Chronic hyperinsulinemia reduces insulin sensitivity and metabolic functions of brown adipocyte

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Abstract

The growing pandemics of diabetes have become a real threat to world economy. Hyperinsulinemia and insulin resistance are closely associated with the pathophysiology of type 2 diabetes. In pretext of brown adipocytes being considered as the therapeutic strategy for the treatment of obesity and insulin resistance, we have tried to understand the effect of hyperinsulinemia on brown adipocyte function. We here with for the first time report that hyperinsulinemia-induced insulin resistance in brown adipocyte is also accompanied with reduced insulin sensitivity and brown adipocyte characteristics. CI treatment decreased expression of brown adipocyte-specific markers (such as PRDM16, PGC1 α , and UCP1) and mitochondrial content as well as activity. CI-treated brown adipocytes showed drastic decrease in oxygen consumption rate (OCR) and spare respiratory capacity. Morphological study indicates increased accumulation of lipid droplets in CI-treated brown adipocytes. We have further validated these findings *in vivo* in C57BL/6 mice implanted with mini-osmotic insulin pump for 8 weeks. CI treatment in mice leads to increased body weight gain, fat mass and impaired glucose intolerance with reduced energy expenditure and insulin sensitivity. CI-treated mice showed decreased BAT characteristics and function. We also observed increased inflammation and ER stress markers in BAT of CI-treated animals. The above results conclude that hyperinsulinemia has deleterious effect on brown adipocyte function, making it susceptible to insulin resistance. Thus, the above findings have greater implication in designing approaches for the treatment of insulin resistance and diabetes via recruitment of brown adipocytes.

Key Words

- ▶ human mesenchymal stem cells
- ▶ differentiation
- ▶ brown adipocytes
- ▶ insulin
- ▶ insulin resistance

Journal of Endocrinology
(2016) **230**, 275–290

Introduction

Obesity has become a world-impending problem consuming \$2 trillion of the world economy (McKinsey 2014 report). According to WHO, nearly one third of the adult world population comes in overweight and obese category (WHO report 2015). Obesity is defined as excess accumulation of body fat or adipose tissue and is considered to be the antecedent of metabolic disorders such as insulin resistance (IR), type 2 diabetes, hypertension, dyslipidemia, cardiovascular diseases, etc. (Ye 2013). Numerous studies, on both animals and humans, have inferred negative correlation between body weight and insulin sensitivity (Cinti 2012). Weight gain in the body is largely due to accumulation of white adipose tissue (WAT); thus, insulin resistance can directly be correlated with WAT (Wajchenberg 2000). In last couple of decades, scientists have explored the composition of different fat pads and their physiological functions. There are mainly two functionally divergent fat tissues present in adults, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is abundant in body, whereas the latter is barely present in adults. Although the association of WAT and insulin resistance is known since three decades, recently scientist have correlated the decrease in BAT with obesity and insulin resistance (Vijgen *et al.* 2011). Lowell and coworkers showed that brown adipose tissue-ablated mice exhibit increased obesity, diabetes, and hyperlipidemia (Lowell *et al.* 1993, Hamann *et al.* 1996). 18 FDG-PET/CT studies have corroborated decreased BAT content in person with increased body mass index (BMI) (Cypess *et al.* 2009, Ouellet *et al.* 2011). Above studies indicate that a balance between WAT and BAT is necessary to maintain energy homeostasis and healthy body composition. Recent evidences have associated BAT with non-shivering thermogenesis, lipolysis, fatty acid oxidation, insulin sensitivity, and improved serum lipid profile (Zhang *et al.* 2014). Study conducted by Gray *et al.* showed that hyperinsulinemia precedes insulin resistance in mice lacking pancreatic leptin signaling (Gray *et al.* 2010). This was further confirmed in obese human by Alemzadeh and coworkers in which authors have shown beneficial effect of diazoxide on obese hyperinsulinemic adults (Shanik *et al.* 2008). Clinical observations have shown higher basal insulin level in obese persons, which indicates inefficiency of adipose tissue to respond to insulin (Erdmann *et al.* 2008). The effect of hyperinsulinemia on WAT has been studied extensively, but not much has been interrogated in BAT. The effect of prolonged hyperinsulinemia on functioning of BAT

in obese person remains to be explored. The beneficial effects of BAT have motivated scientific community and it is now being stipulated as a promising therapeutic strategy to cure obesity and insulin resistance (Liu *et al.* 2013). Before soliciting further, whether this strategy will work in a hyperinsulinemic obese environment needs to be established.

In this study for the first time, we have shown the effects of hyperinsulinemia on differentiated and characterized brown adipocytes. Basal chronic hyperinsulinemia (500 pM insulin exposure for 72 h) causes insulin resistance in both white and brown adipocytes differentiated from hMSC. Surprisingly, we found decreased expression of brown adipocyte markers such as UCP1, PGC1 α , and less active mitochondria on chronic treatment with insulin. Brown adipocytes on CI treatment show decreased oxygen consumption and respiratory capacity. We have also validated these findings in chronic exogenous hyperinsulinemia-induced insulin resistance in mice model. Our results further strengthens the existing notion that hyperinsulinemia acts as a precursor for the development of insulin resistance in both white and brown adipocytes.

Research design and methods

Isolation and characterization of hMSC from liposuction sample

Liposuction samples were obtained from patient undergoing fat reduction surgery with their informed consent. Approval for isolation of adipose-derived stem cells was obtained from Institutional Committee on Stem Cell Research (ICSCR) of Dr Ram Manohar Lohia Institute of Medical Science (RML-IMS), Lucknow (IEC No: 20/14). Stromal vascular fraction (SVF) was isolated from liposuction samples using collagenase digestion as described in Zuk *et al.* (2002) with few modifications. Briefly, lipoaspirate was washed thoroughly in phosphate buffer saline (PBS) containing 1% penicillin/streptomycin solution (Gibco) and digested with 1% collagenase type-Ia (Sigma Chemical) for 30 min at 37°C. Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 20% FBS (Gibco) was added to nullify the action of collagenase and centrifuged at 400g for 10 min. The pellet obtained was suspended in DMEM and filtered through 70 μ M cell strainer. Isolated cells were characterized using human MSC analysis kit (Catalogue: 562245) obtained from BD stem flow. The characterization was done based on surface markers CD73+, CD90+, and CD105+ using flow cytometry.

hMSC culture and differentiation into white and brown adipocytes

hMSC was cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (Gibco) and antibiotics. hMSC was differentiated using a differentiation cocktail containing 500 μ M IBMX, 5 μ g/mL insulin, 1 μ M dexamethasone, and 200 μ M indomethacin (all purchased from Sigma Chemicals). Treatment of differentiation cocktail was given as shown in Fig. 1(C). Fully mature white adipocytes (WA) after 12 days of differentiation were treated as mentioned in the figure legends and in the Results section.

Human mesenchymal stem cell are known to differentiate into white and brown adipocytes; recently, Elabd and coworkers showed that human adipose-derived multipotent stem cell can also be differentiated into functionally active brown adipocytes (Elabd *et al.* 2009). We followed a similar protocol with few modifications. We used induction media containing 0.5 μ M rosiglitazone and 2 nM T₃ (tri-iodothyronine) to differentiate hMSC into brown adipocytes (Fig. 2A). A schematic diagram of white and brown adipocyte differentiation protocol is shown in Figs. 1C and 2A.

Insulin resistance development

To mimic basal hyperinsulinemia, differentiated adipocytes were incubated with 500 pM insulin (Sigma, cat no I5500) in 10 mM glucose media supplemented with 10% FBS for 72 h. Medium was changed every 24 h to replenish the insulin level. After 72 h, cells were washed extensively using KRH buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, and 0.33 mM CaCl₂) supplemented with 5 mM glucose and 0.5% BSA (step-down media) before acute insulin pulsing.

Oil red O staining

Performed as described elsewhere (Beg *et al.* 2015).

[3H] 2-deoxyglucose uptake

Determination of 2-DOG uptake in differentiated adipocyte was performed as described previously (Sashidhara *et al.* 2014). Glucose uptake was measured in triplicate well (three sets of experiments) and normalized with total protein expressed as fold induction with respect to unstimulated cells. Radioactivity retained by the cell lysate was measured by liquid scintillation

counter (Beckman Coulter LS 6500; Beckman Coulter Inc, Brea, CA, USA).

Real-time PCR

Total RNA was isolated from cells using TRIZOL reagent (Invitrogen). First-strand cDNA synthesis was performed using high-capacity cDNA reverse transcription kit (Applied Biosystems) and subsequently used for quantitative real-time PCR analysis on Light Cycler 480 (Roche Diagnostics) using SYBR Green master mix (Roche diagnostics). Statistical analysis of the quantitative real-time PCR was done using ($2^{-\Delta\Delta C_t}$) method, which calculates the relative changes in gene expression. Relative change in gene expression was normalized with endogenous reference gene (18S rRNA). The primers used to check the expression of different genes are presented in Table 1.

JC-1 and tetramethylrhodamine, ethyl ester (TMRE) staining

JC-1 (M34152) and TMRE (T669) dye were procured from Invitrogen Life Technologies and experiments were performed as per the manufacturers' protocol, briefly fully differentiated adipocytes were washed with PBS and incubated with TMRE (500 nm) and JC1 (2 μ M) for 30 min. The cells were washed with PBS and images were taken using high-content screening platform Celloomics (Array Scan V^{II}).

Mitochondrial respiration study

To study mitochondrial respiration activity, hMSC were seeded in eight-well Flux analyzer (Seahorse Biosciences, North Billerica, MA, USA) cell plate at a density of 10,000 cells/well. Cells were allowed to grow till confluence and were differentiated into brown and white adipocytes as mentioned above. Oxygen consumption rate (OCR) of white and brown adipocytes was determined using XFp Extracellular Flux analyzer (Seahorse Bioscience). We used 1 μ M oligomycin, 1 μ M FCCP, and 0.5 μ M rotenone/antimycin A mixture as per manufacturer's protocol. Readings were normalized with total protein concentration.

Western immunoblotting

Western blotting was performed as described previously (Beg *et al.* 2015). Densitometric quantification of protein bands was performed using National Institute of Health (NIH) Image J software.

Figure 1

Insulin resistance model development in adipocyte differentiated from hMSC. hMSC after 1 day of isolation (image a), confluent hMSC (image b), and hMSC differentiated into fully mature adipocyte stained with oil red O (image d), all the images were taken at 10x magnification with Leica DFC450 C (A). hMSC isolated from six different liposuction samples were pooled together for flow cytometry analysis in BD FACS Caliber. Negative cocktail contained CD34, CD45, CD11b, and CD19. 20,000 counts were taken in each reading (B). Schematic diagram of differentiation treatment and western blot analysis of adipocyte-specific transcription factors at different time intervals during the period of 15 days of differentiation treatment, $n=3$ (C). Schematic diagram of chronic insulin treatment in adipocyte (D). Glucose uptake was measured at 24-h intervals during the period of chronic insulin treatment. Control and CI-treated adipocytes were stimulated with 10 nM insulin for 20 min. Data are represented as fold difference, $n=3$, error bars represent s.d., *** $P<0.001$ as compared by Student's t -test (E). Glucose uptake of control and 72-h CI-treated adipocytes with and without 10 nM insulin stimulation for 20 min, $n=3$ error bars represent s.d., ** $P<0.005$ as compared by Student's t -test (F). All glucose uptake readings were normalized with respective protein concentration. Western blot analysis of IRS1 and GLUT4 in control and CI-treated adipocytes normalized with actin. Densitometry of respective blots are shown in left panel, $n=3$, error bars indicate s.d., * $P<0.05$ at tested by Student's t -test (G). Western blot analysis of p-AKT (Ser 473) and pAS160 (Thr 642) in control and CI-treated adipocytes upon varying concentration of insulin stimulation (20 min). Densitometry of blots normalized with total AKT and AS160 protein is shown adjacent to the blots, $n=3$, error bars represent s.d., *** $P<0.001$ as compared by Student's t -test (H).

Figure 2

Differentiation of hMSC into brown adipocytes. Schematic diagram of brown adipocyte differentiation protocol (A). Brown adipocyte differentiated using the above mentioned protocol was subjected to RNA isolation and real-time PCR. Expression profile of brown adipocyte-specific marker genes compared with white adipocytes, $n=3$, error bars represent s.d., *** $P<0.001$ as tested two-way ANOVA and Bonferroni's post-test analysis (B). Real-time PCR analysis of genes related to mitochondrial biogenesis and fatty acid oxidation, $n=3$, error bars represent s.d., *** $P<0.001$ as tested two-way ANOVA and Bonferroni's post-test analysis (C and D). Western blot analysis of PRDM16, PGC1 α , and UCP1 in white and brown adipocytes. Densitometry of representative blots normalized with actin, $n=3$, error bar indicates s.d., *** $P<0.001$, ** $P<0.01$, and * $P<0.05$ as tested by Student's t -test (E). White and brown adipocytes were grown in 96-well eppendroff clear bottom imaging plate and stained with JC-1 dye. Cells were excited with 405 or 488 nm and image was taken at 20 $\times$ magnification using Cellomics (high-content screening platform). Images were analyzed using image J software. The red fluorescence shows high-membrane potential mitochondria (J aggregates). The green fluorescence shows low-membrane potential mitochondria (L monomers). The graph on the right-hand side is the relative quantification of red to green ratio normalized with nuclear stain DAPI. $n=3$, the error bars denote s.d., *** $P<0.001$ (F). Brown and white adipocytes were stained with TMRE dye. The cells were excited at 405 nm. Images were taken at 20 $\times$ magnification and analyzed similar to the above experiment. The graph besides the image shows the relative quantification of mean red intensity normalized with DAPI. $n=3$, error bars represent s.d., *** $P<0.001$ as tested by Student's t -test (G). The oxygen consumption rates (OCR) of brown and white adipocytes at basal level and in the presence of ATP synthase inhibitor (1 μ M oligomycin), proton uncoupler (1 μ M FCCP), and electron transport chain inhibitors (0.5 μ M rotenone/antimycin mix) was measured with Seahorse Bioscience XFp Extracellular Flux analyzer. $n=3$, error bars represent s.d., ** $P<0.005$, *** $P<0.001$ as tested by Student's t -test (H).

Animal care and treatment

The study was conducted in 6 to 8-week-old C57BL/6 male mice at the animal facility of CSIR-Central Drug Research Institute. All experimental procedures were approved by the institutional animal ethics committee and were conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEAs), India. The mouse housing environment includes a temperature of $23 \pm 2^\circ\text{C}$, humidity 50–60%, light 300 Lux at floor level with regular 12h light cycle.

Totally, 16 male mice were housed (eight mice/cage) and given water and normal chow diet *ad libitum*.

Implantation of mini-osmotic pump

Alzet osmotic pumps filled with saline or insulin (glargine) were primed for at least 24h in sterile saline in a 37°C water bath according to the manufacturer's instructions. These saline- or insulin-filled pumps were implanted subcutaneously for 4 weeks and re-implanted the fresh saline- or insulin glargine-filled pump for

Table 1 Primer sequences used for quantitative RT-PCR.

<i>Homo sapeins</i>		<i>Mus musculus</i>	
Gene	Primer sequence	Gene	Primer sequence
<i>PGC1d</i> Reverse	TTATTGGGAAATGCCTCCTG	<i>Elovl3</i> Reverse	GGCACCATCTTTGGCATACT
<i>PGC1d</i> Forward	GGGTCATTTGGTGACTCTGG	<i>Elovl3</i> Forward	AGTGTTCGGTTGTTGTGTGG
<i>PRDM16</i> Reverse	CGGATGTTCCCCAACAAAGTA	<i>Atp5g1-001 Atp synthase</i> Reverse	CATTTGTGTGCCAGTTTCA
<i>PRDM16</i> Forward	CATTTGTA CTGCGCTCCT	<i>Atp5g1-001 Atp synthase</i> Forward	GGGACAGGGATTGATTACAC
<i>NRF1</i> Reverse	CCATCTGGTGGCCTGAAG	<i>Cox IV</i> Reverse	CTCCCTGCCTCCCTCACT
<i>NRF1</i> Forward	GTAGTGCCTGGGTCCATGA	<i>Cox IV</i> Forward	AGCATGGACCATTGGATACG
<i>ATP5G1-001 ATP synthase</i> Reverse	AGGGCTAAAGCTGGGAGACT	<i>Cox III</i> Reverse	TAGCCTCGTACCAACACATGA
<i>ATP5G1-001 ATP synthase</i> Forward	TTAGACCCTGGTACAACAGC	<i>Cox III</i> Forward	AGTGGTGAAATTCCTGTTGGA
<i>CIDEA</i> Reverse	GTCATGGGAAGCGAGCAC	<i>Fatp1</i> Reverse	GACAAGCTGGATCAGGCAAG
<i>CIDEA</i> Forward	TGGGTTTATTGCAGGAGATGA	<i>Fatp1</i> Forward	GAGGCCACAGAGGCTGTTC
<i>DIO2</i> Reverse	TACCCGTCTTAGGCAGCAAC	<i>Cdh15</i> Reverse	CGCCGGAACACTTACTATGC
<i>DIO2</i> Forward	AAACATCTGCTGGGTCTTCG	<i>Cdh15</i> Forward	GCGTCAGTGGCTAGCTGATT
<i>ELOVL3</i> Reverse	AACCTGCAAGGGCCTCTC	<i>Ucp1</i> Reverse	TAAGCCGGCTGAGATCTTGT
<i>ELOVL3</i> Forward	GCCCCAGGATACTGAAGAT	<i>Ucp1</i> Forward	GGCCTCTACGACTCAGTCCA
<i>UCP1</i> Reverse	GGAAGCAAGATTTAGCTGGTCT	<i>Pgc1a</i> Reverse	GAACCTTGGGGTCATTTG
<i>UCP1</i> Forward	GTGGGTTGCCAATGAATAC	<i>Pgc1a</i> Forward	CCCATACACAACCGCAGTC
<i>MPZL2</i> Reverse	TCTGCCTGTGCACTGATGAT	<i>Dio2</i> Reverse	TGAGAAGTCTGGTACATCAGCAA
<i>MPZL2</i> Forward	GAGCTCTTCGGCCCATC	<i>Dio2</i> Forward	TGGCATGCCCTGTAGTTT
<i>SIRT1</i> Reverse	TTCAGACCATTTTGAACATCACT	<i>Nrf1</i> Reverse	GAACAACACAGATTCCATGCTC
<i>SIRT1</i> Forward	GAACAGCTATATTTCAAACCTTTT	<i>Nrf1</i> Forward	GCCTCCCGATAGTTTGAGAAA
Cytochrome oxidase Reverse	TTCTGTTGCGGGAAGCTC	<i>Cpt1a</i> Reverse	TGGCATAGCTGTCAATAGATGC
Cytochrome oxidase Forward	GAAGAACGTAGCCGCTTGG	<i>Cpt1a</i> Forward	GACGAATCGGAACAGGGATA
Cytochrome oxidase III Reverse	GCAGGATTTTCTGAGCCTTT	<i>Prdm16</i> Reverse	CACCTTCGCTTTTCTACCC
Cytochrome oxidase III Forward	GGGATTTAGCGGGGTGAT	<i>Prdm16</i> Forward	CCTAAGGTGTGCCAGCA
Cytochrome C Reverse	ATAGCGTCCACAGGCATAGC	<i>Tnfα</i> Forward	CATCTTCTAAAATTCGACTCACAA
Cytochrome C Forward	ACAGTCCGAAGATGATGGTAAAG	<i>Tnfα</i> Reverse	TGGGAGTAGACAAGGTACAACCC
<i>SLC27A1</i> Reverse	GTGTGAGGTGCCAGGAA	<i>Mapk8</i> Forward	AGAAACTGTTCCCGATGTG
<i>SLC27A1</i> Forward	TCCTTCTCAAGACCTGAGC	<i>Mapk8</i> Reverse	ACAAATCTCTTGCTGACTGG
<i>RhsR-MCAD</i> Reverse	GGAGCCATTGATGTGTGCT	<i>IL1β</i> Forward	AGTTGACGGACCCAAAAG
<i>RhsF-MCAD</i> Forward	CTGCTTTGGTCTTTATACCAGCTA	<i>IL1β</i> Reverse	AGCTGGATGCTCTCATCAGG
<i>CPT1 β</i> Reverse	CCCTATGCTCTACAGCTTCCA	<i>Ccl2</i> Forward	CTTCTGGGCTGCTGTTCA
<i>CPT1 β</i> Forward	GGGCGCACAGACTCTAGGTA	<i>Ccl2</i> Reverse	CCAGCCTACTATTGGGATCA
		<i>IL6</i> Forward	GAGGATACCACTCCCAACAGACC
		<i>IL6</i> Reverse	AAGTGCATCATCGTTGTTATACA
		<i>F4/80</i> Forward	TGTCTCTTGCCTGGAC
		<i>F4/80</i> Reverse	GAGACTTCTGAGCTGACACTGC

additional 4 weeks. So the total duration of the pump implanted with saline or insulin (glargine) was 8 weeks. For insertion of pumps, mice were anesthetized briefly with ketamine (80 mg/kg) and xylazine (10 mg/kg). The insertion site was shaved and disinfected with povidone-iodine and 70% alcohol. Post-surgery and implantation of mini-osmotic pumps, wounds were closed with sterile sutures and monitored throughout experiment. None of the mice exhibited signs of infection at the insertion site. About 10% glucose solution was provided in drinking water for the first 7 days to prevent hypoglycemia. These mice were kept on the normal chow diet.

Serum and organ collection

After 8 weeks, mice were fasted for 6 h and killed using anesthetic ether. Blood was collected from orbital sinus. Serum was isolated by centrifugation at 12,000g for 15 min at 4°C. Epididymal white adipose tissue (eWAT) and interscapular brown adipose tissue (iBAT) were excised from the 8-week continuous saline and CI-treated animals after acute pulsing with insulin (0.75 U/kg).

Serum insulin and lipid profile

Serum levels of total cholesterol, triglyceride, LDL-cholesterol, and HDL-cholesterol were analyzed using kits from Randox Laboratory Ltd (Mumbai, India). Serum insulin concentrations were determined by enzymatic immunoassay (SPI-BIO, Bertin Pharam, Montigny le-Bretonneux, France).

Body weight

Body weight of the mice was recorded weekly.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was performed as described previously. Briefly, mice were fasted for 6 h. Basal fasting glucose was measured with the help of Accu-Check active glucometer. For glucose tolerance test, glucose (2 g/kg) was administered intraperitoneally, and blood glucose was measured at 15, 30, 60, 90, and 120 min from tail tip.

Insulin tolerance test

Insulin tolerance test was performed in 5-h fasted mice. Regular insulin (0.75 U/kg, Sigma Aldrich) was injected

intraperitoneally, and glucose was measured at 0, 15, 30, 60, and 90 min from tail tip.

Energy expenditure analysis by oxymax/comprehensive lab animal monitoring system (CLAMS)

Oxymax/CLAMS (Columbus Instruments, Columbus, OH, USA) were used to quantitate food intake, locomotor activity, oxygen consumption (VO_2), carbon dioxide production (VCO_2), metabolic rate, and heat production. Mice treated with saline or chronic insulin were individually housed in chambers maintained at $24 \pm 1^\circ\text{C}$ and given free access to chow diet and water. All the measurements were taken every 15 min for 3 days after the mice were acclimatized for 1 day. Basal metabolic rate (BMR) was determined by averaging lowest plateau region of oxygen consumption curve corresponding to resting periods. All data collected were averaged from 3 days of monitoring.

Body composition analysis

Body composition was measured after 8 weeks of saline or chronic insulin treatment using ^1H NMR spectroscopy (EchoMRI 3-in-1, Echo Medical Systems Ltd, Houston, TX, USA).

Statistical analysis

Results are expressed as mean $\pm$ s.d. for at least three to five separate determinations for each experiment. Densitometry of western blot data expressed as mean $\pm$ s.d. of three independent experiments. Statistical significance was determined by Student's *t*-test and one-way ANOVA for real-time results (GraphPad Prism version 3). *P* values < 0.05 were considered significant.

Results

Insulin resistance model development in adipocyte differentiated from hMSC

International stem cell association describes hMSC based on surface markers CD73, CD90, and CD105 (Hermida-Gomez *et al.* 2011). hMSC isolated from six different liposuction samples were pooled together for flow cytometry analysis and further experiments. More than 98% of cells were positive for CD105, CD73, and CD90 surface markers. Cocktail containing CD34, CD11b, CD19, and CD45 was used as negative markers (Fig. 1B).

Cells showed fibroblast morphology and differentiated into adipocyte on treatment with differentiation cocktail. On treatment with differentiation cocktail (MDI), most of the cells acquired lipid droplets, which were stained with oil red O (Fig. 1A). hMSCs on treatment with induction media show increased expression of adipocyte-specific markers such as FAS, PPAR γ , C/EBP α , and SREBP1c (Fig. 1C). Fully differentiated white adipocytes were treated with 500pM insulin as shown in experimental design (Fig. 1D). Time-dependent exposure of 500pM insulin on differentiated adipocytes showed decreased insulin-stimulated (10nM) glucose uptake at 24 and 48 h, but significant decrease was obtained only at 72 h (Fig. 1E). We confirmed above result at 72-h time point with/without insulin stimulation in control and CI-treated adipocytes (Fig. 1F). From here onward, 72-h treatment of 500pM is mentioned as CI. We further confirmed insulin resistance at protein level. CI treatment significantly decreased IRS1 and GLUT4 expression compared with control adipocytes (Fig. 2G). One of the major functions of insulin is to translocate cytosolic GLUT4 to membrane that is mediated in part by activation of AKT and downstream substrate AS160 protein. We found that CI treatment significantly decreased insulin-stimulated phosphorylation of AKT and AS160 compared with control adipocytes (Fig. 2H). The above results confirm that adipocytes differentiated from hMSC on treatment with pathophysiological level of insulin (500pM) for 72h (CI) show characteristics of insulin resistance.

Differentiation of hMSC into brown adipocytes

hMSCs were differentiated into brown adipocyte as shown in Fig. 1A. Adipocyte differentiated using the above protocol showed increased expression of brown adipocyte markers such as *PGC1 α* , *PRDM16*, *NRF1*, *DIO2*, *EVA1*, *EVOL3*, and *SIRT1* compared with white adipocytes (Fig. 2B). Expression of mitochondrial and fatty acid oxidation-related genes such as *ATP synthase*, *UCP1*, *COX*, *COX III*, *CYT C*, *FATP1*, and *MCAD* were also increased in brown adipocytes (Fig. 2C and D). We confirmed increase in the expression of UCP1, PGC1 α , and PRDM16 protein levels in brown adipocytes (Fig. 2E). Morphologically brown adipocytes are known to have high number of mitochondria compared with white adipocytes. JC-1 and TMRE staining confirmed significantly high number of functionally active mitochondria in brown adipocytes differentiated from hMSC compared with white adipocytes (Fig. 2F and G). Oxygen consumption rate (OCR) of brown adipocytes

was considerably higher compared with white adipocytes, indicating that the latter is metabolically more active compared with white adipocytes. Basal and maximal respirations of brown adipocytes were also significantly higher compared with white adipocytes, indicating increased functionally active population of mitochondria. Spare respiratory capacity and proton leak were found to be significantly high in brown adipocytes compared with white adipocytes (Fig. 2H). The spare respiratory capacity indicates the amount of extra ATP that can be produced during energy stress. It also demonstrates how well the cell is equipped to moderate energy deficiency. The brown adipocytes differentiated from hMSC thus indicate superior mitochondrial function than white adipocytes.

Chronic insulin treatment decreases expression of brown adipocyte-specific markers and leads to insulin resistance in brown adipocytes

The effect of hyperinsulinemia on white adipocytes has been extensively studied and documented in the literature. To probe the effect of hyperinsulinemia on brown adipocytes, we subjected brown adipocytes to similar CI treatment. We found that brown adipocytes become resistant to insulin-stimulated glucose uptake similar to white adipocytes on CI treatment (Fig. 3A). To further validate insulin resistance in brown adipocytes, we studied insulin signaling level alteration in CI treated white and brown adipocytes. CI treated brown adipocytes showed decreased insulin stimulated phosphorylation of AKT (Ser473) and AS160 (Thr642) similar to CI treated white adipocytes. We also found decrease expression of Glut4 in CI treated brown adipocytes compared to control brown adipocytes (Fig. 3B). The above results prompted us to study the effect of hyperinsulinemia on functioning of brown adipocytes. Our result indicates that CI treatment decreases the expression of brown adipocyte-specific markers and transcription factors *PRDM16*, *PGC1 α* , *DIO2*, *SIRT1*, etc. (Fig. 3C). We also found decreased expression of mitochondria and fatty acid oxidation-related genes such as *UCP1*, *COX*, *COX III*, *CYT C*, *FATP1*, and *MCAD* in CI-treated brown adipocytes (Fig. 3D and E). The above results indicate decrease in metabolic activity and uncoupling property of brown adipocyte on CI treatment. We have further validated CI-induced decrease in brown adipocyte markers such as PGC1 α , UCP1, PRDM16, and SIRT1 at protein level (Fig. 3F). We found increased expression of ER

Figure 3

Chronic insulin treatment decreases expression of brown adipocyte-specific markers and leads to insulin resistance in brown adipocytes. White and brown adipocytes were given CI treatment as indicated in figure (A). 10 nM insulin stimulation was given before glucose uptake. Glucose uptake reading was normalized to total protein concentration and is represented as fold difference. $n=3$, error bars represent s.d., $***P<0.001$, $**P<0.01$, $*P<0.05$ as tested by Student's *t*-test (A). Western blot analysis of p-AKT (Ser473), p-AS160 (Thr 642), and GLUT4 in white and brown adipocytes with and without CI treatment as indicated in figure (B). 10 nM insulin stimulation was given for 20 min before protein isolation. Panel adjacent to the blots show the densitometry of protein normalized with respective total protein and actin in case of GLUT4. $n=3$, error bars denote s.d., $***P<0.001$ and $**P<0.01$ as compared by one-way ANOVA and Bonferroni post-test analysis (B). Real-time PCR analysis of brown adipocyte-specific marker genes, genes related to mitochondrial biogenesis, and fatty acid oxidation. $n=3$, error bars represent s.d., $***P<0.001$ as tested by two-way ANOVA and Bonferroni's post-test analysis (C, D and E). Western blot analysis of PGC1 α , UCP1, PRDM16, and SIRT1 in white and brown adipocytes with and without CI treatment as shown in figure (F). The graphs adjacent to the blots show densitometry of the respective proteins normalized with actin. $n=3$, error bars represent s.d., $***P<0.001$ as tested with Student's *t*-test (F). Western blot analysis of protein related to ER stress PERK, IRE1 α , PDI, BIP, and CHOP in control and CI-treated white and brown adipocytes. The graphs adjacent to the blots show densitometry of respective proteins normalized with actin. $n=3$, error bars indicate s.d., $***P<0.001$, $**P<0.01$ and $*P<0.05$ as tested by Student's *t*-test (G). Brown adipocytes were grown in 96-well eppendorf clear bottom imaging plate and CI treatment was given for 72 h before staining with JC-1 dye. Cells were excited with 405 or 488 nm and image was taken at 20 $\times$ magnification using Cellomics (high-content screening platform). Images were analyzed using Image J software. The red fluorescence shows high-membrane potential mitochondria (J aggregates) and green fluorescence shows low-membrane potential mitochondria (L monomers). The graph on the right-hand side is the relative quantification of red to green ratio normalized with nuclear stain DAPI. $n=3$, the error bars denote s.d., $***P<0.001$ as tested by Student's *t*-test (H). Similar to the above experiment, brown and brown CI (72 h)-treated adipocytes were stained with TMRE dye. Cells were excited at 405 nm and image was taken at 10 $\times$ using Cellomics (high-content screening platform). The graph beside the image shows the relative quantification of mean red intensity normalized with DAPI. $n=3$, error bars represent s.d., $***P<0.001$ as tested by Student's *t*-test (I). The oxygen consumption rates (OCR) of brown and brown CI (72 h)-treated adipocytes at basal level and in the presence of ATP synthase inhibitor (1 μ M oligomycin), proton uncoupler (1 μ M FCCP), and electron transport chain inhibitors (0.5 μ M rotenone/antimycin mix) was measured with Seahorse Bioscience XFp Extracellular Flux analyzer, $n=3$, error bars represent s.d., $**P<0.005$, $***P<0.001$ as tested by Student's *t*-test (J). Oil red O image of brown adipocytes with and without 72 h CI treatment taken with Leica DFC450 C microscope at 40 $\times$ magnification. The graph beside the image shows the relative oil red O absorbance at 492 nm. $n=3$, error bars indicate s.d., $***P<0.001$ as tested by Student's *t*-test (K).

stress-related proteins PERK, IRE α , PDI, BIP, and CHOP in CI-treated white and brown adipocytes (Fig. 3G). To assess whether the previous results translate to mitochondria biogenesis and functioning, we stained the CI-treated brown adipocytes with JC-1 and TMRE dye. TMRE staining indicates decreased mitochondrial content in IR brown adipocytes (Fig. 3H). The number of functionally active mitochondria was also significantly decreased in CI-treated brown adipocytes (Fig. 3I). Mitochondrial respiration rate measured in terms of OCR using extracellular flux analyzer further validated the decrease in functionally active mitochondria in CI treated brown adipocytes. The decreases in basal and maximal respiration on CI treatment in brown adipocytes indicate decrease in mitochondrial content. Spare respiratory capacity and proton leak were also significantly reduced on CI treatment, depicting functionally impaired mitochondria (Fig. 3J). We further analyzed the morphological changes induced by CI on brown adipocytes. There was significant increase in lipid accumulation with increase in droplet size on CI treatment in brown adipocytes (Fig. 3K). The above results indicate that hyperinsulinemia affects brown adipocyte functions and are susceptible to insulin resistance similar to white adipocytes.

Chronic insulin exposure increased obesity and induces insulin resistance in C57BL/6 mice

To verify the *in vitro* findings, we next investigated whether CI exposure affects insulin sensitivity and glucose metabolism *in vivo* in C57BL/6 mice. To do this, the mini-osmotic pumps filled with saline or long-acting insulin analogue glargine (0.6 U/day) were implanted subcutaneously for 8 weeks (hereafter referred as CI treatment). The continuous chronic exposure of insulin increased body weight gain and weight of eWAT (Fig. 4A and B). We found increased fat mass without any change in lean mass on CI-treated C57BL/6 mice (Fig. 4C and D). Moreover, glucose tolerance and insulin tolerance (GTT and ITT) tests revealed profound increase in glucose intolerance with decreased insulin sensitivity (Fig. 4E and F). In parallel to the above findings, we also found increased level of serum insulin and lipid profile in CI-treated animals (Fig. 4G and H). To determine whether the chronic insulin treatment affects insulin signaling in adipose tissue, we investigated phosphorylation levels of AKT and AS160 in eWAT. CI exposure leads to decreased phosphorylation of both AKT (Ser 473)

and AS160 (Thr 642) in eWAT (Fig. 4I). Collectively, CI treatment in mice leads to reduced insulin sensitivity and impaired glucose metabolism.

Hyperinsulinemia reduces insulin sensitivity, characteristics, and functioning of brown adipose tissue

It is well recognized that reduced energy expenditure is associated with enhanced obesity and thereby insulin resistance. Thus, we next investigated the role of hyperinsulinemia on energy expenditure by indirect calorimetric analysis. Indeed, CI treatment in mice increased food intake and decreased locomotor activity (Fig. 5A and B). CI-treated mice also showed less O₂ consumption and CO₂ production compared with saline-treated mice (Fig. 5C and D). Consistent with the reduced VO₂ and VCO₂, CI-treated mice have less metabolic rate and generated less heat compared with saline-treated mice (Fig. 5E and F). Taking clues from the *in vitro* findings and energy expenditure studies, we were keen to validate and further study the effect of CI on BAT. We found that CI treatment leads to decreased insulin-stimulated phosphorylation of AKT (Ser 473) and AS160 (Thr 642) and with less expression of Glut4 in iBAT (Fig. 5G). Literature suggests that UCP1, PRDM16, and PGC1 α negatively regulate obesity and metabolic syndrome (Liang & Ward 2006, Kozak & Anunciado-Koza 2008, Feldmann *et al.* 2009, Becerril *et al.* 2013). To assess whether the decreased insulin sensitivity in CI-treated animals also affects brown adipose markers in iBAT, we analyzed mRNA expression of brown adipocyte-specific genes involved in mitochondrial biogenesis and fatty acid oxidation. The continuous exposure of insulin decreased the mRNA expression of brown adipocyte-specific transcription factors such as *Prdm16*, *Sirt1*, *Dio2*, and *Pgc1 α* in iBAT (Fig. 5H). The decrease in expression of mitochondrial biogenesis and fatty acid oxidation-related genes such as *Atp5G*, *Cox IV*, *Cox III*, *Ucp1*, *Fatp1*, and *Mcad* in iBAT of CI-treated animals suggests decreased metabolic activity and loss of uncoupling property (Fig. 5I and J). We have also validated expression of UCP1, PGC1 α , SDH, and PRDM16 at protein level (Fig. 5K). Decreases in mitochondrial biogenesis and fatty acid oxidation are well implicated in obesity and metabolic disorders. The above results suggest that hyperinsulinemia not only reduces brown adipocyte capacity to utilize fatty acid via uncoupled respiration but also diminishes brown adipocyte characteristics, making it susceptible to

Figure 4

Chronic insulin exposure increased obesity and insulin resistance in C57BL/6 mice. Body weight gain of saline- and CI-treated C57BL/6 mice monitored up to 8 weeks. $n=6$, error bars represent s.d., * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as tested by two-way ANOVA and Bonferroni's post-test analysis (A). Epididymal white adipose tissue weight of saline- and CI-treated C57BL/6 mice were taken after killing. $n=6$, error bars indicates s.d., * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as tested by Student's t -test (B). Total fat and lean mass of saline- and CI-treated C57BL/6 mice were assessed using Echo MRI after 8 weeks. $n=6$, error bars represent s.d., * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as tested by Student's t -test (C and D). Glucose tolerance was assessed by IPGTT and glucose profiles calculated from IPGTT as area under the curve (E). Insulin tolerance was assessed by ITT and glucose profile calculated from ITT as area under the curve (F). Serum insulin, triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol were measured in 6-h fasted animals (G and H). ** $P<0.01$, *** $P<0.001$ as tested by two-way ANOVA and Bonferroni's post-test analysis. Proteins isolated from subcutaneous WAT of three control and CI-treated C57BL/6 mice were subjected to western blot analysis for p-AKT (Ser473) and p-AS160 (Thr 642). The total level of respective proteins is given below each blot (I).

insulin resistance. To gain further insights into how CI treatment impairs insulin sensitivity and functioning of BAT, we investigated inflammatory and ER

stress-associated marker. We found increased expression of inflammatory markers (TNF α , IL6, and F4/80) in CI-treated mice (Fig. 5L). CI treatment also increased

Figure 5

Hyperinsulinemia reduces insulin sensitivity in brown adipose tissue and affects characteristic and functioning of BAT. Food intake during CLAMS (A) locomotor activity (B), volume of O₂ consumption (C), CO₂ production (D), metabolic rate (E), and heat production (F). Data from figure A–F were taken and analyzed from CLAMS studies. The data represented here are mean of 6 days, $n=6$, error bars indicate s.d., $*P<0.05$, $**P<0.01$, $***P<0.001$ as tested by Student's *t*-test analysis. Western blot analysis of p-AKT (Ser473), p-AS160 (Thr 642), and Glut4 protein expression was done in protein isolated from interscapular BAT of three control and CI-treated C57BL/6 mice along with their total level of respective proteins, $n=3$ (G). Real-time PCR analysis of brown adipocyte-specific markers, genes involved in mitochondrial biogenesis, and fatty acid oxidation in interscapular BAT of control and CI-treated C57BL/6 mice. $n=3$, error bars represent s.d., $*P<0.05$, $**P<0.01$, $***P<0.001$ as tested by one-way ANOVA and Bonferroni's post-test analysis (H, I and J). Western blot analysis of PGC1 α , UCP1, SIRT1, and SDH in protein isolated from interscapular BAT of three control and CI-treated mice, $n=3$ (K). Real-time PCR analysis of inflammatory markers in interscapular BAT of control and CI-treated C57BL/6 mice. $n=3$, error bars represent s.e.m., $*P<0.05$, $**P<0.01$, $***P<0.001$ as tested by two-way ANOVA and Bonferroni's post-test analysis (L). Western blot analysis of Chop and Bip in protein isolated from interscapular BAT of three control and CI-treated mice, $n=3$ (M).

expression of ER stress markers Chop and Bip at protein level. These data suggest that part of impaired insulin sensitivity and functioning of BAT in CI-treated mice might be due to ER stress and inflammation.

Discussion

Hyperinsulinemia is considered as a common factor among the metabolic syndrome including obesity and diabetes (Butler *et al.* 1990, Kim *et al.* 2015b). A number of studies have reported that hyperinsulinemia alone is

sufficient to cause/drive insulin resistance (Rizza *et al.* 1985, Kahn & Flier 2000, Yang *et al.* 2014, Pedersen *et al.* 2015). Moreover, hyperglycemia (Liu *et al.* 2009), free fatty acids (Ning *et al.* 2011), or the amino acids (Zhang *et al.* 2007) alone are insufficient to induce insulin resistance. Clinical and rodent studies have successfully correlated decrease in BAT volume with increased body mass index (BMI), but remained to be explored the detailed inter-relationship. All obese animal models show defective BAT activity and lower level of UCP1 expression (Ferre *et al.* 1986, Yoneshiro *et al.* 2011). Thus, it was logical to study the effect of hyperinsulinemia on

BAT function. Various *in vitro* insulin resistance models of white adipocytes have been reported in the literature to study the disease pathophysiology. Recent advancement in stem cell research and the feasibility to differentiate human adipose-derived stem cells (hADSCs) to white and brown adipocytes have made hADSC as a desired model to study adipocyte biology (DeLany *et al.* 2005). Obese prediabetic subjects have been reported to have 400–600 pM/L serum insulin and 7–11 mmol/L of blood glucose (Dileepan & Feldt 2013). Recently, Guanfeng Xu and coworkers exposed human primary adipocytes to 600 pM insulin/25 mM glucose for 48 h to induce insulin resistance (Xu *et al.* 2015). This inspired us to develop IR adipocyte model by decreasing the insulin exposure concentration to 500 pM and glucose 10 mM for 72 h to corroborate with pathophysiological level. Our results showed that hMSC-derived both white and brown adipocytes developed insulin resistance on chronic exposure to 500 pM insulin for 72 h and collate very well with the findings of Xu and coworkers (Fig. 1H). hMSC-derived white adipocyte is increasingly being used to study insulin resistance, but there is still considerable knowledge gap in context of brown adipocytes and hyperinsulinemia-induced insulin resistance. Recently, two independent research groups have reported differentiation of mesenchymal stem cells into functionally active brown adipocytes (Pisani *et al.* 2011, Ahfeldt *et al.* 2012). These differentiated adipocytes showed all the distinct characteristics of brown adipocytes such as increased expression of PRDM16, PGC1 α , UCP1, and high mitochondrial content (Fig. 2B, C, D, E, F, G and H). This also extended us the opportunity to probe the effect of hyperinsulinemia on brown adipocytes. Our *in vitro* results suggest that hyperinsulinemia reduces insulin sensitivity of brown adipocytes by decreasing brown adipocyte-specific characteristics such as expression of UCP1, PGC1 α , mitochondrial biogenesis, and spare respiratory capacity, which signifies the metabolic activity of mitochondria. Study conducted by Ruidan Xue and coworkers have used spare respiratory capacity to characterize biomarkers with thermogenic potential with strong correlation with UCP1 expression (Xue *et al.* 2015). The decrease in spare respiratory capacity and proton leak in IR brown adipocytes confirms the loss of uncoupling function, which may be due to decrease in UCP1 expression (Fig. 3D and F).

Taking clues from these studies, we developed insulin resistance model *in vivo* in C57BL/6 mice. Excess energy intake plays an important role in causing obesity and insulin resistance (Frederich *et al.* 1995, Considine *et al.* 1996). CI treatment for 8 weeks in mice increased

nocturnal food intake significantly. We have also observed increased hypertrophied adipose tissue mass after completion of treatment period. Hypertrophied adipocytes are known to secrete increased leptin, which in turn causes leptin resistance (Ren 2004, Knight *et al.* 2010). The cumulative outcome, although it does not clarify cause or effect, but results into increased food intake as reflected in our studies. Consistent with the previous studies, chronic insulin treatment in C57BL/6 mice leads to increased obesity and decreased insulin sensitivity (Fig. 4A, B, C, D, E, F, G, H and I). Surprisingly, we also found that CI treatment in brown adipocytes significantly reduced insulin sensitivity (Fig. 5G), expression of brown adipocyte-specific genes (Fig. 5H, I and J), and decreased mitochondrial content and function (Fig. 5K). CI treatment also led to decrease in mitochondrial respiration and proton leak in hMSC-derived brown adipocytes (Fig. 3J), energy expenditure (Fig. 5C, D and E), heat production *in vivo* (Fig. 5F), thereby affecting the function of brown adipocytes. Our studies concludes that although brown adipose tissue has been known to protect from diet-induced obesity and improve insulin sensitivity, but in continuous hyperinsulinemic milieu as observed in clinical setups, brown adipocyte capacity to utilize the excess energy is compromised. The above results, to certain extent, explain the negative correlation of functionally active brown adipose tissue with increase in BMI. Negative correlation between decreases in brown fat content with increase in BMI is well reported except in context of the effects of hyperinsulinemia on the trans/differentiation and functional aspects of BAT. BAT activity has been detected in lean person with low fasting blood glucose level and show negative correlation with increase in BMI (Lee *et al.* 2010). Clinical study conducted by Orva and coworkers showed that brown adipose tissue in obese subject does not respond to cold and insulin stimulation (Orava *et al.* 2013). Similar results were also observed in 18FDG-PET/CT scan study by Pfannenberger *et al.* (2010). The decrease in basal and resting metabolic rate along with heat production in CI-treated animals further strengthens the *in vitro* results and our hypothesis. Our results can be interpreted in concordance to the *in vivo* studies conducted by different group in obese subjects depicting to have defective BAT function (Heilbronn *et al.* 2007, Kim *et al.* 2015). Hyperinsulinemia has been reported to cause mitochondrial dysfunction in white adipocytes, muscle, and heart through generation of reactive oxygen species, free radicals, and ER stress. Earlier Shabalina and coworkers have reported the effect of reactive oxygen species (ROS) in overall functioning of brown adipocytes

(Shabalina *et al.* 2014). We found increased expression of ER stress markers in BAT of both *in vitro* and *in vivo* CI conditions (Figs. 3G and 5M). Another interesting observation is the significant increase in the expression of pro-inflammatory markers such as Tnf α , IL6, and F4/80 in BAT micro milieu of CI-treated animals (Fig. 5L). All these results give possible explanation to decreased activity of BAT function and characteristics upon CI treatment, but may not be implicated for changed BAT phenotype. Our results collates very well with the study conducted by Matamala and coworkers, where authors have demonstrated that lack of BAT response in obese rat is due to the change in mitochondria subpopulation and it was different from lean rat (Matamala *et al.* 1996). The above literature provides coherent evidence to suggest that CI treatment may also change the mitochondrial subpopulation leading to decrease the metabolic activity of brown adipocytes. Loss of uncoupling property on CI treatment will lead to less utilization of lipid droplets and increase lipid accumulation (hypertrophy), which makes brown adipocytes susceptible to insulin resistance.

Our *in vitro* and *in vivo* results suggest that development of insulin resistance in brown adipocytes is associated with decrease in expression of brown adipocyte markers and loss of uncoupling property (Fig. 5A, B, C, D, E, F, G, H, I, J, K, L and M). Recent study demonstrated that high-fat diet-fed C57BL/6 mice show increased lipid droplet accumulation in brown adipose tissue and insulin resistance (Roberts-Toler *et al.* 2015). The study conducted by Mehran and coworkers showed that *Ins1*^{+/-} and *Ins2*^{-/-} knockdown mice have increased energy expenditure and lipid mobilization resulting from increased UCP1 expression at both mRNA and protein level. One of the mechanisms suggested for increased UCP1 expression is increased sympathetic activity in *Ins1*^{+/-} and *Ins2*^{-/-} knockdown mice (Mehran *et al.* 2012). From these studies, it is rational to conclude that peripheral hyperinsulinemia plays a negative role in UCP1 expression. Other report indicates that the expression of UCP1 is dependent on SIRT1-mediated deacetylation of PPAR gamma (Qiang *et al.* 2012). We found decreased expression of SIRT 1 in CI-treated white and brown adipocytes, which may act as a possible connecting link between insulin and UCP1 expression. The excess ectopic lipid accumulation has deleterious effect leading to inhibition of genes involved in mitochondrial biogenesis and thermogenesis. Many studies have reported reduced thermogenesis in HFD-fed obese rodents. Oil red O staining of IR brown adipocytes yielded similar results as mentioned above, and there was a significant increase in lipid accumulation (Fig. 3K).

BAT of genetically obese ob/ob mice show insulin resistance and reduced cold-induced activation of thermogenesis (Thurlby & Trayhurn 1980). In concurrence, our studies imply the role of hyperinsulinemia in defective BAT function in obese subjects. Vascular refraction has been shown to mediate whitening of brown adipocyte due to hypoxia (Shimizu *et al.* 2014). Even though increase in lipid accumulation and decrease in brown adipocyte characteristics are evident from our results, it will be inconclusive to suggest whitening of brown adipocytes. As human brown fat is known to possess molecular signatures similar to white adipocytes (Sharp *et al.* 2012), it is not surprising to observe the adaptation of BAT according to the physiological needs.

In conclusion, we for the first time here with report that hyperinsulinemia reduces insulin sensitivity of brown adipocytes both *in vitro* and *in vivo* conditions. Our study also confirms the negative correlation of BAT function along with decrease in mitochondrial content and respiration with hyperinsulinemia. Our study has greater implication in respect to use of brown adipocyte for the treatment of insulin resistance and related metabolic syndrome. Our findings imply that browning alone may not be sufficient to permanently revert insulin resistance as its effect dissipates in hyperinsulinemic obese micro milieu on longer run. Above results compel us to re-evaluate the strategy of recruiting brown adipocyte for the treatment of obesity and insulin resistance unless beforehand we correct micro milieu.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research work is supported by the Department of Biotechnology and Department of Science and Technology DBT Government of India Project GAP1079, CSIR-CDRI Network project: "Towards holistic understanding of complex diseases: Unraveling the threads of complex disease (THUNDER)", Project No: BSC0102 and project Understanding of disease dynamics and to accelerate drug discovery. Project No: UNDO-BSC0103.

Acknowledgment

We acknowledge the help provided by Prof. Nuzhat Husain (Director), Dr Ram ManoharLohia, Institute of Medical Science, for approval from stem cell ethics committee. We also acknowledge the help and support provided by staff of the Sushrut Institute of Plastic Surgery (SIPS) Hospital for collection of liposuction sample. We acknowledge the help provided by A L Vishwakarma and staffs in the Sophisticated Analytical Instrumentation Facility (SAIF) in performing flow cytometry experiments. Research work is supported by the CSIR-CDRI Network project: "Towards holistic understanding of complex diseases: Unraveling the threads of

complex disease (THUNDER)", Project No: BSC0102 and by the Department of Biotechnology (DBT) project (GAP0179). MB is supported by S R F D B T; S R, A G, and K S are supported by SRF-UGC; D K and A S are supported by SRF-CSIR, New Delhi. S V is supported by THUNDER project. This manuscript bears CSIR-CDRI manuscript number: 9262.

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Received in final form 21 June 2016

Accepted 22 June 2016

Accepted Preprint published online 23 June 2016