vehicle, 10, 50 or 100 μg/mL heparin for 12 hrs. N=6/group. Results are presented as mean ± SEM. Different letters between bars mean $P \leq 0.05$ in one way ANOVA analyses followed by post hoc Tukey's tests. (C) Immunoblots and quantification of AgRP secretion in culture medium and AgRP protein expression in N38 cells treated with vehicle or 100 μg/mL heparin for 1, 3, 6, 12 hrs. N=6/group. Results are presented as mean ± SEM. *, $P \leq 0.05$ or **, $P \leq 0.01$ in two way ANOVA analyses followed by post hoc Bonferroni tests.

Figure S5

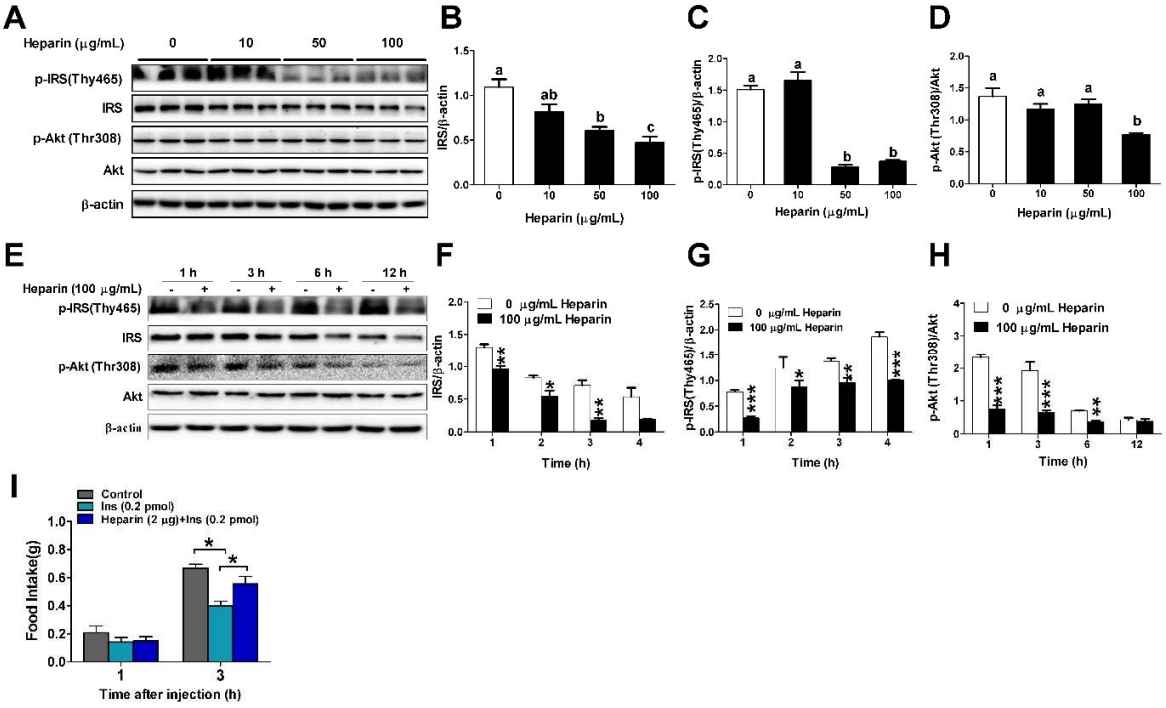

Figure S5. Heparin inhibits insulin/insulin receptor signaling pathway in N38 cells and high dose of heparin attenuates anorexigenic effect of insulin. Related to Figure 6. (A-D) Immunoblots (A) and quantification (B-D) of IRS, p-IRS and p-Akt/Akt protein expression in N38 cells cultured with vehicle, 10, 50 or 100 μg/mL heparin for 12 hrs. N=6/group. Results are presented as mean ± SEM. Different letters between bars mean P≤0.05 in one way ANOVA analyses followed by post hoc Tukey's tests. (E-H) Immunoblots (E) and quantification (F-H) of IRS, p-IRS and p-Akt/Akt protein expression expression in N38 cells treated with vehicle or 100 μg/mL heparin for 1, 3, 6, 12 hrs. N=6/group. (I) Dark cycle food intake of male C57BL6/J mice after i.c.v. injection of saline, 0.2 pmol insulin or heparin (2 μg) + insulin (0.2 pmol). N=4/group. Results are presented as mean ± SEM. *, P≤0.05, **, P≤0.01 or ***, P≤0.001 in two way ANOVA analyses followed by post hoc Bonferroni tests.

Figure S6

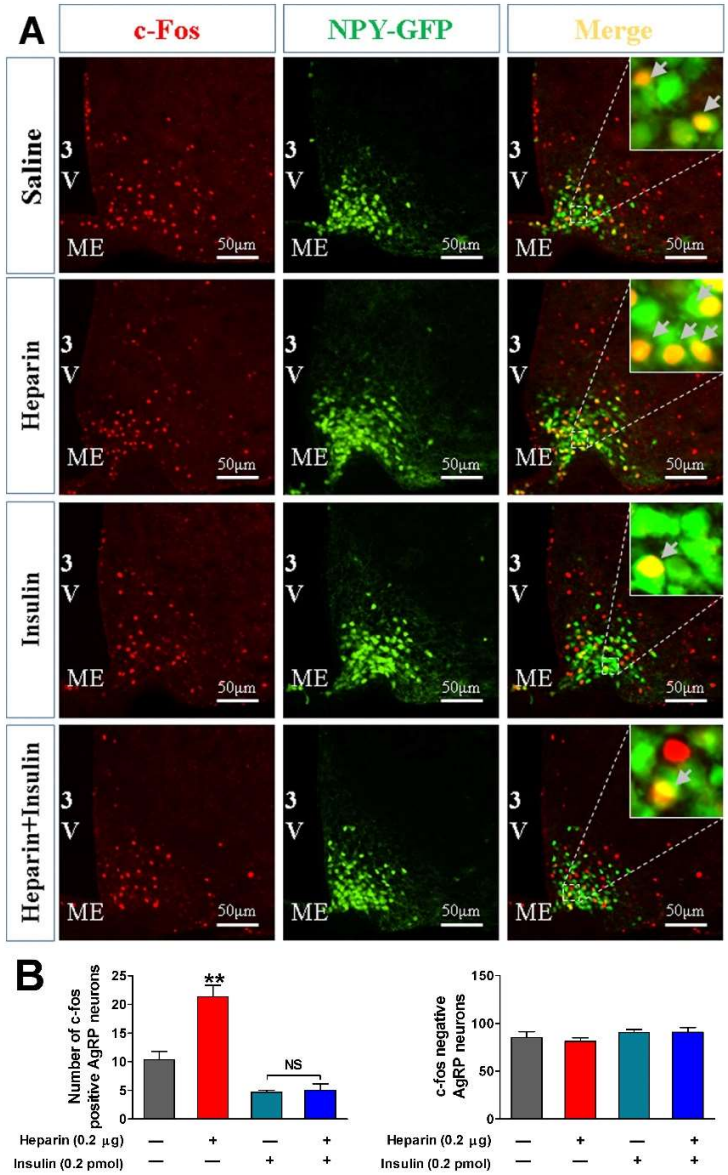

Figure S6. Insulin block stimulatory effects of heparin on AgRP activation. Related to Figure 6. (A) Representative images and quantification (B) of c-Fos expression in the ARC of NPY-GFP mice 3 hr after i.c.v. injection of saline, 0.2 μg heparin, 0.2 pmol insulin or heparin+insulin. N=3/group. Results are presented as mean ± SEM. **, P≤0.01 in two way ANOVA analyses followed by post hoc Bonferroni tests.

Figure S7

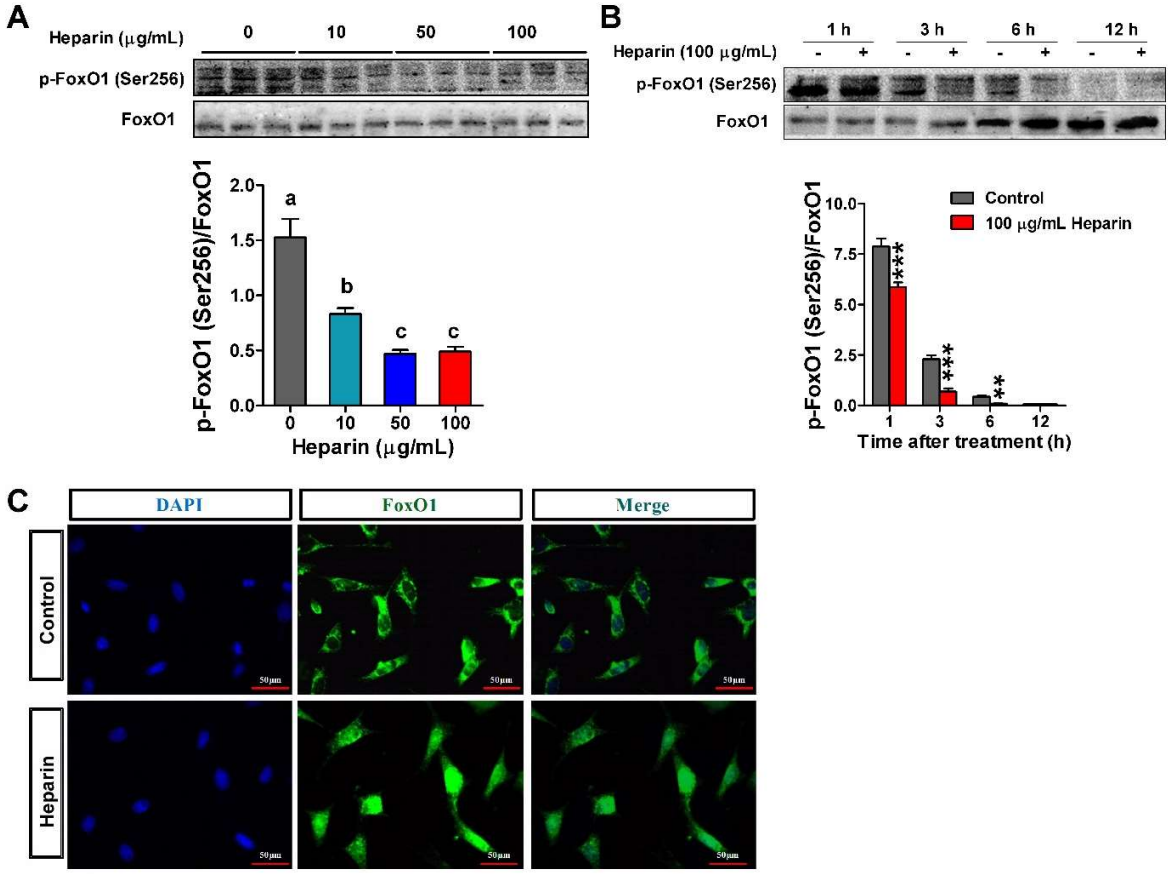

Figure S7. Heparin reduces FoxO1 activity in N38 cells *in vitro*. Related to Figure 7. (A) Immunoblots and quantification of p-FoxO1/FoxO1 protein expression in N38 cells cultured with vehicle, 10, 50 or 100 μg/mL heparin for 3 hrs. N=6/group. (B) Immunoblots and quantification of p-FoxO1/FoxO1 protein expression in N38 cells cultured with vehicle or 100 μg/mL heparin for 1, 3, 6 or 12 hrs. N=6/group. (C) Representative immunocytofluorescent images of FoxO1 transposition in N38 cells cultured with vehicle or 100 μg/mL heparin for 3 hrs. Dapi (blue, nucleus) and FoxO1 (green).

Supplemental Experimental Procedures

Animals

Both Qunmin and C57BL6/J mice were used for acute feeding studies. Long-term heparin effects were characterized in C57BL6/J mice. Several transgenic mouse lines including AgRP-Cre, NPY-GFP and tdTOMATO (#012899, #006417 and #007419, Jackson Laboratory, Bar Harbor, ME) were maintained on a C57BL6/J background and used for electrophysiological recording or DREADD specific activation of AgRP neurons. Qunmin and C57BL6/J mice were purchased from Animal Experiment Center of Guangdong Province (Guangzhou, Guangdong, China).

Cell culture

Embryonic mouse hypothalamic cell line N38 (mHypoE-38, CLU118, Cellutions, Burlington, NC, United State) was cultured in low glucose DMEM (11885092, Thermo Fisher Scientific, Carlsbad, CA, United State), supplemented with 10% Fetal Bovine Serum (16000044, Thermo Fisher Scientific), 100000 units/L of penicillin sodium, and 100 mg/L of streptomycin sulfate (11860038, Thermo Fisher Scientific) at 37 °C in a humidified atmosphere that contained 5% CO_2.

Heparin levels at different nutritional states

Male C57BL6/J mice at 10-week-old age were randomly divided into three groups according to body weight. These mice were maintained on normal chow diet, switched to calorie-restricted diet (60% calorie intake) for one week, or fasted for 24 hr. At the end of experiment, mice were deeply anesthetized and sacrificed. Blood serum samples from all three groups were collected to determine serum heparin level.

Peripheral effects of heparin on food intake

The effect of unfractionated heparin (S12004, ShangHai YuanYe Biotechnology, Shanghai, China) on food intake was assessed in both Qunmin and C57BL6/J mice. Following a 6-h food deprivation prior to the onset of dark cycle (6pm), Qunmin mice were weighed and randomly assigned to receive either 0 (saline), 0.1, 1 or 3 mg/kg heparin by intravenous (i.v.) injection. Food intake was measured 1, 3, 6 and 12 hr after i.v. injection. Hypothalamic samples were collected 3 hr after i.v. injection to determine the mRNA and protein expression of AgRP, POMC and NPY. In another separate trial, C57BL6/J mice were subjected to the same procedure but received 0, 0.1, 1 or 3 mg/kg heparin by intraperitoneal (i.p.) injection. Hypothalamic samples were collected 3 hr after i.p. injection for western-blot analysis of AgRP, POMC, p-IRS (Thy465), IRS, p-Akt (Thr308) and Akt. Additionally, another cohort of male C57BL6/J mice were i.p. injected with 1 mg/kg heparin and serum samples were collected 0, 1, 3, 6 and 12 hr after injection to measure serum heparin levels. Serum glucose, insulin, non-essential fatty acid (NEFA), triglyceride and ghrelin levels were also evaluated using Glucose and TG kit (BioSino, Beijing, China), NEFA Kit (Wako, 294-63601, Osaka, Japan), Insulin ELISA kit (ALPCO, 80-INSMSU-E01, Boston, United State) and Ghrelin Kit (Mskbio, Wuhan, Hubei, China) following the manufacturer's instructions. Similarly, to investigate the effects of heparin cleavage enzyme heparinase (H3917, Sigma, St. Louis, MO, United State) on food intake, singly housed C57BL6/J mice were i.p. injected with 0 (saline) or 5 U/kg heparinase after 6 hr fasting before dark cycle (6 pm). Food intake was measured for 1, 3 and 12 hr after injection. Additionally, the effects of low molecular weight heparin (LMWH, Z140647, ShangHai YuanYe Biotechnology, Shanghai, China) on food were also tested. As described above, 3, 6, 12 hr food intake was monitored after i.v. injection of 0 (saline), 30 or 60 IU/kg LMWH in Qunmin mice, while 1, 3 hr food intake was measured after i.p. injection of 0 or 100 IU/kg LMWH in C57BL6/J mice.

Central effects of heparin on food intake

To exam the central effect of heparin on food intake, a guide cannula with 2.3 mm projection (#62003, Plastics One, Roanoke, VA, United State) was stereotaxically implanted in the lateral ventricle. Specifically, 10-week-old male and female C57BL6/J mice were anesthetized with inhaled isoflurane, and an indwelling i.c.v. guide cannula was stereotaxically inserted to target the lateral ventricle (bregma, anterior-posterior: -0.4 mm; lateral: +1.3 mm; dorsal-ventral: -2.3 mm). One week after surgery, the cannulation accuracy was validated by the increase of drinking and grooming behavior after administration of 10 ng angiotensin II (A107852, Shanghai Aladdin Bio-Chem Technology, Shanghai, China) as we did before (Zhu et al., 2015). After validation, mice were fasted for 6 hrs before dark cycle (6 pm). In the beginning of dark cycle, mice received i.c.v. injections of saline or heparin (0.2 μg in 2 μL). Food intake was then measured for 1, 3, 6 and 12 hr after injection. Similarly, central effects of Desulphated heparin (0.2 μg in 2 μL, DSH003/N, Iduron, Nether Alderley, Cheshire, England) were tested in another cohort of male C57BL6/J mice. To exam if AgRP is required for orexigenic effects of heparin, female C57BL6/J mice were subjected to the same procedure but received saline, heparin (0.2 μg in 2 μL), Anti-AgRP IgG (1 μg in 2 μL, sc-50299, Santa Cruz, Dallas, TX, United State) or heparin+Anti-AgRP IgG (0.2 μg+1 μg) by i.c.v. injection. To exam if FoxO1 mediates the

orexigenic effects of heparin, another cohort of female C57BL6/J mice received saline, heparin (0.2 μg in 2 μL), AS1842856 (20 pmol in 2 μL, #344355, Millipore, Temecula, CA, United State) or heparin+AS1842856 (0.2 μg+20 pmol) by i.c.v. injection. To exam if heparin promote feeding by competing for insulin binding to the insulin receptor, female C57BL6/J mice received saline, heparin (0.2 μg in 2 μL), insulin (0.2 pmol, 91077C-100MG, Sigma) or heparin+insulin (0.2 μg+0.2 pmol). Another cohort of male C57BL6/J mice received saline, insulin (0.2 pmol) or heparin+insulin (2 μg+0.2 pmol).

CLAMS metabolic chambers

Male chow-fed C57BL6/J mice with matched body weight were singly housed in home cage. Heat production and O_2 consumption were monitored for 24 hrs after i.p. injection of saline or 1 mg/kg heparin at 6 pm in home cage by CLAMS (Promethion Metabolic Screening Systems, Sable systems International, North Las Vegas, NV, USA).

Long-term effects of heparin on body weight, food intake and body composition

Both male and female C57BL6/J mice were randomly divided into two groups according to body weight. Each group received either heparin (1mg/kg) or saline i.p. injection once per two days for 16 days. Food intake and body weight were measured every other day. At the end of the experiment, body composition was determined using quantitiative magnetic resonance (QMR, Niumag Corporation, Shanghai, China). Then mice were deeply anesthetized and sacrificed. Brown adipose tissue (BAT), gonadal WAT (gWAT) and inguinal WAT (iWAT) were isolated and weighed. An aliquot of iWAT was collected for hematoxylin and eosin (HE) staining. The hypothalamic samples from half of the male mice were collected and the protein expression of FoxO1 and p-FoxO1 was determined. Another half of the male mice were perfused and brain sections were collected for immunofluorescent staining of FoxO1.

Heparin effects on c-Fos expression of AgRP neurons

The expression of c-Fos in AgRP neurons after heparin treatment was examined in NPY-GFP reporter mice, which have NPY/AgRP neurons labelled with green fluorescence. Specifically, male NPY-GFP mice were i.p. injected with saline or 1 mg/kg heparin at 9 am in the morning. Three hours after injection, mice were perfused and brain sections were collected for c-Fos staining. Similarly, brain sections from another cohort of male NPY-GFP mice were used for c-Fos staining 3 hour after i.c.v. injection of saline, 0.2 μg heparin, 0.2 pmol insulin or heparin+insulin at 9 am in the morning. In a separate trial, after 6 hrs of fasting, male NPY-GFP mice were i.p. injected with saline or heparinase (i.p. 5U/kg) at 6 pm. Three hours after injection, mice were perfused and brain sections were collected for c-Fos staining.

Electrophysiology

Whole-cell patch clamp recordings were performed on TOMATO-labelled neurons (AgRP/NPY neurons) in the ARH-containing hypothalamic slices from AgRP-IRES-Cre/Rosa26-tdTOMATO mice. Six to twelve-week old mice, were deeply anesthetized with isoflurane in early morning and transcardially perfused with a modified ice-cold sucrose-based cutting solution (adjusted to pH 7.3) containing (in mM) 10 NaCl, 25 $NaHCO_3$, 195 Sucrose, 5 Glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 Na pyruvate, 0.5 $CaCl_2$, 7 $MgCl_2$ bubbled continuously with 95% O_2 and 5% CO_2, as we did before (Cao et al., 2014). The mice were then decapitated, and the entire brain was removed and immediately submerged in ice-cold sucrose-based cutting solution. The ARH-containing hypothalamic slices (250 μm) were cut with a Microm HM 650V vibratome (Thermo Fisher Scientific). The slices were recovered for 1 h at 34℃ in artificial cerebrospinal fluid (aCSF, adjusted to pH7.3) containing (in mM) 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11.1 glucose, and 21.4 $NaHCO_3$) saturated with 95% O_2 and 5% CO_2 before recording.

Slices were transferred to the recording chamber and allowed to equilibrate for at least 10 min before recording. The slices were perfused at 34°C in oxygenated aCSF at a flow rate of 1.8-2 mL/min. TOMATO-labelled AgRP/NPY neurons in the ARH were visualized using epifluorescence and IR-DIC imaging on an upright microscope (Eclipse FN-1, Nikon, Tokyo, Japan) equipped with a moveable stage (MP-285, Sutter Instrument, Novato, CA, United State). Patch pipettes with resistances of 3-5 MΩ were filled with intracellular solution (adjusted to pH 7.3) containing (in mM) 128 K gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 $MgCl_2$, 0.05 Na-GTP and 0.05 Mg-ATP. Recordings were made using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, United State), sampled using Digidata 1440A and analyzed offline with pClamp 10.3 software (Molecular Devices). Series resistance was monitored during the recording, and the values were generally < 10 MΩ and were not compensated. The liquid junction potential (LJP) was +12.5 mV, and was corrected after the experiment. Data would be excluded if the series resistance increased dramatically during the experiment or without overshoot for action potential. Current clamp was engaged to test neural firing and resting membrane potential before and after bath perfusion of heparin (100 μg/mL, 4 minute bath perfusion),

and after wash (4 minute). During the recording of each neuron, a lucifer yellow dye was injected into the recorded neuron via the pipette. Slices were fixed with 4% formalin in PBS at 4°C overnight and then subjected to post hoc identification of the anatomical location of the recorded neurons within the ARH.

In some experiment, the aCSF solution also contained 1 μM tetrodotoxin (TTX, a sodium channel blocker, #1078, R&D system, Minneapolis, MN, USA) and a cocktail of fast synaptic inhibitors, namely bicuculline (50 μM, a GABA receptor antagonist, Tocris.inc), AP-5 (30 μM, an NMDA receptor antagonist, Tocris.inc) and CNQX (30 μM, an NMDA receptor antagonist, Tocris.inc) to block the majority of presynaptic inputs. AP-5 (30 μM) and CNQX (30 μM) were used to block glutamatergic inputs, while bicuculline (50 μM) was included to block GABAergic inputs. For the miniature excitatory postsynaptic current (mEPSC) and miniature inhibitory post synaptic current (mIPSC) recordings, the internal recording solution contained (in mM): CsCH3SO3 125; CsCl 10; NaCl 5; MgCl2 2; EGTA 1; HEPES 10; (Mg)ATP 5; (Na)GTP 0.3 (pH 7.3 with NaOH). The mEPSC in AgRP/NPY neurons was measured in the voltage clamp mode with a holding potential of −60 mV in the presence of 1 μM TTX and 50 μM bicuculline. The mIPSC in AgRP/NPY neurons was measured in the voltage clamp mode with a holding potential of −70 mV in the presence of 1 μM TTX, 30 μM CNQX and 30 μM AP-5.

In other cohort of mice, slice was incubated with insulin (100 nM) pre-recording. During the recording, heparin (100 μg/mL) perfused (2 mL/min) application in the presence of 100nM insulin.

DREADD manipulation of AgRP neurons

To determine if AgRP neurons mediate the orexigenic effects of heparin, DREADD technology was used to inhibit AgRP neurons activity. Inhibitory DREADD vector (pAAV-hSyn-DIO-hM4D(Gi)-mCherry) was a gift from Bryan Roth (Addgene plasmid # 44362) (Krashes et al., 2011) and packaged in serotype 9 by Hanbio Biotechnology (Shanghai, China). At 8 weeks of age, AAV9-hM4Di-mCherry was stereotaxically delivered into the ARH of male AgRP-Cre (AgRP-hM4Di) or WT (control) littermates (bregma, anterior-posterior: -1.4 mm; lateral: ±0.2 mm; dorsal-ventral: -5.9 mm; 200 nL/side). After 2 weeks of recovery, 0.3 mg/kg CNO was i.p. injected in both control and AgRP-hM4Di mice at 6 pm. Food intake was monitored at 2, 4, 12 hr after i.p. injection. After a rest for 3 days, both control and AgRP-hM4Di mice were subjected to the same procedure but received saline + CNO or CNO + heparin (1 mg/kg). Food intake was recorded 4 hr after i.p. injection. On day 30 after virus injection, all mice were perfused with 10% formalin and brain sections were collection. Brain sections were then mounted and mCherry signals were monitored under fluorescent microscope for validation of injection accuracy. Only those mice with mCherry signals exclusively in the ARH were included in analyses for feeding behavior.

Another cohort of AgRP-Cre mice were injected with AAV9-hM4Di-mCherry into the ARH and effects of saline + CNO (0.3 mg/kg) or CNO + heparin (3 mg/kg) on food intake were monitored following the same procedure. The AgRP-Cre mice injected with same virus but with target missed were used as control (AgRP-hM4Di$^{-/-}$).

To validate that hM4Di-infected AgRP neurons can be inhibited by CNO, AAV9-hM4Di-mCherry was stereotaxically delivered into the ARH of another cohort of AgRP mice as described above. One week after virus infection, mice were sacrificed and ARH-containing brain slices were prepared. Effects of CNO (10 μM, bath superfusion) and CNO + heparin (100 μg/mL) or CNO (10 μM) + heparin (1 mg/mL) on resting membrane potential and firing rate of mCherry-labelled AgRP neurons were electrophysiological recorded.

In a separate trial, a Gs-linked DREADD (GsD) (Wess et al., 2013) was selectively expressed in AgRP neurons to exclusively increase the release of AgRP (Nakajima et al., 2016). GsD vector (pAAV-hSyn-DIO-rM3D(Gs)-mCherry) was a gift from Bryan Roth (Addgene plasmid #50458) and packaged in serotype 9 by Hanbio Biotechnology. Following the same procedures as in inhibitory DREADD experiments, effects of CNO (0.3 mg/kg, i.p.), CNO (0.3 mg/kg) co-injected with saline or heparin (1 mg/kg), or CNO (0.3 mg/kg) co-injected with saline or heparinase (5 U/kg) on dark cycle food intake were measured in male WT or AgRP-Cre mice receiving GsD vector infection in the ARH.

Heparin response in N38 cells *in vivo*

To determine the effects of heparin on FoxO1 phosphorylation, N38 cells were treated with 0, 10, 50 or 100 μg/mL heparin for 3 hrs and collected for western-blot analysis of FoxO1 and p-FoxO1 protein expression. Similarly, in another separate trial, N38 cells were treated with 0 or 100 μg/mL heparin for 1, 3, 6 and 12 hrs and collected for FoxO1 and p-FoxO1 immunoblotting. To determine the effects of heparin on translocation of FoxO1, N38 cells were

collected for FoxO1 staining after 3 hrs incubation with 0 or 100 μg/mL heparin.

N38 cell was used to determine if FoxO1 is required for heparin's stimulatory effects on AgRP neurons. The concentration and time of heparin treatment were first tested to optimize the stimulatory effects on AgRP expression and secretion. To optimize the concentration, N38 cells were treated with 0, 10, 50 or 100 μg/mL heparin for 12 hrs and collected for mRNA and protein analysis of AgRP. To optimize the time, N38 cells were treated with 0 or 100 μg/mL heparin for 1, 3, 6 and 12 hrs and both N38 cells and supernatant medium were collected to determine AgRP protein expression and secretion. To test the requirement of FoxO1, N38 cells were treated with vehicle (0.1% DMSO), 100 μg/mL heparin, 10 μM AS1842856 (FoxO1 antagonist) or 100 μg/mL heparin + 10 μM AS1842856 for 12 hrs and AgRP protein expression was determined in N38 cells after treatment.

To determine the effects of heparin on insulin-insulin receptor interaction, N38 cells cultured with vehicle or 100 μg/mL heparin for 3 hrs and then incubated with 100 nM insulin for 30 min. Half of the cells were lysed and immunoprecipitated with the mouse-anti-insulin (1:500, 8138, Cell signaling). Immunoprecipitates were then subjected to immunoblotting with rabbit-anti-insulin receptor (1:500, bs-0681R, Bioss). A portion of the total lysates (20 μg) was used as input and immunoblotted with anti-insulin receptor as above. Another half of the cells were collected for insulin immunocytofluorescence staining.

To determine heparin's effects on insulin signaling pathway, N38 cells were collected for p-IRS (Thy465), IRS, p-Akt (Thr308) and Akt immunoblotting after vehicle, 10, 50 or 100 μg/mL heparin treatment for 12 hrs or vehicle or 100 μg/mL heparin treatment for 1, 3, 6, 12 hrs.

Immunofluorescence
Brain sections were incubated with primary rabbit-anti-c-Fos antibody (1:1000, 2250, Cell Signaling Technology, Danvers, MA, United State) or rabbit-anti-FoxO1 (1:1000, 2880, Cell signaling) at room temperature overnight, followed by incubation in goat-anti-rabbit Alexa Fluor 555 (1:1000, bs-0295G-AF555, Bioss) or goat-anti-rabbit FITC conjugated secondary antibody (1:1000, bs-0295G, Bioss) for 1 hr. Sections were mounted on slides and cover slipped with Mounting Medium with DAPI (H-1200, Vector Laboratories, Burlington, ON, Canada). At least five coronal sections containing the ARH were imaged from each mouse brain.

Immunocytofluorescence
N38 cells were fixed using 4% paraformaldehyde and permeabilitized in a PBS solution containing 0.4% Triton X-100. Immunofluorescence staining was performed using either rabbit-anti-FoxO1 (1:1000, 2880, Cell signaling) and FITC-conjugated goat-anti-rabbit secondary antibodies (1:1000, bs-0295G, Bioss) or mouse-anti-Insulin (1:1000, 8138, Cell signaling) and FITC-conjugated goat-anti-mouse secondary antibodies (1:500, A32723, Thermo Fisher Scientific). Images were acquired using a Nikon imaging system (Nikon).

CHIP-PCR
FoxO1-flag (Addgene plasmid #12148) was transfected into N38 cells as previously described (Kitamura et al., 2006). Transfected N38 cells were treated with vehicle, 100 μg/mL heparin, 100 nM insulin or heparin+insulin (100 μg/mL+100nM). Three hrs after treatment, N38 cell was collected for immunoprecipitation using anti-Flag antibody (F1804, Sigma). Chromatin-immunoprecipitation (Chip)-PCR was performed using Chip Assay Kit (P2078, Beyotime, Haimen, Jiangsu, China) according the instructions provided by the manufacturer. AgRP binding activity was tested by using published primers (Kitamura et al., 2006) as follows: 5'-CCTGAAAGCTTTGTCCTCTGAAGC-3' and 5'-GCAGAACCTAGGGATGGGTCATGC-3' (-401 to -378 and -8 to +16 of AgRP, respectively).

Luciferase assay
To test heparin's effects on AgRP promoter activity, AgRP driven luciferase Agrp-pGL3 plasmid (17552, -401 to +16 of AgRP, addgene) was transfected into N38 cell along with control vectors as previously described (Kitamura et al., 2006). Twenty-four hrs after transfection, the cells were treated with vehicle, 100 μg/mL heparin, 10 μM AS1842856 or 100 μg/mL heparin + 10 μM AS1842856. Three hrs after treatment, the cells were lysed and promoter activity was measured by using Dual-Luciferase® Reporter Assay Kit (E1910, Promega) according to the manufacturer's instructions. In a separate trial, AgRP promoter activity was measured after vehicle (control), 100 μg/mL heparin, 100 nM insulin or 100 μg/mL heparin + 100 nM insulin treatment as described above.

Spectrophotometric determination of serum heparin

Serum heparin level was determined as previously described (Xu et al., 2006). Specifically, serum heparin concentration was evaluated by incubation with Pyronine G (107518, Merck Millipore, Darmstadt, Germany) in the Britton-Robinson buffer solution (a mixture of 0.04 M H_3PO_4, 0.04 M CH_3COOH, 0.04 M H_3BO_3 that was titrated to pH 4.1 with 0.2 M NaOH). Ten μL various reference heparin samples with known concentration (10, 8, 5, 2.5, 1.25, 0.0625, 0 mg/L) or serum were incubated with 25 μL 0.05% Pyronine G in pH=4.1 Britton- Robinson buffer and 165 μL tri-distilled water at 37 °C for 15 min. Pyronine G absorption at 575 nm was measured by FLx800 Fluorescence Reader (BioTek, Winooski, VT, United State). The reduction of Pyronine G absorption at 575 nm was directly proportional to the concentration of heparin. All measurements were conducted in duplicate. The final serum heparin concentration was calculated based on a standard curve generated from the reference samples using relative absorbance ΔA= A-A0 (A0: 0 mg/L heparin absorbance).

HE staining

An aliquot of iWAT was fixed with 10% formalin for overnight and then store in 50% ethanol. The fixed iWAT pads were embedded with paraffin, sectioned and stained with hematoxylin and eosin (HE). Pictures of stained iWAT tissue were obtained using a Leica Dm6000 M Microscope (Leica, Wetzlar, Germany). Adipocyte size in iWAT was analyzed using Image-Pro Plus software. Up to six fields of view were captured from the same location within each iWAT. Thirty adipocytes were measured per section. Data from 5 mice were collected and averaged for each group.

qPCR analyses

Real Time PCR assay was performed as described before (Cai et al., 2016). Briefly, total mRNA was extracted using TRIzol Reagent (15596-026, Thermo Fisher Scientific) according to the manufacturer's instructions. After DNase I digestion (2270A, Takara Bio, Kusatsu, Shiga, Japan), the total mRNA was reverse-transcribed to cDNA using the M-MLV Reverse Transcriptase (M1705, Promega, Madison, WI, United State) and oligo (dT) 18 primer (3806, Takara Bio) according the instructions provided by the manufacturer. SYBR Green Real-Time PCR was performed according to published protocols (Bookout and Mangelsdorf, 2003). Results were normalized by the expression of house-keeping gene β-actin. The primer sequences are shown as follows: β-actin, S: 5'-GGTCATCACTATTGGCAACGAG-3', A: 5'-GAGGTCTTTACGGATGTCAACG-3'; AgRP, S: 5'-CTTTGGCGGAGGTGCTAGAT-3', A: 5'-TGCGACTACAGAGGTTCGTG-3'; POMC, S: 5'-TTAAGCCGGTGGGCAAGA-3', A: 5'-GGACCTGCTCCAAGCCTAAT-3'.

Western blot analysis

Total protein were extracted from hypothalamic or N38 cells using RIPA lysis buffer (1 mM PMSF, P0100, Solarbio, Beijing, China) and protein concentration was determined using BCA protein assays (23225, Thermo Fisher Scientific). Equivalent amounts of protein (20 μg) were separated by 10% SDS PAGE and the samples were transferred onto nitrocellulose membranes (BioRad, Hercules, CA, United State). Membranes were then subjected to immunoblotting with rabbit-anti-AgRP (1:500, sc-50299, Santa cruz), rabbit-anti-POMC (1:1000, ab180766, abcam), rabbit-anti-NPY (1:500, sc-28943, Santa cruz), rabbit-anti-β-actin (1:2000, bs-0061R, Bioss, Woburn, MA, United State), goat-anti-p-IRS (1:500, sc-17194, Santa Cruz), rabbit-anti-IRS (1:1000, 3407, Cell signaling), rabbit-anti-p-Akt (1:1000, 9275S, Cell signaling), rabbit-anti-Akt (1:2000, 9272S, Cell signaling), rabbit-anti-FoxO1 (1:1000, 2880, Cell signaling) or rabbit-anti-p-FoxO1 (Ser256, 1:1000, 9461, Cell signaling), followed by donkey-anti-goat HRP conjugated secondary antibody or goat-anti-rabbit HRP conjugated secondary antibody (1:50000, bs-0294D or bs-0295G, Bioss). Western blots were visualized with SuperSignal West Pico Chemoluminescence substrate (Thermo Fisher Scientific) and qualified by Image J software.

Heparin-sepharose chromatography

Heparin-sepharose or sepharose (Wsac, CS-A13-0B, Beijing) was packed into a column and washed with the phosphate buffer (100 mM, pH7.4). N38 cells were lysed and applied to the heparin-sepharose or sepharose column. Proteins that interact with immobilized heparin were retained and all other proteins were washed out. The retained proteins were eluted from the column with the phosphate buffer (100 mM pH7.4) containing 2 M NaCl. The retained proteins were then subjected to immunoblotting with the rabbit-anti-insulin receptor (1:500, bs-0681R, Bioss) or rabbit-anti-β-actin (1:2000, bs-0061R, Bioss). A portion of the total lysates (20 μg) was used as input and immunoblotted with anti-insulin receptor and anti-β-actin as above.

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