ONLINE DATA SUPPLEMENT

Leptin restores endothelial function via endothelial PPARγ- Nox1 mediated mechanisms in a mouse model of congenital generalized lipodystrophy.

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MATERIAL AND METHODS

Animals

Lipodystrophic mice deficient in Bscl2 (gBscl2$^{-/-}$) were generated, characterized and provided by Dr. Weiqin Chen (Augusta University)[1]. 10-12 week-old male animals were compared to their wildtype littermate control (gBscl2$^{+/+}$). Mice with inducible deletion of the Bscl2 gene were generated by Dr. Weiqin Chen by crossing floxed Bscl2 mice on the C57BL/6 background with Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J mice, which enables a temporal control of floxed gene expression by tamoxifen induction *in vivo*. Bscl2 deletion was induced via activation of cre recombinase in 10-week-old mice by 5 consecutive days of tamoxifen administration via gavage (0.1mL of a 20mg/mL solution in corn oil). Inducible endothelial specific leptin receptor deficient mice (LepR$^{EC-/-}$) were generated by crossing Cdh5-CreERT2 mice (from R. Adams, Max-Planck-Institute) with LepR$^{flox/flox}$ (Jackson Laboratory). Endothelial specific deletion of LepR was induced via activation of the endothelial specific cre [2] in 8-week-old mice by 14 consecutive daily intraperitoneal injections of tamoxifen (0.1mL of a 20mg/mL solution in corn oil)[3]. Animal receiving tamoxifen injections ($^{-/-}$) were compared to vehicle (corn oil)-treated animals ($^{+/+}$). All animals were fed standard mouse chow, and tap water was provided ad libitum. Mice were housed in an American Association of Laboratory Animal Care-approved animal care facility at Augusta University. Augusta University Institutional Animal Care and Use Committee approved all protocols (IACUC protocol #2011-0108).

Treatments

Animals were treated with saline or leptin (0.3mg/kg/day, ProSpec, Israel) via subcutaneous osmotic mini-pumps (ALZET, Cupertino, Calif; model 1007D, 0.5 µL/h) for 7 days as previously described [4-6]. Another set of animals was treated with a Sodium-Glucose Cotransporter 2 Inhibitor (Dapagliflozin, Selleckchem TX-USA, 1mg/Kg/day in drinking water) for 7 days [7].

Metabolic characterization and Interleukin 1β (IL-1β) measurement

At the end of the 7-day treatment, mouse body composition was analyzed by nuclear magnetic resonance spectroscopy (EchoMRI)[8]. Mice were then anesthetized (isoflurane 5%) and euthanized via decapitation, in accordance with our approved animal protocol. Trunk blood was collected for plasma isolation. Non-fasting blood glucose was measured using a glucometer (AlphaTRAK, Abbott, USA). Plasma total cholesterol and triglycerides were measured with enzymatic colorimetric assays (Wako, Richmond, VA, USA). Plasma leptin and IL-1β levels were determined using ELISA kit from ALPCO Diagnostics (Salem, NH, USA) and R&D System (Mineapolis, MN, USA) respectively.

Vascular function

Thoracic aortas were dissected surgically, cleaned of surrounding fat, cut in four rings and mounted on a wire myograph (DMT) as described previously [8]. Briefly, two tungsten

wires were inserted into the lumen of the arteries and fixed to a force transducer and a micrometer. Arteries were bathed in a physiological salt solution and arterial viability was determined with a potassium-rich solution (40 mmol/l). Endothelium-dependent and independent relaxations were respectively tested with concentration response curves (CRC) to acetylcholine (ACh, Sigma Aldrich, MO); (0.1 nmol/l to 100 µmol/l) and sodium nitroprusside (SNP, Sigma Aldrich, MO-USA); (0.1 nmol/l to 10 µmol/l) in vessels preconstricted with serotonin (5HT, 0.1-1 µM, Sigma Aldrich, MO). CRC to ACh were repeated in the presence of inhibitor N-nitro-l-arginine methyl ester [l-NAME; 100 µmol/L, unspecific nitric oxide synthase (NOS) Sigma Aldrich, MO-USA], tempol (100 µmol/l; SOD mimetic, Sigma Aldrich, MO-USA), polyethylene glycol (PEG) catalase (200 U/ml, Sigma Aldrich, MO-USA), GKT137831 (10 µmol/l; NOX1 and 4 inhibitor, GenKyoTex, Switzerland), GKT771 (10 µmol/l; specific Nox1 inhibitor, Genkyotex, Switzerland), pioglitazone (10 µmol/l; peroxisome proliferator-activated receptor gamma, PPAR-γ activator, Tocris, United Kingdom), GW9962 (5 µmol/l, PPAR-γ antagonist, Cayman, MI-USA) or leptin (10µg/mL for 2 hours) to determine the involvement of Nox1-derived reactive oxygen species (ROS) and PPAR-γ to the endothelial dysfunction, as well as to investigate the direct vascular effects of leptin. CRCs to ACh and SNP are presented as percent of 5HT-induced constriction. The individual CRC were fitted by nonlinear regression analysis. pD2 (defined as the negative logarithm of the EC50 values) and maximal response (Emax) were determined.

Oxidative stress measurement

Vascular oxidative stress was measured by dihydroethidium (DHE, Thermo Fisher Scientific, NH-USA) and Amplex Red (Thermo Fisher Scientific, NH-USA) in thoracic aortas as previously described. [9, 10]

Real-time PCR

Total aortic mRNA was extracted (Trizol Plus, Invitrogen, Carlsbad, Calif) and the concentration was established with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, Del). Complementary DNA was generated by RT-PCR with SuperScript III (Thermo Fisher Scientific, NH-USA). Reverse transcription was performed at 50 °C for 50 min; the enzyme was heat inactivated at 85 °C for 5 min, and real-time quantitative RT-PCR was performed with the SYBR-Green Supermix (Bio-Rad Laboratories, Hercules, Calif). Genes analyzed were Nox family members (Nox1, Nox2, Nox4, NoxA1 and NoxO1), as well as PPAR-γ, Tumor Necrosis Factor–α (TNF- α), Interleukin 1β (IL-1β), Forkhead box protein P3 (FOXP3), F4/80, Bscl2, Leptin Receptor (LepR) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as housekeeping gene. The sequence of the primers is included in Table S1.

Western blot

Aorta homogenates were separated via gradient SDS/PAGE (4–11% gel) (Bio-Rad Laboratories) and transferred to Immobilon-P poly (vinylidene fluoride) membranes. To determine the expression of relevant proteins, immunoblots were probed with antibodies targeting endothelial nitric oxide synthase (eNOS, BD Transduction Laboratories, San

Jose, CA-USA, catalog nº 610297), phosphorylated eNOS (Ser^{1177}, BD Transduction Laboratories, San Jose, CA-USA, catalog nº 612393), Signal transducer and activator of transcription 3 (STAT3; BD Transduction Laboratories, San Jose, CA-USA, catalog nº 610190), phosphorylated STAT3 (Tyr^{705}, Cell Signaling, Danvers, MA-USA, catalog nº 9145S and β-actin (Sigma-Aldrich, MO-USA, catalog nº A3854). Protein expression was normalized by β-actin.

Pulmonary endothelial cells isolation

Mice were sacrificed and their lungs excised and pooled, washed in PBS and diced into small pieces, which were incubated in Dulbecco's Modified Eagles Medium (DMEM; Gibco, Thermo Fisher Scientific, NH-USA) containing 10% fetal bovine albumin (FBS), 2 mg/mL of collagenase II and 40 mg/ml dispase-II at 37°C for 1 hour while shaking. Cell suspension was vigorously vortexed and meshed through 40 µm nylon cell strainers (Fisherbrand, Thermo Fisher Scientific, NH-USA). After centrifugation, the cell pellet was resuspended in 1X PBS with 0.5% bovine serum albumin (BSA) and 2 mM ethylendiaminetetraacetic acid (EDTA). Endothelial cells were labeled with CD31-conjugated magnetic MicroBeads and sorted using magnetic separation LS columns (Miltenyi Biotech, German).

Human Umbilical Endothelial Cells (HUVEC)

In order to analyze whether leptin regulates PPARγ expression, HUVEC cells (Lonza, MD-USA) were stimulated with metreleptin, a synthetic analog of the hormone leptin, (10ng/mL, Aegerion, Cambridge, MA-USA). In order to check the ability of PPARγ to modulate Nox1 activity and the efficacy of GKT771 to inhibit Nox1, HUVECs were transfected with Nox1/NoxO1/NoxA1[11] and incubated either with pioglitazone (10 µmol/l) or GKT771 (10 µmol/l) 2h before superoxide production measurements. Superoxide production was measured by using the luminol-based chemiluminescent probe, L012, 24h after transfection[12].

Aortic human samples

In order to investigate whether human vessels develop leptin resistance, which would limit the potential beneficial effects of leptin, discarded aortic and vein specimens were obtained from human patients undergoing open-heart-surgery at Augusta University Medical Center. Human subject protocol was approved by Institutional Review Board at the Medical College of Georgia at Augusta University (#1200473-1). Tissue sections were separated in small section about 5 mm x 5mm, placed in DMEM containing 20% FBS and stimulated with metreleptin (10ng/mL) from 15-60 minutes to analyze STAT3 phosphorylation by western blot and 8-24 hours to check PPARγ gene expression.

Reference

1. Chen W, Chang B, Saha P, Hartig SM, Li L, Reddy VT, Yang Y, Yechoor V, Mancini MA, Chan L. Berardinelli-seip congenital lipodystrophy 2/seipin is a cell-autonomous regulator of lipolysis essential for adipocyte differentiation. *Mol Cell Biol*. 2012;32:1099-1111
2. DeFalco J, Tomishima M, Liu H, Zhao C, Cai X, Marth JD, Enquist L, Friedman JM. Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. *Science*. 2001;291:2608-2613
3. Lanahan AA, Lech D, Dubrac A, Zhang J, Zhuang ZW, Eichmann A, Simons M. Ptp1b is a physiologic regulator of vegf signaling in endothelial cells. *Circulation*. 2014
4. Bruder-Nascimento T, Butler BR, Herren DJ, Brands MW, Bence KK, Belin de Chantemele EJ. Deletion of protein tyrosine phosphatase 1b in proopiomelanocortin neurons reduces neurogenic control of blood pressure and protects mice from leptin- and sympatho-mediated hypertension. *Pharmacol Res*. 2015;102:235-244
5. Belin de Chantemele EJ, Muta K, Mintz J, Tremblay ML, Marrero MB, Fulton DJ, Stepp DW. Protein tyrosine phosphatase 1b, a major regulator of leptin-mediated control of cardiovascular function. *Circulation*. 2009;120:753-763
6. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature*. 1999;401:73-76
7. Joubert M, Jagu B, Montaigne D, Marechal X, Tesse A, Ayer A, Dollet L, Le May C, Toumaniantz G, Manrique A, Charpentier F, Staels B, Magre J, Cariou B, Prieur X. The sglt2 inhibitor dapagliflozin prevents cardiomyopathy in a diabetic lipodystrophic mouse model. *Diabetes*. 2017
8. Bruder-Nascimento T, Ekeledo OJ, Anderson R, Le HB, Belin de Chantemele EJ. Long term high fat diet treatment: An appropriate approach to study the sex-specificity of the autonomic and cardiovascular responses to obesity in mice. *Front Physiol*. 2017;8:32
9. Silva MA, Cau SB, Lopes RA, Manzato CP, Neves KB, Bruder-Nascimento T, Mestriner FL, Montezano AC, Nguyen Dinh Cat A, Touyz RM, Tostes RC. Mineralocorticoid receptor blockade prevents vascular remodelling in a rodent model of type 2 diabetes mellitus. *Clin Sci (Lond)*. 2015;129:533-545
10. da Costa RM, Fais RS, Dechandt CRP, Louzada-Junior P, Alberici LC, Lobato NS, Tostes RC. Increased mitochondrial ros generation mediates the loss of the anti-contractile effects of perivascular adipose tissue in high-fat diet obese mice. *Br J Pharmacol*. 2017;174:3527-3541
11. Chen F, Barman S, Yu Y, Haigh S, Wang Y, Black SM, Rafikov R, Dou H, Bagi Z, Han W, Su Y, Fulton DJ. Caveolin-1 is a negative regulator of nadph oxidase-derived reactive oxygen species. *Free Radic Biol Med*. 2014;73:201-213
12. Jagnandan D, Church JE, Banfi B, Stuehr DJ, Marrero MB, Fulton DJ. Novel mechanism of activation of nadph oxidase 5. Calcium sensitization via phosphorylation. *J Biol Chem*. 2007;282:6494-6507

Table S1: List of primers		
IL-1β	**Forward**	5’ TCA CAG CAG CAC ATC AAC AAG 3’
	Reverse	5’ CCA GCA GGT TAT CAT CAT CAT CC 3’
TNF-α	**Forward**	5’ GCA AGC AGC CAA CCA G 3’
	Reverse	5, ACA AGC AGG AAT GAG AAG AG 3’
F4/80	**Forward**	5’ TCCTGCTGTGTCGTGCTGTTC 3’
	Reverse	5’ GCCGTCTGGTTGTCAGTCTTGTC 3’
FOXP3	**Forward**	5’ GGCCCTTCTCCAGGACAGA 3’
	Reverse	5’ GCTGATCATGGCTGGGTTGT 3’
CD36	**Forward**	5’ GATGACGTGGCAAAGAACAG 3’
	Reverse	5’ TCCTCGGGGTCCTGAGTTAT 3’
CYP27a1	**Forward**	5’ GCCTTGCACAAGGAAGTGACT 3’
	Reverse	5’ CGCAGGGTCTCCTTAATCACA 3’
Nox1	**Forward**	5’ CATGGCCTGGGTGGGATTGT 3’
	Reverse	5’ TGGGAGCGATAAAAGCGAAGGA 3’
Nox2	**Forward**	5’ CAAGATGGAGGTGGGACAGT 3’
	Reverse	5’ GCTTATCACAGCCACAAGCA 3’
Nox4	**Forward**	5’ TGTTGCATGTTTCAGGTGGT 3’
	Reverse	R 5’ AAAACCCTCGAGGCAAAGAT 3’
NoxA1	**Forward**	5’ ACGGTGGATGTTCTGTGTGA 3’
	Reverse	5’ AAGCATGGCTTCCACATAGG 3’
NoxO1	**Forward**	5’ ACACGTCGGGGGCATACTGGTC 3’
	Reverse	5’ GGCTGCCTCTGGGTTGGGATA 3’
PPAR-γ (mouse)	**Forward**	5’ TGCCAAAAATATCCCTGGTTTC 3’
	Reverse	5’ GGAGGCCAGCATCGTGTAGA 3’
PPAR-γ (human)	**Forward**	5’ TTCAAGAGTACCAAAGTGCAATCAA 3’
	Reverse	5’ AATAAGGTGGAGATGCAGGCTC 3’
Bscl2	**Forward**	5’ GCTCTTCTGCACCATCCTTC 3’

	Reverse	5’ CGGTGGAGGAATCACAGTC 3’
Leptin Receptor	**Forward**	5’ TGATTTGTGTTTGATTTATTGTTT 3’
	Reverse	5’ TATTCGTAGACGTGGGTTGG 3’
GADPH	**Forward**	5’ ACCCAGAAGACTGTGGATGG 3’
	Reverse	5’ CACATTGGGGGTAAGGAACAC 3’
GADPH	**Forward**	5’ ACCCAGAAGACTGTGGATGG 3’
	Reverse	5’ CACATTGGGGGTAAGGAACAC 3’

Table S2. Metabolic Characteristics in non-fasting mice

Parameters	**Vehicle**		**Leptin (10ug/day for 7 days)**	
	gBscl2+/+	***gBscl2-/-***	***gBscl2+/+***	***gBscl2-/-***
Cholesterol (mg/dL)	165.7 ± 12	162.7 ± 6.9	153.4 ± 16	177.8 ± 16.1
Triglycerides (mg/dL)	234.15 ± 16	239.2 ± 22	271.6 ± 23	262.6 ± 29
Glucose (mg/dL)	170.5 ± 6.9	339.6 ± 30*	153 ± 25	192 ± 25†
Leptin (ng/mL)	4.03 ± 0.3	0.38 ± 0.1*	9.70 ± 4.2	3.01 ± 1.0

Data are presented as means ± standard error of the mean (SEM). *P < 0.05 (versus gBscl2+/+ treated or not with leptin). †P < 0.05 (versus gBscl2-/-) n= 6 to 8.

Table S3. Characteristics of patients

Patient	Sex	Age	Weight (kg)	Height (cm)	BMI	Diabetes
1	Male	77	85	181	25.9	No
2	Female	63	61.5	173	20.5	No
3	Female	65	180	187	51.5	No
4	Male	61	86	180	26.5	Yes

BMI: Body Mass Index

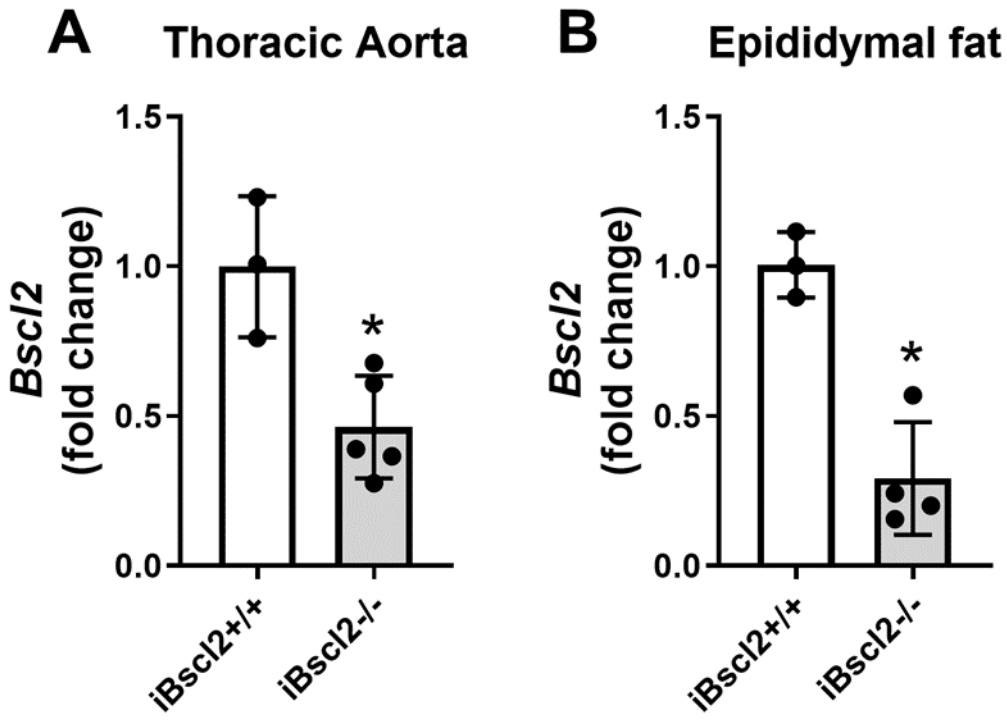

Figure S1. *Bscl2 gene expression in vascular and adipose tissue.* Bscl2 gene expression in thoracic aorta (A) and epididymal fat depot (B) measured by RT-PCR from Gt(ROSA)26Sortm1 (cre/ERT2)Tyj/J mice treated with corn oil (iBscl2+/+) or tamoxifen (iBscl2-/-). Mice were treated for five consecutive days. Experiments were performed one week after the last injection. Data are presented as means ± standard error of the mean (SEM). N=4-5 *P<0.05 vs gBscl2+/+; *P<0.05 vs iBscl2+/+. T-test was used for comparison between two groups.

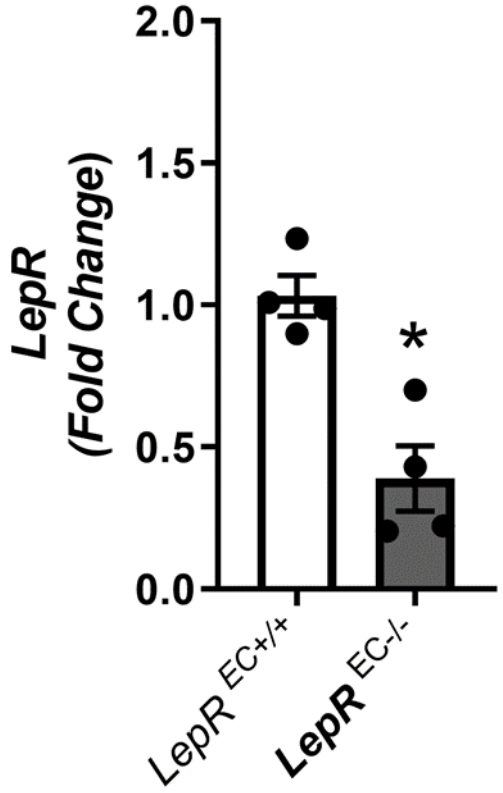

Figure S2. *Knockdown in leptin receptor expression in endothelial cells.* Leptin receptor gene expression in pulmonary endothelial cells from LepR EC+/+ or LepR EC-/- mice measured by real time-PCR. Data are presented as means ± standard error of the mean (SEM). N=4. *P<0.05 vs LepR $EC^{+/+}$. *T-test* was used for comparison between two groups.

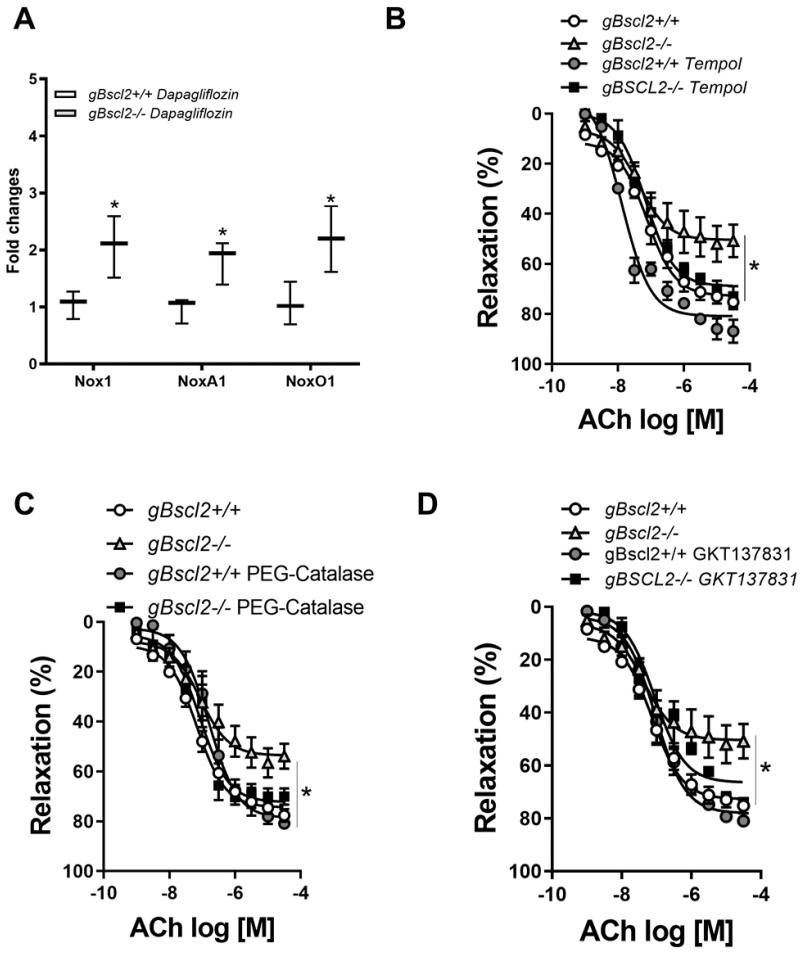

Figure S3. *Reactive oxygen species induce endothelial dysfunction in lipodystrophy independent of high glucose levels.* Effects of Sodium Glucose Co-transporter 2 (SGLT2, dapagliflozin 1mg/Kg/day for 7 days) on Noxs gene expression in thoracic aorta from gBscl2+/+ and gBscl2-/- (A). Concentration Response Curve (CRC) to acetylcholine (Ach) in presence of Tempol (100 µM) (B), PEG-Catalase (200U/mL) (C) or (GKT137831 (10µM) (D) in aortic rings from gBscl2+/+ and gBscl2-/-. Data are presented as means ± standard error of the mean (SEM). Real Time PCR graphs are presented as interleaved from minimal to maximal. N=4-6 *P<0.05 vs gBscl2+/+. Two-way ANOVA test followed by Tukey's multiple comparation test was used for comparison between four groups.

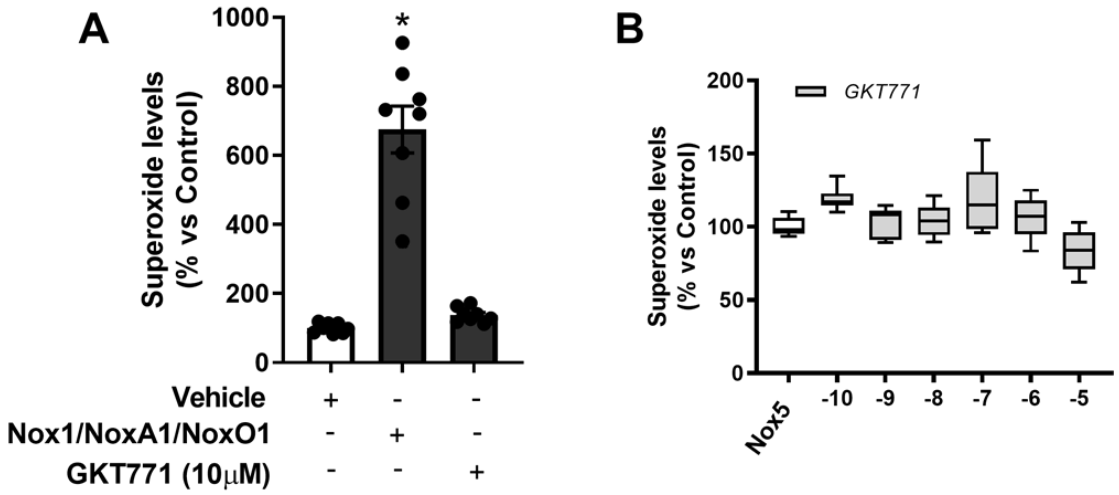

Figure S4. *GKT771 selectively inhibits NOx1 activity*. Superoxide production was measured by the chemiluminescent probe, L012 in HUVEC transduced with Nox1/NoxA1/NoxO1 (A) or Nox5 (B). Graph A is presented as means ± standard error of the mean (SEM). Graph B is presented as interleaved from minimal to maximal. N= 6-8. *P<0.05 vs Nox1/NoxA1/NoxO1 transduced HUVEC. One-way ANOVA followed by Tukey's multiple comparation test was used to comparison between different groups.

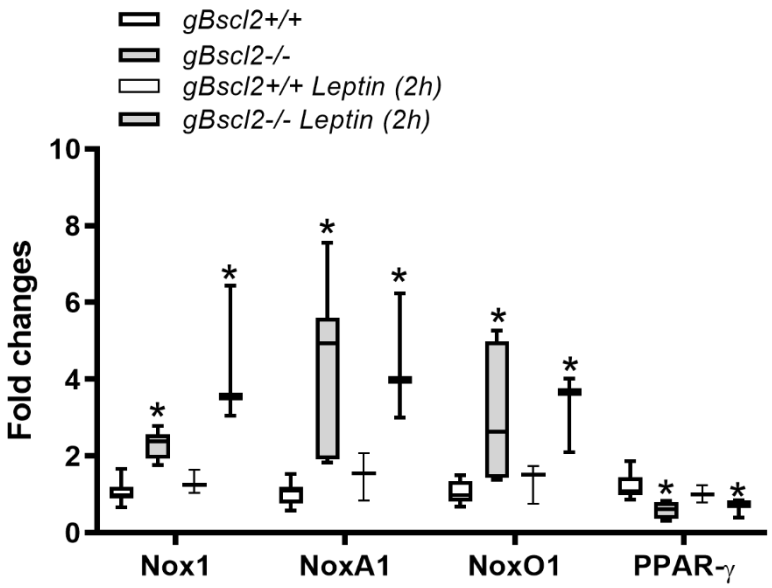

Figure S5. *Short term incubation with leptin does not alter Noxs enzymes and PPAR γ expression in thoracic aorta.* Effects of leptin incubation (10µg/mL, 2h) on Noxs enzymes and PPAR γ gene expression in thoracic aorta from gBscl2+/+ and gBscl2-/- mice. Data are presented as interleaved from minimal to maximal. N= 3-9. *P<0.05 vs gBscl2+/+. Two-way ANOVA test followed by Tukey's multiple comparation test was used for comparison between four groups.

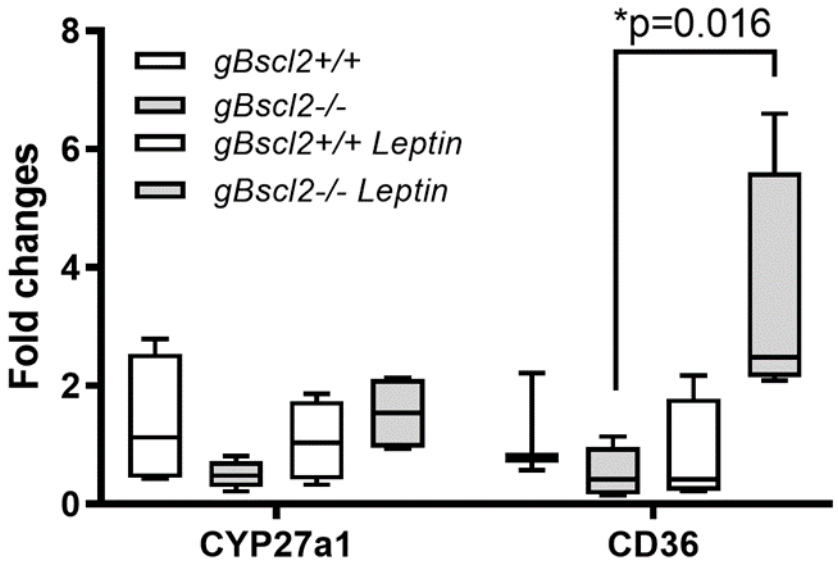

Figure S6. *Leptin treatment increases vascular PPARγ target genes in lipodystrophy.* Effects of leptin treatment (10µg/day for 7 days, osmotic mini-pump) on PPARγ target genes in aortas from gBscl2+/+ and gBscl2-/- mice. Data are presented as interleaved from minimal to maximal. N= 4-6. *P<0.05 vs gBscl2+/+. Two-way ANOVA test followed by Tukey's multiple comparation test was used for comparison between four groups.

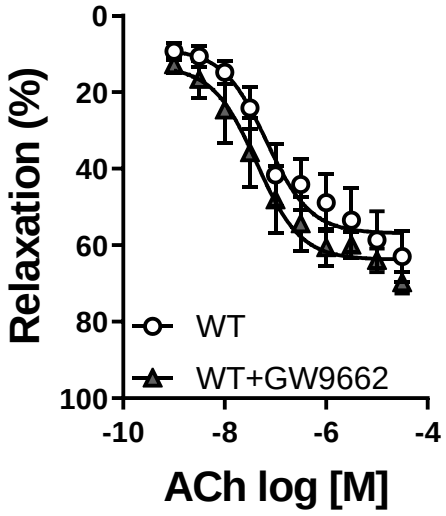

Figure S7. *PPARγ blockade in vitro does not affect the vascular relaxation.* Concentration Response Curve (CRC) to acetylcholine (Ach) in presence of GW9662 (2h, 5 μM) in aortic rings from wild type mice. Data are presented as means ± standard error of the mean (SEM). N=6. T-test was used for comparison between the groups.