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Thermogenic regulation of human abdominal and neck area derived adipocytes by mitophagy, irisin and BMP7

by

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The PhD Defence takes place at the Library of the Department of Biochemistry and Molecular Biology (Room Number: F 3.009-010) on 23rd August 2022 from 13:00 hours.

Introduction and theoretical background

Obesity and the role of thermogenic adipocytes

Obesity defined by disproportionate body weight for height with an excessive accumulation of adipose tissue is considered by many as a 21st century epidemic. Incidence of obesity is increasing globally over the past decades. It is the sixth important risk factor contributing to the overall disease burden worldwide. Obesity is a major risk factor for various metabolic diseases, type 2 diabetes, coronary heart disease and certain cancers. Obese patients have up to 3 times higher risk for relative morbidity than people with normal body weight for other components of metabolic syndrome. Although the prevalence of obesity is increasing worldwide, available effective therapeutic targets are limited. Thermogenic beige and brown adipocytes play an important role in combating obesity. Recent studies using radiolabelled substrates like ^{18}F-FDG and ^{123}I-MIBG identified metabolically active brown adipose tissue (BAT) in healthy humans that can dissipate energy into heat. PET/CT found these thermogenic fat depots to be interspersed into six anatomic depots: cervical, supraclavicular, axillary, paraspinal, mediastinal, and abdominal. These thermogenic fat depots are predicted to account for up to 5% of basal metabolic rate in healthy adults that would result in 4kg of fat loss per year. Studies have also shown a strong negative corelation between obesity or glucose intolerance and amount of active BAT in humans. Hence stimulation of BAT might be an important therapeutic application for obesity treatment.

Types of adipocytes

Adipocytes can be broadly classified into three types: white adipocytes, brown adipocytes, beige adipocytes.

White adipocytes containing an unilocular lipid droplet are the most abundant type of adipocytes. They primarily function as a storehouse of excess energy in the form of lipid droplets. Major white adipose tissue (WAT) depots in humans are generally identified as subcutaneous and visceral. Excess of visceral WAT has been linked to various metabolic dysfunctions.

Brown adipocytes are distinguished by their abundant mitochondria and presence of multilocular lipid droplets. White and brown adipocytes both accumulate triglycerides in their cytoplasm; while white adipocytes form an unilocular lipid droplets, brown adipocytes form multilocular lipid droplets. Brown adipocytes have high expression of uncoupling protein 1 (UCP1), an inner mitochondrial membrane protein that when activated is involved in non-shivering thermogenesis.

Studies have shown the presence of “brown adipocyte-like” cells in murine WAT depots upon cold exposure that possessed multilocular morphology and mitochondria with high UCP1 content. In the recent period considerable attention has been paid in understanding this group of adipocytes termed as “beige” adipocytes. Earlier formation of beige adipocytes was assumed to be reversible transdifferentiation of white to brown adipocytes. However recent studies using mouse models showed that beige adipocytes arose from a distinct precursor in response to stimuli like cold, diet, exercise and it was partially regulated by the β3-adrenergic signalling pathway.

Beige and white adipocytes are derived from the same mesenchymal precursors, but the beige adipocytes exist in a masked condition in subcutaneous WAT depots when adrenergic stimulation is absent. Studies have shown that certain genetic factors like rs1421085 T-to-C single-nucleotide variant can influence the differentiation of mesenchymal precursors into

either beige or white adipocytes. Obese individuals possess less BAT but high amount of brownable fat enriched with beige adipocytes. However, these beige adipocytes are undergoing transition to white adipocytes as a result of high level of ongoing autophagy/mitophagy, as indicated by the higher expression of ATG genes in subcutaneous WAT of obese individuals. A recent study has shown that beige adipocytes can transition to white upon removal of adrenergic stimulus and mitophagy plays an important role in the transition.

Uncoupling protein 1 (UCP1) mediated thermogenesis

UCP1 is an inner mitochondrial membrane protein that is primarily responsible for the thermogenic function of BAT. The mitochondrial electron transport chain transports protons to the intermembrane space, thereby creating a proton gradient across the inner membrane, which drives ATP synthesis. UCP1 is a member of mitochondrial carrier protein family that can uncouple mitochondrial ATP synthesis from the respiratory chain, thereby decreasing the proton gradient across the inner mitochondrial membrane and generate heat. β3-adrenergic stimulation triggers the formation of long-chain fatty acids from triglycerides upon cleavage by hormone sensitive lipase, that in turn leads to activation of UCP1. Fatty acids permanently associated with UCP1 by hydrophobic interactions function as proton carriers though UCP1 across the inner mitochondrial membrane. The protons are released in the mitochondrial matrix; however, the fatty acid anion stays associated with UCP1 to continue the proton transfer cycle. Thus, UCP1 leads to energy dissipation as heat generated from β-oxidation of fatty acids.

Futile creatine cycle mediated thermogenesis and other UCP1-independent thermogenic mechanisms

UCP1 has been well established as an important component of non-shivering thermogenesis. However, UCP1 knockout mice were seen to be able to tolerate chronic cold exposure if the temperature change was gradual. This created a gap in understanding the mechanism of thermogenesis. Recent studies indicated that proteins involved in creatine synthesis and phosphorylation were elevated in beige fat mitochondria. The findings hinted towards a futile creatine phosphorylation and dephosphorylation cycle that has the capacity to mediate thermogenesis in beige fat. In this cycle, first creatine is phosphorylated by creatine kinase using ATP. Following that, the high energy phosphate is immediately hydrolysed generating heat. Recent studies indicated that the phosphatase involved in the dephosphorylation may most likely be the tissue-nonspecific alkaline phosphatase (TNAP). Studies showed that the level of mitochondrial creatine kinase 2 (CKMT2) was elevated in beige fat. A very recent study indicated that the cytosolic creatine kinase B (CKB) is targeted to mitochondria and plays a vital role in the creatine cycle. Further research is necessary to identify all the components involved in this newly discovered mechanism for thermogenesis.

A similar thermogenic futile cycle is mediated by the calcium ion (Ca^{2+}) cycling by the Ca^{2+} ATPase SERCA. SERCA pumps calcium ions into the sarcoplasmic reticulum that is followed by leakage of Ca^{2+} into the cytoplasm. Sarcolipin uncouples SERCA-mediated ATP hydrolysis from calcium ion pumping resulting in generation of heat. Although SERCA mediated thermogenesis is mostly active in skeletal muscle cells, studies have shown this mechanism to be active in BAT as well.

Mitophagy and maintenance of thermogenic beige adipocytes

Mitochondria play an important role in driving thermogenesis of beige and brown adipocytes. Mitochondria can change their morphology via fusion or fission to shift between large networks of elongated and fragmented units. Rodent brown adipocytes develop high rate of mitochondria fragmentation upon adrenergic stimulation that results in enhanced mitochondrial uncoupling

energy expenditure. UCP1 enriched mitochondria in humans are mostly fragmented. Mitochondrial turnover is essential for maintaining cellular homeostasis that is tightly regulated by biogenesis and selective degradation. Mitochondrial biogenesis is precisely controlled by several nuclear-coded transcriptional regulators like the peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α), the mitochondrial transcription factor A (Tfam), the nuclear respiratory factor (Nrf) 1 and Nrf2.

Autophagy is an evolutionary conserved mechanism by which unwanted and damaged cellular components are digested and recycled intracellularly in eukaryotic cells. During autophagy, unwanted or damaged proteins or organelles are engulfed by a double membrane vesicle called autophagosome in order to be delivered to the lysosome for degradation. Autophagosome biogenesis is mediated by proteins encoded by autophagy-related genes (ATG). During autophagosome formation the most important step is the conjugation of microtubule-associated protein 1 light chain 3 (LC3-I) to the phosphatidylethanolamine (PE) that results in the formation of the lipidated LC3-II, which is then inserted into autophagosomes. LC3 is the most commonly used marker for autophagosomes as the amount of LC3-II corelates with the number of autophagosomes. A well accepted method to monitor autophagic activity is the conversion of LC3-I to LC3-II by western blot analysis. Increased ratio of LC3-II/LC3-I indicates a high level of ongoing autophagy. Autophagic flux is a measure of the autophagic degradation activity. Chloroquine (CQ) inhibits the last stage of autophagy, hence preventing the degradation of autophagic cargo and LC3-II. Thus, application of CQ is a convenient tool to measure autophagic flux. p62 is an autophagy adapter protein that functions as a link between LC3 and the ubiquitinated targets. p62 is continuously degraded during autophagy, hence a lower level of p62 indicates higher autophagic activity and vice versa.

Mitophagy or the selective degradation of unwanted and damaged mitochondria by autophagy is an essential quality control mechanism that helps to maintain a healthy and functional mitochondrial network. Mitophagy is triggered by the reduction of mitochondrial membrane potential and occurs primarily via parkin dependent and independent mechanisms. The parkin mediated mitophagy mechanism is initiated by stabilization of PINK1 (PTEN (phosphatase and tensin homolog)-induced putative kinase 1) on the outer mitochondrial membrane (OMM). This is followed by the recruitment of parkin that in turn ubiquitinates OMM proteins. Selective autophagy adaptor proteins like, NBR1 (neighbour of Brca1 gene 1), p62 (SQSTM1), OPTN (optineurin) and NDP52 (nuclear dot protein 52) act as bridges between the ubiquitinated mitochondrial proteins and LC3 to mediate the engulfment of mitochondria into autophagosomes. Mitophagy can also occur independent of parkin mediated ubiquitination through the direct interaction of LC3 with mitochondria-localized proteins like, FUNDC1 (FUN14 domain-containing protein1), BNIP3 (BCL2 Interacting Protein 3), BCL2L13 (Bcl-2-Like Protein 13) and NIX (BNIP3 homology NIP3-like protein X). Recent studies in mice have indicated that beige adipocytes can convert to white upon removal of adrenergic stimulation via mitophagy mediated selective clearance of the mitochondria.

Brown adipose tissue as an endocrine organ

BAT is an important mediator of adaptive thermogenesis and increased BAT activity has been shown to exert a protective role against obesity and other metabolic disorders like type 2 diabetes. Recent studies suggest a secretory role of BAT that can contribute to the systemic effects of BAT activity. Specific adipokines released by BAT termed as batokines, mediate their effect in autocrine, paracrine or endocrine manner. Batokines have been shown to promote hypertrophy and hyperplasia of BAT, vascularization, innervation and blood flow that will assist in improving thermogenesis. Vascular endothelial growth factor A (VEGFA) secreted by brown adipocytes promotes angiogenesis and vascularization of BAT. Fibroblast growth factor

(FGF) 21 is another important batokine that enhances the beiging of WAT in animal studies and improves BAT thermogenesis.

Browning inducers and thermogenic activators

Upon cold exposure in animals, the cold is perceived by transient receptor potential (TRP) channels. TRPs are membrane proteins whose major function is to communicate the changes in the environment. Cold activated TRPs activate the sympathetic nerves entering BAT. Noradrenaline (NA) is released from the sympathetic nerve endings which activates the β-adrenoreceptors, which in turn activates the adenyl cyclase leading to increased intracellular cAMP levels. cAMP activates intracellular events like hydrolysis of triglyceride, oxidation of fatty acids and activation of UCP1 leading to improved thermogenesis in both mouse and humans.

Insulin signalling has been shown to play a crucial role in glucose uptake and storage of lipid droplets in both white and brown adipocytes, which occurs via the upregulation and transfer of Glucose transporter type 4 (GLUT4) to the plasma membrane. Leptin that acts as an appetite supressing hormone secreted by adipocytes has been shown to enhance BAT thermogenesis by activating SNS via the release of melanocyte-stimulating hormone in the hypothalamus. Thyroid hormones have also shown to elevate heat production that was suggested to be peripherally mediated. However, recent studies indicated that T3 inhibits AMPK in the ventromedial hypothalamus resulting in SNS activation leading to improved thermogenic capacity in mice. The central effects of thyroid hormones seem to be more significant than their peripheral action on non-shivering thermogenesis mediated regulation of energy balance.

Irisin

Physical exercise has been shown to mediate beneficial metabolic effects and offer protection against various pathological conditions, neurodegenerative disorders and certain cancers. Physical exercise stimulates the release of certain hormones from skeletal and cardiac muscles that are termed myokines that mediates the crosstalk between brain, muscle and adipose tissue and in turn regulating browning. Irisin was discovered as a myokine that is cleaved from Fibronectin Type III Domain Containing 5 (FNDC5) transmembrane protein and was able to induce a beige differentiation program in mice WAT. Physical exercise upregulates PGC-1α in skeletal myocytes, that in turn upregulates FNDC5; cleavage of FNDC5 leads to the release of irisin in circulation. A recent study has indicated that irisin exerts its effect via the integrin receptors integrin subunit alpha V (ITGAV) and integrin subunit beta (ITGB) 1/3/5 (ITGB1/3/5). Irisin mediates its positive effects on browning via the p38 MAPK and ERK pathways.

BMP7

Bone morphogenic protein (BMP) 7 acts as an auto/paracrine mediator that drives classical brown adipocyte differentiation in mice. BMP7 has been shown to promote differentiation of brown preadipocytes from embryonic stem cells even in absence of an adipogenic differentiation medium. BMPs exert its effect via heterotetrametric complexes of transmembrane receptors which are type I or type II serine/threonine kinase receptors. Type I BMP receptors can be classified into BMPR1A, BMPR1B, Activin A Receptor (ACVR)-like (L) 1, and ACVR1. Studies showed BMP7 to interact with BMPR1A, BMPR1B, and ACVR1. BMP7 also interacts with Type II BMP receptors like BMPR2, ACVR2A, and ACVR2B. BMP7 increases the expression of UCP1, PGC1α, and PRDM16 via the activation of p38 MAPK pathway. Previous studies indicated BMP7 can upregulate UCP1 expression in selected clones of human neck derived immortalized white and brown preadipocytes.

Aim of the study

- **Recent studies indicated that mitophagy is elevated during beige to white adipocyte transition in mice upon removal of adrenergic stimulation. So, I aim to understand how autophagy/mitophagy is regulated during the thermogenic response associated with a cAMP mediated adrenergic stimulus.**

- **Irisin has been shown to induce a beige differentiation program in mouse subcutaneous white adipose tissue. It can also induce a thermogenic response in human subcutaneous abdomen derived differentiating adipocytes. Hence, I intend to elucidate the effect of irisin in human subcutaneous and deep neck derived differentiating adipocytes.**

- **BMP7 is a potent browning inducer and has been shown to drive classical thermogenic brown adipocyte differentiation in mice. BMP7 can promote brown adipocyte like differentiation in human subcutaneous abdomen derived differentiating adipocytes. Hence, I intend to elucidate the effect of BMP7 in human subcutaneous and deep neck derived differentiating adipocytes.**

Material and methods

Source of cells and ethics statement

Human adipose derived mesenchymal stem cells (hADMSCs) were isolated from the stromal vascular fractions (SVF) of subcutaneous abdominal adipose tissue of healthy volunteers undergoing planned liposuction for experiments evaluating the regulation of mitophagy upon thermogenic stimulus. In case of treatments with Irisin and BMP7, hADMSCs were obtained from SVF of subcutaneous neck (SC) and deep neck (DN) tissues of healthy volunteers. A pair of biopsies was obtained from SC and DN areas of same donor to avoid inter-individual variations. Isolations were performed from donors aged between 18-65 years.

The study protocol was approved by the Medical Research Council of Hungary via approval number: 20571-2/2017/EKU. All experiments were carried out strictly under approved ethical guidelines and regulations.

Materials

All materials were obtained from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

Isolation, cultivation, and differentiation of human adipose-derived mesenchymal stem cells (hADMSCs) ex vivo

Adipose tissue biopsies were transported immediately to our laboratory following elective surgeries. Fibrous material and blood vessels were removed from the biopsies, minced into small pieces, and digested with collagenase (120 U/ml) in PBS (1 hour, 37°C) with gentle agitation. hADMSCs were centrifuged down from the cell suspension at 1300rpm for 10 minutes; then resuspended and cultured in T75 flasks with DMEM-F12 medium containing 10% FBS (Thermo Fisher Scientific, MA, USA), 100 U/ml penicillin-streptomycin, 33 µM biotin and 17 µM panthothenic acid at 37°C in 5% CO_2. Absence of mycoplasma was confirmed by PCR analysis (PCR Mycoplasma Test Kit I/C, PromoCell Gmbh, Germany). hADMSCs from fully confluent flasks were trypsinized and seeded (15000 cells/cm^2) to 6 well, 12 well, µ-Ibidi or 96 well Seahorse plates in the same medium under identical conditions.

Once the cell culture became confluent, differentiation was initiated by either the white or beige adipogenic differentiation medium for a period of 14 days (unless indicated otherwise). The white adipogenic differentiation medium consisted of DMEM-F12 supplemented with 33 µM biotin, 17 µM panthothenic acid, 10 µg/ml human apo-transferrin, 20 nM human insulin, 100 nM hydrocortisone, 200 pM T3, 2 µM rosiglitazone, 25 nM dexamethasone and 500 µM IBMX for the first four days, following which rosiglitazone, dexamethasone and IBMX were omitted from the differentiation medium. The beige adipogenic differentiation medium consisted of DMEM-F12 containing 33 µM biotin, 17 µM panthothenic acid, 10 µg/ml apo-transferrin, 0.85 µM human insulin, 200 pM T3, 1 µM dexamethasone and 500 µM IBMX for the first four days, after that, dexamethasone and IBMX were omitted, and 500 nM rosiglitazone was added to the differentiation medium. The medium was replaced every fourth day till the end of the 14-day differentiation period.

Treatments of differentiating hADMSCs and mature adipocytes with browning-inducers or activators

Where indicated, SC and DN derived preadipocytes were differentiated following the white adipogenic differentiation protocol in the presence of known browning inducers: human recombinant irisin (Cayman Chemicals, MI, USA, 11451) at 250 ng/mL or human recombinant

BMP7 (R&D Systems, MN, USA, 354-BP) at 50 ng/mL throughout the 14-day differentiation period (unless indicated).

Human subcutaneous abdominal derived preadipocytes were differentiated following the white or beige adipogenic differentiation protocol for 14 days. The differentiated adipocytes were subjected to dibutyryl-cAMP (500μM) treatment for 6, 10 or 14 hours respectively.

Gene silencing treatments in subcutaneous abdominal differentiated hADMSCs

Transfection and gene silencing were performed on differentiated white and beige adipocytes of abdominal origin using DharmaFECT1 transfection reagent (Dharmacon, CO, USA, T-2001-03). Differentiated adipocytes were incubated with a mixture of DharmaFECT1 and 50 nM of PARK2-targeted siRNA (Dharmacon, ON-TARGETplus SMARTpool Human Parkin siRNA, L-003603-00-0005) or non-targeted negative control siRNA (Dharmacon, ON-TARGETplus Non-targeting Control Pool, D-001810-10-05) for 4 days in white or beige differentiation medium devoid of penicillin/streptomycin.

RNA Isolation, RT-qPCR, and RNA-Sequencing

For RNA isolation, following differentiation and treatments adipocytes were collected using TRI Reagent. Total cellular RNA was isolated manually by chloroform extraction and isopropanol precipitation.

For RT-PCR, RNA quality was ascertained by spectrophotometry, followed by cDNA generation by TaqMan reverse transcription reagents kit (Thermo Fisher Scientific). qPCR was performed with LightCycler 480 (Roche Diagnostics, IN, USA) using the probes (Applied Biosystems, MA, USA) listed in Table. Human GAPDH was used as the endogenous control. Samples were run in triplicate and gene expression values were calculated by the comparative cycle threshold (Ct) method. ΔCt values were obtained by deducting GAPDH Ct from the Ct of target; the normalized gene expression values were calculated by $2^{-\Delta Ct}$. Probes used for the studies are listed below:

Table 1: List of TaqMan probes used in the study

Gene name	Assay ID
ITGAV	Hs00233808_m1
CXCL1	Hs00236937_m1
NFKB1	Hs00765730_m1
RELA	Hs00153294_m1
IL6	Hs00985639_m1
CCL2	Hs00234140_m1
CX3CL1	Hs00171086_m1
IL32	Hs00992441_m1
GAPDH	Hs99999905_m1
ID1	Hs00357821_g1
CKMT2	Hs00176502_m1
UCP1	Hs00222453_m1
ACAN	Hs00153936_m1
CRYAB	Hs00157107_m1
PPARGC1A	Hs01016719_m1
PARK2	Hs01038322_m1
SQSTM1	Hs00177654_m1

OPTN	Hs00184221_m1
NDP52	Hs00977443_m1
BNIP3	Hs00969291_m1
BNIP3L	Hs00188949_m1
FKBP8	Hs01014664_m1
BCL2L13	Hs00209789_m1
FUNDC1	Hs00697693_m1

For RNA-Sequencing, RNA sample quality was validated by Agilent Bioanalyzer using Eukaryotic Total RNA Nano Kit and samples with RNA integrity number >7 were used for library preparation. Libraries were prepared by NEBNext Ultra II RNA Library Prep (New England BioLabs, MA, United States), followed by sequencing runs on Illumina NextSeq500 using single-end 75 cycles sequencing. The reads were aligned to GRCh38 (with EnsEMBL 95 annotation) by STAR aligner. Reads were quantified by featureCounts and R was used for gene expression analysis. Outlier genes, having Cook's distance>1 were removed from further analysis. After removal of the outlier genes, the obtained expression profile of the transcripts is considered as normalized RNA counts. PCA analysis could not show any batch effect considering donor, sex or tissue origin. Differentially expressed genes were obtained using DESeq2 algorithm with a log2 fold change threshold of more than 0.85. Pathway analysis was performed using PANTHER Reactome pathways and heatmaps were visualized using Morpheus Webtool based on calculated z-scores. The interaction networks were determined using STRING and constructed using Gephi 0.9.2; node size illustrated the fold change.

Quantification of mitochondrial DNA by qPCR

Total DNA was isolated by manual phenol–chloroform extraction from Trizolyzed samples. mtDNA was quantified by qPCR in triplicates on diluted DNA using Ct method. qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) with the following primers:

i) **mtDNA specific PCR**

forward 5'-CTATGTCGCAGTATCTGTCTTTG-3',

reverse 5'-GTTATGATGTCTGTGTGGAAAG-3'

ii) **nuclear specific PCR (SIRT1 gene),**

forward 5'-CTTTGTGTGCTATAGATGATATGGTAAATTG-3',

reverse 5'-GATTAAACAGTGTACAAAAGTAG-3'

Western blotting and antibodies

Cells were collected in 1x Laemmli loading buffer, boiled at 100°C for 10 min, separated by SDS-PAGE and finally transferred to PVDF Immobilon-P transfer membrane (Merck-Millipore, Germany). Following transfer, membrane was blocked by 5% skimmed milk for 1 hour and then kept for primary antibody incubation overnight in 1% milk solution. The primary antibodies used are listed below:

Table 2: List of primary antibodies used in the study

Primary antibody	Dilution	Manufacturer
anti-p50	1:1000	Cayman Chemicals, 13755
anti- IκBα	1:1000	Cell Signalling Technology, 4812
anti-β-actin	1:5000	Novus Biologicals, A2066
anti-UCP1	1:750	R&D Systems, MAB6158
anti-pCREB	1:1000	Merck-Millipore, 05-667
anti-CREB	1:1000	Abcam, ab31387
anti-PGC1α	1:1000	Santa Cruz Biotechnology, H-300
anti-CKMT2	1:1000	Novus Biologicals, NBP2-13841
anti-CKB	1:1000	Novus Biologicals, A2066
anti-OXPHOS	1:1000	Abcam, ab110411
anti-Aggrecan	1:1000	Novus Biologicals, NB100-74350
anti-ID1	1:1000	Novus Biologicals, JM92-13
anti-p62	1:5000	Novus Biological, NBP1-49956
anti-LC3	1:2000	Novus Biological, NB100-2220
anti-Parkin	1:750	Santa Cruz Biotechnology, sc-32282

HRP-conjugated goat anti-rabbit IgG antibody (1:10,000, Advansta, CA, USA, R-05072-500) and HRP-conjugated goat anti-mouse IgG antibody (1:5000, Advansta, R-05071-500) were used as the respective secondary antibodies. Immobilon western chemiluminescence substrate (Merck-Millipore) was used to visualize the immunoreactive proteins. Densitometry was performed by FIJI (ImageJ).

Immunostaining and image acquisition by laser scanning cytometry (LSC) and confocal microscopy

Preadipocytes were plated and differentiated in 8-well Ibidi µ-slides followed by the mentioned treatments. Cells were washed once with PBS and fixed by 4% PFA for 5 minutes. 0.1% saponin was used for membrane permeabilization, followed by blocking with 5% skimmed milk. Incubations were kept overnight with the following primary antibodies: anti-LC3 (1:200, Novus Biological, NB100-2220), anti-TOM20 (1:75, WH0009804M1), anti-CXCL1 (1:100, 712317, Thermo Fisher Scientific) or anti-UCP1 (1:200, U6382). Secondary antibody incubation was kept for 3 hours with the following antibodies: Alexa 647 goat anti-mouse IgG (1:1000, Thermo Fischer Scientific, A21236) and Alexa 488 goat anti-rabbit IgG (1:1000, Thermo Fischer Scientific, A11034). Propidium Iodide (1.5 µg/mL, 1 h) was used to label the nuclei. Images were acquired either with Olympus FluoView 1000 confocal microscope or iCys Research Imaging Cytometer.

Image analysis

i) CXCL1 immunostaining intensity: The boundaries of preadipocytes and differentiated adipocytes were marked based on brightfield (BF) images and nuclear staining. The intensity of CXCL1 immunostaining was determined for each cell type manually using FIJI.

ii) LC3 punctae and fragmented mitochondria content (TOM20): LC3 punctae was quantified using FIJI with size (pixel^2) 50–infinity AU. Fragmented mitochondria were

quantified from TOM20 immunostaining using FIJI with size (pixel^2) 0–100 AU. Both LC3 punctae and fragmented mitochondria content was normalized to per nucleus.

iii) Co-localization of LC3 and TOM20 immunostaining: Colocalization was quantified by Pearson's correlation coefficients (PCC) between the pixel intensities of the two detection channels.

iv) UCP1 immunostaining intensity, differentiation rate and texture sum variance calculation: Images were obtained by iCys Research Imaging Cytometer and analysed by high throughput automatic cell recognition protocol using the iCys companion software (iNovator Application Development Toolkit, CompuCyte Corporation) and CellProfiler (The Broad Institute of MIT). The analysis was performed using technology developed by Dr. Endre Kristóf, Dr. Quang-Minh Doan-Xuan and Dr. Zsolt Bacsó of University of Debrecen.

Determination of cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

Cells were seeded and differentiated on XF96 (Seahorse Biosciences, MA, USA). assay plates and OCR, ECAR were measured by XF96 oximeter (Seahorse Biosciences). Baseline respiration and acidification were measured for 30 min followed by cAMP stimulated values. A single bolus dose of dibutyryl-cAMP (at 500 μM final concentration) was added to mimic adrenergic stimulation leading to the stimulated OCR and ECAR values, that were measured every 30 mins. Oligomycin at 2 μM final concentration was added 3 hours post treatment to block ATP synthase activity for proton leak OCR measurement. Creatine cycle related OCR was measured by addition of β-GPA (2mM) after cAMP stimulation. Antimycin A (10 μM) was added at the end for baseline correction. OCR was normalized to protein content.

Determination of released factors

Supernatants were collected from cell culture experiments during regular replacement of media on days 4,12,18,21 of the differentiation period, as indicated. For SC and DN differentiating adipocytes, supernatants collected from the same set of donors were considered as one repetition. In case of tissue biopsies, 10-20 mg of SC and DN tissue samples from the same donors were floated for 24 hours in DMEM-F12-HAM medium in the presence or absence of 250 ng/mL irisin. The release of CXCL1, CX3CL1, IL-32, TNFα, and IL1-β were analysed using ELISA Kits (R&D systems) following manufacturer's instructions.

Human umbilical vein endothelial cell (HUVEC) adhesion assay

The HUVEC cell line was generated from endothelial cells isolated from human umbilical cord vein of a healthy newborn by collagenase treatment. M199 medium (Biosera, France) supplemented with 10% FBS (Thermo Fisher Scientific), 10% EGM2 (Lonza, Switzerland), 20 mM HEPES, 100 U/mL Penicillin, 100 μg/mL Streptomycin and 2.5 μg/mL Amphotericin B (Biosera) was used for cell culture. Cells were immortalized by viral delivery of telomerase gene using pBABE-neo-hTERT. Prior to the assay, EGM2 was omitted from the medium and FBS content was reduced to 1% and maintained for 24 hours, that will make proliferation unlikely (hereafter starvation media). 96-well plate was precoated with fibronectin (1.25 μg/mL) for 1 hour at 37°C, washed twice with PBS and seeded with HUVEC cells at 1000 cells/well density. Cells were allowed to adhere for 2 hours at 37°C and 5% CO_2 with the medium consisting of starvation media and conditioned media (collected during Day 12 of differentiation period) in 1:1 ratio. Recombinant human CXCL1 (R&D Systems, 275-GR) was added to the starvation media at 2500 pg/mL for its respective assay. Unattached cells were removed by PBS wash and adhered cells were incubated with starvation media containing CellTiter-Blue Cell Viability reagent (resazurin; Promega, WI, USA; 36 times dilution). To

determine the ratio of attached cells in various conditions, the fluorescence intensity change of each well (Ex:530 nm/Em:590 nm), due to the conversion of resazurin to resorufin by cellular metabolism, was measured 2, 4, 6, 18, and 24 hours after adding resazurin. Slope of fluorescence intensity values plotted against time showed a linear slope, suggesting negligible cell proliferation. The relative adhesion values were obtained after subtraction of control values (wells containing only starvation media without cells).

Statistical analysis and figure preparation

Results are expressed as mean ± SD for the number of independent repetitions indicated. For multiple comparisons, statistical significance was determined by one- or two-way ANOVA followed by Tukey post hoc test. For comparisons between two groups two-tailed t-test was used. Graphpad Prism 9 was used for figure preparation and evaluation of statistics.

Results

Regulation of thermogenesis via mitophagy

Adrenergic stimulus elevates expression of thermogenic genes in human primary abdominal preadipocytes differentiated to mature white and beige adipocytes

hADMSCs isolated from the SVF of abdominal subcutaneous adipose tissue were differentiated to mature white and beige adipocytes following the respective white or beige differentiation protocol for a period of 14 days. Differentiated white and beige adipocytes were subjected to cAMP mediated adrenergic stimulus for a period of 6,10 and 14 hours respectively. Beige adipocytes possessed significantly higher UCP1 protein expression than white adipocytes. Adrenergic stimulus elevated UCP1 gene expression starting 6 hours post treatment and was found to be significantly elevated at 10 hours post treatment in both white and beige differentiated adipocytes. A similar pattern was observed for the UCP1 protein expression upon adrenergic stimulus with significant upregulation at 14 and 6 hours post treatment in white and beige differentiated adipocytes. Blocking lysosomal degradation by chloroquine (CQ) did not affect UCP1 protein expression.

cAMP stimulus further upregulated the total mitochondrial content, that was found to be significantly upregulated 6 hours post treatment in white and beige differentiated adipocytes. The gene expression of PGC1α, a mitochondrial biogenesis regulator was also significantly elevated 6 hours post cAMP treatment in both white and beige differentiated adipocytes. Hence, cAMP mediated adrenergic stimulus elevated the thermogenic potential of human primary abdomen derived white and beige differentiated adipocytes.

The high mitophagy rate in human abdominal derived white and beige differentiated adipocytes is downregulated upon adrenergic stimulus

A recent study in mice showed that autophagy-dependent mitochondrial clearance was low in beige adipocytes which upregulated during whitening upon removal of the β3-AR agonist. Our study intended to investigate how autophagy is regulated upon adrenergic stimulation by cAMP. Gene expression of general autophagy markers ATG5, ATG7 and ATG12 were significantly reduced 6 hours post cAMP mediated adrenergic stimulus in both white and beige differentiated adipocytes. LC3-II/ LC-I ratio is a well-accepted method for monitoring ongoing autophagic activity; an increased ratio indicates a high level of ongoing autophagy. CQ was administered to measure the autophagic flux. Under basal conditions a high LC3-II/LC3-I ratio was obtained for white differentiated adipocytes as compared to beige, that was further elevated upon CQ treatment, which indicated an increased autophagic flux in the white adipocytes. The cAMP mediated adrenergic stimulus significantly reduced the LC3-II/LC3-I ratio in both white and beige differentiated adipocytes starting 6 and 10 hours post treatment, respectively. This clearly indicated an inhibition of autophagy following adrenergic stimulus. Protein expression of p62, a well-known autophagy adapter protein, was significantly elevated following 6 hours cAMP treatment in both types of adipocytes that indicated reduced autophagy. Together, the data suggested that cAMP mediated adrenergic stimulus downregulates the ongoing autophagic activity in both white and beige differentiated adipocytes.

Next, co-immunostaining of LC3 (labelling autophagosomes) and TOM20 (labelling mitochondria) were performed in white and beige differentiated adipocytes without treatment or with cAMP and CQ treatments. Quantification of LC3 punctae counts is an important measure of ongoing autophagy and colocalization of LC3, TOM20 identifies mitophagy. CQ treatment increased the number of LC3 punctae count in white differentiated adipocytes indicating a higher autophagic flux. cAMP mediated adrenergic stimulus significantly reduced

LC3 punctae count in both white and beige differentiated adipocytes further confirming that autophagy is repressed following the stimulus. Co-localization measurements for LC3 and TOM20 revealed that CQ increased co-localization in white and beige differentiated adipocytes indicating an enhanced mitophagic flux, whereas cAMP treatment led to a significant reduction in co-localization, further indicating repressed mitophagy upon its stimulus.

A recent study revealed that mitochondria with fragmented morphology are mostly abundant in UCP1. Quantification of fragmented mitochondria content from TOM20 immunostaining showed that, cAMP treatment significantly increased the content of fragmented mitochondria in both white and beige differentiated adipocytes. These data collectively suggest that in unstimulated adipocytes, most of the fragmented mitochondria are eliminated because of high mitophagy rate, which is rescued upon adrenergic stimulus, leading to a greater availability of UCP1 rich fragmented mitochondria capable of increased thermogenesis.

Adrenergic stimulus downregulates mitophagy via both parkin dependent and independent mechanisms

Mitophagy occurs in both parkin dependent and independent mechanisms. cAMP mediated adrenergic stimulus significantly downregulated PARK2 gene expression starting 10 hours post treatment in both white and beige differentiated adipocytes. Parkin protein expression was significantly downregulated 6 and 14 hours post cAMP treatment in beige differentiated adipocytes. Significant reduction in gene expression of OPTN and CALCOCO2 (NDP52), the most prominent adapters of parkin-dependent pathway, was observed following the cAMP treatment. These results indicated prompt repression of parkin dependent mitophagy pathway upon adrenergic stimulus.

Hence, as a next step, silencing of Parkin by siRNA was performed after white and beige differentiation, to check if that results in a stronger thermogenic response. The silencing resulted in 60% reduction of Parkin protein expression in both white and beige differentiated adipocytes. The Parkin silencing by itself did not alter the levels of LC3-II/LC3-I ratio or the p62 protein expression, indicating the ongoing mitophagy rate remained largely unchanged. The silencing resulted in increased UCP1 protein expression only in beige differentiated adipocytes. cAMP could similarly upregulate UCP1 protein expression in cells incubated with Parkin and control siRNA, indicating that Parkin deficiency did not lead to the appearance of a more active beige phenotype.

Since, silencing of parkin alone could not repress autophagy or improve the cAMP mediated thermogenic response, the expression of parkin-independent mitophagy genes were also evaluated. In response to adrenergic stimulus, the gene expression of BCL2L13, FKBP8, FUNDC1 and BNIP3 was promptly repressed. Together, these results indicated a complex mechanism involving both parkin-dependent and independent mitophagy pathways by which mitophagy is repressed in white and beige adipocytes following adrenergic stimulus.

Effect of irisin on neck depot derived adipocytes

Human primary subcutaneous neck (SC) and deep neck (DN) derived preadipocytes differentiated equally to mature white adipocytes irrespective of irisin treatment

hADMSCs were isolated from SVFs of SC and DN biopsies of nine independent donors, maintained and then differentiated following the white adipogenic differentiation protocol with or without the presence of irisin for 14 days. Samples were collected, followed by RNA isolation and RNA-Sequencing. Heatmap illustrating gene expression patterns of general adipocyte markers like FABP4, PLIN1 and ADIPOQ were elevated in all differentiated adipocytes compared to preadipocytes. Adipocyte differentiation rate quantification by laser scanning cytometry showed that more than 50% cells were differentiated following the 14-day differentiation and irisin did not exert any effect on the differentiation rate. Gene expression of the proposed irisin receptors ITGAV and ITGB1,3,5 were expressed at a high extent at preadipocyte level and in all differentiated adipocytes. Hence SC and DN derived preadipocytes differentiated equally irrespective of irisin treatment.

Irisin treatment cannot upregulate characteristic thermogenic genes in SC and DN derived differentiating adipocytes in contrast to adipocytes obtained from abdominal subcutaneous tissue depot

RNA Sequencing identified 79 upregulated genes upon irisin treatment that are visualized by Volcano Plot. 50 and 66 genes were significantly upregulated in SC and DN adipocytes in response to irisin, 37 of which are commonly upregulated between the depots.

Previously our research group has shown that irisin treatment at 250 ng/mL can upregulate UCP1 gene expression in human primary subcutaneous abdominal differentiating adipocytes. Now, we have confirmed that irisin treatment can significantly upregulate UCP1 protein expression in abdominal differentiating adipocytes. Strikingly, irisin could not upregulate characteristic thermogenic marker genes like UCP1 in SC and DN derived differentiating adipocytes.

Genes related to chemokine and interleukin signalling pathways are upregulated in response to irisin in SC and DN derived differentiating adipocytes

Panther enrichment analysis revealed that irisin treatment commonly upregulated pathways involving cytokine signalling (NFKB2, CXCL1, CXCL2, IL32, IL34, IL6, CCL2), interleukin-4 and 13 signalling (IL6, CCL2, JUNB, ICAM1), and class A/1 rhodopsin like receptors (CXCL3, CXCL5, CX3CL1, CXCL2, CCL2, CXCL1) in SC and DN adipocytes. Gephi diagrams clearly illustrate the interactions of upregulated genes belonging to several pathways. IL10 signalling and G-alpha-I signalling pathways were amongst the upregulated pathways in SC and DN adipocytes respectively.

CXCL1 is primarily released from SC and DN derived differentiating adipocytes in response to irisin

The chemokine (C-X-C motif) ligand 1 (CXCL1) belongs to the CXC chemokine family acts as a chemoattractant for several immune cells, especially neutrophils. Earlier termed as GROα, in humans this protein is encoded by the gene CXCL1. CXCL1 was found to be the highest upregulated gene as per RNA-Sequencing, that was further validated by RT-qPCR to be significantly upregulated in both SC and DN adipocytes upon irisin treatment. CXCL1 was found to be released upon irisin treatment from the conditioned medium collected on days number 4 and 12 of the differentiation period of both SC and DN derived differentiating adipocytes.

We aimed to further investigate the dependence of CXCL1 release on the presence of irisin. For this SC and DN derived hADMSCs were differentiated for a period of 21 days with or without irisin, along with a set where irisin treatment was discontinued for the last 7 days. CXCL1 was found to be consistently released on days number 4,12,18,21 of the differentiation period only in presence of irisin; removal of irisin on day 14 promptly lowered the release of CXCL1 on days number 18 and 21.

Inhibition of the proposed irisin receptors by RGDS, a pan integrin inhibitor, could reduce the irisin-stimulated CXCL1 release on day 12 of the differentiation period only for DN adipocytes. This indicated presence of irisin stimulates the CXCL1 release but not prominently on its presumed integrin receptors.

Release of CXCL1 throughout the differentiation period raised the possibility that both undifferentiated preadipocytes and differentiated adipocytes might release the chemokine. To investigate this, Brefeldin A was used to block the secretion machinery of the mixed cell population, followed by CXCL1 immunostaining and image acquisition by confocal microscopy. Irisin treatment resulted in accumulation of increased CXCL1 immunostaining in both SC and DN differentiated adipocytes than their preadipocyte counterparts. This clearly showed that the release of CXCL1 occurred primarily from SC and DN differentiated adipocytes.

Irisin stimulates the CXCL1 release via upregulation of NFκB pathway

According to RNA-Sequencing data, irisin treatment significantly elevated NFKB2 and a very modest increasing trend was observed for NFKB1 and RELA that pointed towards the involvement of NFκB pathway.

RT-qPCR validation confirmed a significant increase of NFKB1 (p50 subunit), RELA (p65 subunit) in DN and an increasing trend in SC adipocytes. Protein expression of p50 subunit was significantly elevated in DN, with an increasing trend in SC adipocytes. IκBα (inhibitor of NFκB transcription factor) protein expression was significantly decreased by irisin treatment in SC, while a decreasing trend was observed in DN adipocytes. These data collectively indicated an upregulation of the NFκB pathway.

To further prove the involvement of the NFκB pathway, a cell permeable inhibitor of NFκB nuclear translocation, SN50 was applied. SN50 significantly reduced the CXCL1 release from both SC and DN adipocytes treated with irisin. Hence, irisin stimulates the release of CXCL1 via the upregulation of NFκB pathway.

The observed effect of the CXCL1 release upon irisin treatment was not likely to be caused by an endotoxin contamination which was supported by the negligible expression of TNFα and CCL3 genes and decreasing trend for IL1β gene in irisin treated adipocytes. Furthermore, TNFα and IL1β could not be detected in the conditioned media of SC and DN derived differentiating adipocytes with or without irisin treatment.

CXCL1 released from irisin stimulated adipocytes and adipose tissue improves adhesion property of human umbilical vein endothelial cells (HUVEC)

SC and DN tissue biopsies were floated for 24 hours in the presence or absence of irisin dissolved in empty media, followed by quantification of CXCL1 release from the conditioned media. CXCL1 release was found to be significantly stimulated from DN tissue biopsies upon irisin treatment.

CXCL1 plays an important role in wound repair and angiogenesis. Angiogenesis is vital for the thermogenic function of BAT. Therefore, we intended to detect the adhesion improving capacity of the released CXCL1. Conditioned media collected on the twelfth day of differentiation, from untreated and irisin treated SC and DN derived adipocytes, were added to HUVECs followed by a resorufin based adhesion assay. The conditioned medium from irisin treated adipocytes, containing various released factors (including CXCL1) significantly increased the number of attached viable HUVECs, compared to the medium collected from untreated adipocytes. HUVECs treated with recombinant CXCL1 at 2500 pg/mL (highest observed concentration of CXCL1 from SC and DN differentiating adipocytes) significantly improved the adhesion property of the endothelial cells. This suggested a potentially beneficial role of CXCL1 in promoting endothelial cell function and adipose tissue remodelling to indirectly promote efficient thermogenesis by enhanced vascularization.

BMP7 stimulates thermogenesis of deep neck derived adipocytes

Human primary SC and DN derived preadipocytes differentiated equally to mature white adipocytes irrespective of BMP7 treatment

hADMSCs were isolated from SVFs of SC and DN biopsies of nine independent donors, maintained and then differentiated following the white adipogenic differentiation protocol with or without the presence of BMP7 for 14 days. Samples were collected, followed by RNA isolation and RNA-Sequencing. Heatmap illustrating gene expression patterns of general adipocyte markers were elevated in all differentiated adipocytes compared to preadipocytes. Adipocyte differentiation rate quantification by laser scanning cytometry showed more than 50% cells were differentiated following the 14-day differentiation; BMP7 did not exert any effect on the differentiation rate. Gene expression of BMPR subunits (BMPR1A, BMPR1B, ACVR1, BMPR2, ACVR2A, ACVR2B) were abundant at the preadipocyte stage in both SC and DN adipocytes, BMP7 treatment did not alter their expression. Hence SC and DN derived preadipocytes differentiated equally irrespective of BMP7 treatment.

BMP7 elevates UCP1 dependent thermogenesis in SC and DN derived differentiating adipocytes

A recent in-depth analysis of murine and human white, beige and brown adipocyte transcriptomes has been utilized to develop BATLAS, an algorithm that can calculate brown/beige adipocyte content in cell and biopsy samples. Analysis of the RNA Sequencing data with BATLAS algorithm showed that browning content of DN adipocytes was significantly higher than SC. BMP7 treatment significantly elevated browning content in both SC and DN derived differentiating adipocytes. Quantification of texture sum variance by laser scanning cytometry revealed that BMP7 treatment slightly reduced the size of lipid droplets in both types of adipocytes suggesting increased capacity for lipolysis and energy expenditure. UCP1 gene expression showed a tendency to increase upon BMP7 treatment as per RNA Sequencing in both SC and DN adipocytes, that was found to be significant in DN adipocytes by RT-qPCR. UCP1 protein expression showed that DN adipocytes have a significantly higher UCP1 than SC. BMP7 treatment significantly elevated UCP1 protein expression in both SC and DN adipocytes. Confocal microscopy pictures of UCP1 immunostaining clearly showed that BMP7 increased the immunostaining intensity in neck derived adipocytes. Laser-scanning cytometry assisted quantification of UCP1 immunostaining intensity showed a significant increase of intensity in DN adipocytes upon BMP7 treatment. Together these data suggest the upregulation of browning marker genes (including UCP1) in SC and DN adipocytes upon BMP7 treatment.

BMP7 elevated mitochondrial biogenesis leading to increased mitochondrial content, OXPHOS complex subunits and fragmented mitochondria

BMP7 treatment upregulated the protein expression of PGC1α in both SC and DN derived adipocytes. PGC1α can be induced by the transcription factor CREB, that binds to a functional CRE in the PGC-1 promoter region. A significant induction of CREB phosphorylation (p-CREB) was observed in DN adipocytes. Together the upregulation of PGC1α and p-CREB suggested an increase of mitochondrial biogenesis upon BMP7 treatment. This fact was further supported by an elevation of the protein expression of mitochondrial respiratory chain complexes upon BMP7 treatment; amongst which Complex I, II and III subunits showed a significant elevated level.

Quantification of fragmented mitochondria based on TOM20 immunostaining revealed a significant upregulation of its content following BMP7 treatment. The treatment also

significantly increased the total mitochondrial DNA content in both types of adipocytes. These data indicated that BMP7 treated differentiated adipocytes possess an enhanced thermogenic potential.

Next, we intended to investigate the functional consequences of improved thermogenic potential. OCR data revealed that both basal and cAMP stimulated OCR was significantly elevated upon BMP7 treatment. Importantly, proton leak related OCR was also significantly elevated following treatment in both SC and DN derived differentiated adipocytes; this indicated increased UCP1 mediated thermogenesis. Basal ECAR showed an increasing trend, while cAMP stimulated ECAR was significantly elevated in DN derived adipocytes differentiated in the presence of BMP7. Together, the data suggests that BMP7 treatment can significantly upregulate thermogenesis in SC and DN derived differentiating adipocytes.

BMP7 elevated creatine driven substrate cycle related thermogenesis in neck area derived adipocytes

BMP7 treatment significantly increased the creatine driven substrate cycle related OCR in both SC and DN derived adipocytes. RNA expression of mitochondrial creatine kinase 2 (CKMT2), one of the possible kinases acting in the futile cycle, was slightly elevated as per RNA-sequencing dataset and was found to be significantly elevated by RT-qPCR analysis in both types of BMP7 treated adipocytes. CKMT2 protein expression followed a similar pattern. RNA-sequencing data revealed an increasing trend for CKB gene expression upon BMP7 treatment, that was found to be significant in terms of protein expression also in both types of adipocytes. TNAP, the phosphatase identified to hydrolyse phosphocreatine to creatine, was highly expressed in both types of adipocytes. Together, these data suggest that BMP7 upregulates thermogenesis via both UCP1 and futile creatine phosphate cycle in human neck area adipocytes.

BMP7 upregulated novel genes in SC and DN derived differentiating adipocytes that might play role in thermogenesis

Next, we aimed to further explore the BMP7 regulated pathways in neck area derived adipocytes that have so far not been linked to thermogenesis. RNA-sequencing data revealed that 121 and 60 genes were upregulated in BMP7 treated SC and DN derived adipocytes, while 190 and 87 genes were downregulated, respectively. 38 genes were commonly upregulated between SC and DN derived adipocytes, while 45 genes were commonly downregulated, respectively. Panther pathway analysis illustrated that BMP signalling involving SMAD group of transcription factors (SMAD6, SMAD7, SMAD9) was commonly upregulated in both types of adipocytes that is consistent with previous findings. Integrin cell surface interactions (ITGA9, COMP, ITGA8), GPCR ligand binding (ADRA2A, ADRA2C, FZD1, FZD5, ACKR1) and extracellular matrix proteins (ACAN) were among the significantly elevated pathways only in SC derived adipocytes upon BMP7 treatment.

According to the RNA-sequencing analysis, ACAN, CRYAB, and ID1 were strongly upregulated in BMP7 treated adipocytes. Gene and protein expression of ACAN was confirmed to be significantly higher in BMP7 treated SC adipocytes. Gene and protein expression of CRYAB, a member of small heat shock protein (HSP) 20 family, was significantly increased in BMP7 treated SC and DN adipocytes. ID1 gene expression was also significantly higher in both types of BMP7 treated adipocytes, while in case of protein expression ID1 was significantly elevated in SC and followed an increasing trend in DN treated adipocytes.

Discussion

Downregulation of mitophagy contributes to the thermogenesis-inducing effect of cAMP mediated adrenergic stimulus in human abdominal white and beige differentiated adipocytes

Subcutaneous abdominal preadipocytes differentiated to white and beige adipocytes following a 14-day long differentiation protocol responded to cAMP mediated thermogenic stimulus starting 6 hours post exposure. The thermogenic stimulus led to increased UCP1 gene and protein expression both in white and beige differentiated adipocytes. The total mitochondria DNA content was also elevated following the cAMP stimulus, that partially resulted from an increased mitochondrial biogenesis driven by elevated PPARGC1A gene expression. The cAMP stimulus resulted in increased amount of mitochondria with fragmented morphology in both white and beige differentiated adipocytes, which possess the capacity to deliver thermogenesis on demand because of their increased UCP1 content.

The increased fragmented mitochondria content may not be solely attributed to increased mitochondrial biogenesis, since mitophagy too plays an important role in the process. The cAMP mediated thermogenic stimulus downregulated gene expression of general autophagy markers like ATG5, ATG7 and ATG12. It also resulted in decreased protein expression of LC3-II/LC3-I, that was further verified by a reduced count of LC3 punctae upon immunostaining. This indicated a repression of the autophagy machinery upon thermogenic stimulus.

The binding of NA to adrenergic receptors increases intracellular cAMP levels, that in turn leads to activation of the cAMP-PKA signalling pathway. The activation of this pathway leads to the induction of mitochondrial biogenesis and genes related to thermogenesis. PKA has also shown to inhibit autophagy through various mechanisms in response to adrenergic stimulation in mammalian cells. PKA can directly phosphorylate LC3 or indirectly via activating the mammalian target of rapamycin complex 1 (mTORC1), that serves as a major repressor of autophagy. Consistent with recent mouse studies, we have shown that the cAMP mediated thermogenic stimulus represses the autophagic activity via activation of cAMP-dependent PKA pathway in primary human abdominal differentiated beige adipocytes.

Altshuler-Keylin et al. identified autophagy-mediated removal of mitochondria as a novel mechanism for beige adipocyte maintenance and energy expenditure in mice. Here, mitophagy rate was evaluated by colocalization of LC3 and TOM20 immunostaining, that indicated a repression of mitophagy upon thermogenic stimulus. Parkin is one of the major mediators of mitophagy, with decrease in parkin-dependent mitophagy reported to drive WAT browning in mouse 3T3-L1 adipocytes. Parkin downregulation was observed in response to thermogenic induction of mouse BAT, and high thermogenic activity was exhibited by parkin knockout mice. However, Corsa et al. published opposing results, claiming parkin is dispensable for adipogenesis, differentiation and mitochondrial quality control in mice beige adipocytes. In our study, parkin gene and protein expression was repressed upon thermogenic stimulus in human white and beige differentiated adipocytes. Gene expression of parkin mediated mitophagy adapter proteins OPTN and NDP52 were also downregulated upon thermogenic stimulus. However, silencing of parkin could neither result in an improved thermogenic response nor it repressed autophagy, as was observed upon cAMP mediated adrenergic stimulus. These results show that Parkin may not play a pivotal role in regulating mitophagy in white and beige adipocytes of abdominal origin. In addition to adapter proteins, parkin independent mitophagy pathways have also been shown to mediate adipose tissue specific thermogenic effects. BCL2L13 has been shown to contribute in beige adipocyte biogenesis and BNIP3 in regulating mitochondrial fragmentation in mouse adipocytes. Here, gene expression of BCL2L13, FKBP8

and FUNDC1 were repressed upon thermogenic stimulus in white and beige adipocytes. Our results suggest that both parkin dependent and independent mitophagy mechanisms are important for mediating mitochondrial maintenance in white and beige adipocytes.

In addition to primary adipocytes, a non-immortalized human adipocyte cell line model, Simpson-Golabi-Behmel syndrome (SGBS) cells were also used to rule out possible effects of heterogenicity and donor dependency. All the experiments performed on primary adipocytes were subsequently repeated on SGBS adipocytes. cAMP mediated adrenergic stimulus exerted similar effects on SGBS white and beige differentiated adipocytes. These further strengthened our findings that cAMP mediated adrenergic stimulus activates the thermogenic potential of subcutaneous abdominal fat by repressing mitophagy via both parkin dependent and independent mechanisms.

The study raises the possibility of obtaining thermogenically active subcutaneous abdominal beige adipocytes from masked beige adipocytes in vivo by adipocyte specific inhibition of mitophagy. Recent studies indicated the presence of high amount of "brownable fat" in obese adults. These depots might remain dormant as a result of high autophagy and mitophagy. Further studies are necessary for better understanding of the mechanisms regulating physiological mitophagy and its pathologic dysregulation that contribute to the cellular homeostasis of active beige adipocytes and the development of obesity, respectively.

Irisin as an inducer of browning

Irisin was discovered as a cleaved product of FNDC5 and primarily released by cardiac and skeletal muscles. It has been shown to induce a beige differentiation program in mouse subcutaneous WAT. In human FNDC5 gene adenine has shown to be replaced by guanine in the start codon, that might result in a shorter precursor protein lacking the part where irisin is cleaved. Despite this, irisin has been detected from human blood plasma at 3-4 ng/mL by mass spectrometry. A recent publication determined the level of circulating irisin to be 0.3 ng/mL in mice, that was previously estimated to be 800 ng/mL. Irisin when applied at 25-1250 ng/mL, significantly increased UCP1 gene and protein expression in rat primary adipocytes. The protein expression of BAT markers like PGC1α, PRDM16, and UCP1 were increased when irisin was applied at 250 ng/mL on 3T3L1 adipocytes. Irisin (625 ng/mL) elevated mitochondrial respiration of human visceral and subcutaneous WAT-derived and perirenal BAT-derived adipocytes. Our research group previously reported that recombinant irisin at above 50 ng/mL induced a beige program in human primary abdominal subcutaneous and SGBS adipocytes which were differentiated following the white adipogenic differentiation protocol. Irisin treatment at 250 ng/ml significantly increased UCP1 protein expression in differentiating abdominal derived adipocytes. Adipocytes from the DN region has the ability to perform continuous non-shivering thermogenesis and hence play an important role in maintaining whole body energy expenditure. The effect of irisin treatment on differentiating human SC and DN derived adipocytes have not been fully elucidated. Recent publications indicated that irisin may induce a different degree of browning response in humans based on the adipose tissue origin. As per our RNA-Sequencing results, irisin was unable to directly influence the expression of characteristic thermogenic marker genes in SC and DN area adipocytes.

Irisin plays a regulatory role, unrelated to thermogenesis upon acting on human neck derived differentiating adipocytes

In addition to their role in thermogenesis, active thermogenic adipose tissues secrete certain adipokines termed "batokines" that acts in an autocrine, paracrine or endocrine manner. Several recently discovered batokines like NRG4, FGF21, CXCL14, BMP8b and adiponectin have

been shown to exert a protective role against obesity by enhancing beiging of WAT, sympathetic innervation, lipolysis and polarization of M2 macrophages. Our previous publication has shown that irisin treatment stimulated the release of batokines like IL6 and MCP1 from human abdominal subcutaneous and neck area derived adipocytes. The released IL6 was shown to improve browning of human subcutaneous abdominal adipocytes. Here, using a RNA-Sequencing based approach, CXCL1 was found to be a novel adipokine released from both SC and DN derived differentiating adipocytes upon irisin treatment. The release of CXCL1 occurred primarily from differentiated adipocytes via upregulation of the NFκB pathway. Irisin has been shown to exert its effect on mouse osteocytes via a subset of integrin receptors, which are assembled from ITGAV and either of ITGB1, ITGB3 or ITGB5. Our data showed a high expression of ITGAV, ITGB1, ITGB3 and ITGB5 in both preadipocytes and differentiated adipocytes. However, RGDS peptide could exert only a mild effect on the irisin stimulated CXCL1 release only in DN adipocytes. This suggests that irisin might exert its effect via receptors other than the proposed integrin receptors.

CXCL1 binds to CXCR2 and act as a chemoattractant of several immune cells, majorly neutrophils. CXCL1 has been shown to initiate migration of immune and endothelial cells upon injury to facilitate tissue repair. Conditioned medium containing CXCL1 collected from irisin treated SC and DN differentiating adipocytes significantly improved the adhesion of HUVECs. Similar observation was made when HUVECs were treated with recombinant CXCL1. Together these showed a possible beneficial paracrine role of the released CXCL1 from irisin treated adipocytes.

BMP7 upregulates thermogenesis in human neck derived adipocytes via both UCP1 dependent and independent mechanisms

BMP7 acts as an auto/paracrine mediator which promotes murine beige and brown adipocyte differentiation. BMP7 pre-treatment before differentiation of immortalized human neck derived white and brown preadipocyte clones showed significant elevation of UCP1 gene expression. Our previous research showed that BMP7 can upregulate UCP1 gene expression and of other classical browning markers like ZIC1 in SGBS adipocytes. BMP7 has been shown to exert its effect via activation of p38 MAPK and PGC1α that will upregulate mitochondrial biogenesis and UCP1 expression. BMP7 also exerts its effect via phosphorylation of SMAD1, SMAD5 and SMAD8.

Our study showed that receptors involve in BMP signalling were abundantly expressed in the preadipocyte stage of human neck area derived adipocytes. BMP7 treatment could significantly upregulate UCP1 dependent thermogenesis in SC and DN derived adipocytes, that was clearly validated by an increase in UCP1 gene, protein expression and immunostaining intensity. BMP7 increased the total and fragmented mitochondria content in neck derived adipocytes via upregulation of the PGC1α and p-CREB pathways that is in accord with previously published studies. The increase of fragmented mitochondria content further indicated an increased thermogenic potential, the functional aspect of which was confirmed by an increased basal, stimulated, and proton leak OCR in the neck derived BMP7 treated adipocytes.

A creatine mediated futile cycle was recently discovered that enhanced mitochondrial respiration in beige fat. Further an adipose tissue specific knockout of glycine amidinotransferase, the rate limiting enzyme for creatine biosynthesis, made mice susceptible to diet-induced obesity. Creatine driven thermogenesis was also shown to play an important in both UCP1 negative and positive murine beige adipocytes. Mitochondrial creatine kinases, CKMT1 A/B, CKMT2, and recently CKB have been shown to play an important role in the creatine cycle driven thermogenesis. Our data showed an increase of CKMT2 and CKB protein

expression in BMP7 treated neck derived adipocytes. As a functional consequence, creatine cycle related OCR was significantly elevated in both SC and DN adipocytes upon BMP7 treatment. This clearly showed that BMP7 elevates thermogenesis by UCP1-independent mechanisms also.

BMP7 holds a strong potential in therapeutic approaches targeting obesity. A recent study in mouse has shown that, liver directed adeno-associated viral (AAV)-BMP7 vectors could increase long lasting presence of BMP7 in circulation. The increase in circulating BMP7 in high-fat-diet-fed and genetically obese mice induced browning of WAT and activation of BAT, which in turn normalized body weight and improved insulin resistance in these mice.

BMP7 upregulates certain novel genes that may serve as positive regulators of thermogenesis

Our results indicated BMP7 upregulates certain genes like ACAN, CRYAB and ID1 that are unrelated to browning of adipocytes so far but show a strong corelation with increased thermogenesis.

ACAN encoding aggrecan protein, a chondroitin sulphated proteoglycan, functions as an important structural component of cartilage. It is also found in brain exclusively in the perineuronal net where it assists in its formation and function. A similar function is also expected for the innervation of brown adipose tissue. ACAN gene and protein expression was significantly elevated in SC adipocytes upon BMP7 treatment.

CRYAB (Crystallin Alpha B) is a major structural proteins of eye lens but expressed in other tissues also. CRYAB belongs to the small HSP family and functions as a chaperone protecting against oxidative stress and apoptosis. In eye lens, CRYAB serves as a substrate for TGM2 (transglutaminase 2) mediated crosslinking. Our group has previously shown that gonadal WAT from TGM2 KO mice showed reduced expression of characteristic beige marker genes like UCP1, TBX1. Our previous publication showed that CRYAB gene expression was higher in the thermogenic DN adipocytes as compared to SC. In this study, CRYAB gene and protein expression was significantly increased in neck derived adipocytes upon BMP7 treatment.

The ID family comprising of ID1, ID2, ID3 and ID4 belongs to the helix–loop–helix (HLH) transcription factor family. They act primarily via dimerization with transcriptional regulators like basic-HLH (bHLH) factors which fails to bind to DNA. Hence the ID proteins function as negative regulators of bHLH proteins. A recent study indicated that ID1 protein is highly expressed in both murine WAT and BAT, with BAT showing the highest expression. In this study, ID1 gene and protein expression was significantly upregulated in BMP7 treated neck derived adipocytes.

Further experiments are necessary to validate the direct effect of ACAN, CRYAB and ID1 in thermogenesis of human beige and brown adipocytes.

Summary

- Adrenergic stimulus elevates thermogenesis in human primary abdominal white and beige differentiated adipocytes. This effect was found to be mediated by increased availability of fragmented morphology mitochondria that possess higher thermogenic capacity. Adrenergic stimulus resulted in increased mitochondrial biogenesis and reduced mitophagy that protected the fragmented mitochondria from degradation leading to their increased availability.
- cAMP mediated adrenergic stimulus resulted in repression of mitophagy via both parkin dependent and independent mechanisms.
- Human SC and DN derived preadipocytes differentiated equally in presence or absence of irisin. However, unlike in abdominal subcutaneous derived adipocytes, the presence of irisin failed to upregulate characteristic thermogenic genes in the neck derived adipocytes.
- Irisin upregulated several common genes in both SC and DN derived differentiating adipocytes which mostly pointed towards cytokine and interleukin signalling pathways. The topmost upregulated gene CXCL1 was found to be released by both SC and DN adipocytes throughout the differentiation period upon irisin treatment. The release of CXCL1 occurred primarily from differentiated adipocytes via the upregulation of NFκB pathway. The released CXCL1 increased adhesion of HUVEC cells.
- Human SC and DN derived preadipocytes differentiated equally in presence or absence of BMP7. BMP7 upregulated thermogenesis in SC and DN derived differentiating adipocytes via both UCP1 dependent and independent (futile creatine cycle mediated thermogenesis) mechanisms.
- BMP7 treatment led to increased availability of thermogenically competent fragmented morphology of mitochondria. The increased amount of mitochondria was a result of upregulation of mitochondrial biogenesis via PGC1α and CREB pathways.
- BMP7 treatment upregulated certain genes like ID1, ACAN and CRYAB that correlated with increased thermogenesis and may represent a novel function in assisting thermogenesis.

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List of publications related to the dissertation

1. **Shaw, A.**, Bartáné Tóth, B., Arianti, R., Csomós, I., Póliska, S., Vámos, A., Bacsó, Z., Győry, F., Fésüs, L., Kristóf, E.: BMP7 increases UCP1-dependent and independent thermogenesis with a unique gene expression program in human neck area derived adipocytes. *Pharmaceuticals (Basel). 14* (11), 1-21, 2021.
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Total IF of journals (all publications): 46,357
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