OSPEDALE Casa Sollievo della Sofferenza
ISTITUTO DI RCOVERO E CURA A CARATTERE SCIENTIFICO
OPERA DI SAN PIO DA PIETRELCINA
Viale Cappuccini snc -SAN GIOVANNI ROTONDO (FG)

DICHIARAZIONE DI UNICITA'

PER PROGETTI FINANZIATI DAI FONDI SIE-FSC-FESR E DA ENTI NAZIONALI ED INTERNAZIONALI

San Giovanni Rotondo, 22/11/2024

Al Responsabile Amministrativo
Dott. Michele Giuliani

DICHIARAZIONE DI UNICITA'
(resa ai sensi del D.P.R. n. 445/2000)

Il sottoscritto Salvatore Alessandro De Cosmo C.F. DCSSVT57E020643B, in qualità di Responsabile Scientifico del Progetto: "TRYPTOPHAN-RELATED METABOLIC PATHWAYS AND MORTALITY RATE IN PEOPLE WITH DIABETES. A COMPREHENSIVE APPROACH BASED ON OMICS ANO CELL BIOLOGY STUDIES"
- Codice Identificativo: RPNRR22DE01_Progetto PNRR-MAD-2022-12375970 (RPNRR22DE01),
- CUP I23C22000720007,
con riferimento alla richiesta di acquisto presentata in data 22.07.2024 ed in relazione agli articoli di seguito elencati:

- Cod. AC-A57868-1ML, Anti-L-Kynurenine Anticorpo [KYNU4A6] - CUSTOMIZED PRODUCT
- Cod. AC-A283963-1ML, Anti-Tryptophan Hydroxylase Anticorpo - CUSTOMIZED PRODUCT
- Cod. AC-A8946-1ML, Anti-IDO1 Anticorpo - CUSTOMIZED PRODUCT
- Cod. AC-A15476-1ML, Anti-Tryptophan rich protein Anticorpo

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Dott. Salvatore A. De Cosmo
C.M. 7/2514
Ricercatore S.C. Medicina Int.

Visto e approvato
Il Direttore Scientifico

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- Protocollo ELISA
- Stralcio pubblicazione scientifica: International Journal of Obesity (2019) 43:2448–2457

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Overview of the used antibodies, procedures, and protocols

Primary antibody	Supplier	Clonality/ strain	Species	Primary antibody incubation time	Antibody dilution	Second antibody* incubation time	Specificity method	Protocols
Anti_L-kynurenine	ANTIBODIES.COM	Polyclonal	Rabbit	60 min RT	1/400	30 min	Western blotting	PROTOCOLS 1
Anti-Tryptophan Hydroxylase	ANTIBODIES.COM	Polyclonal	Rabbit	60 min RT	1/1000	30 MIN	Western blotting	PROTOCOL 2
Anti-IDO-1	ANTIBODIES.COM	Polyclonal	Goat	60 min RT	WB:0.5-1.5µg/ml ELISA: 1:128,000	30 MIN	Western blotting/ELISA	PROTOCOLS 2 and 3
Anti-Tryptophan rich protein	ANTIBODIES.COM	Polyclonal	Rabbit	60 min RT	1/1000	30 MIN	Western blotting	PROTOCOL 2

PROTOCOL 1: Western blot analysis of extracts of various cell lines, using Anti-L-Kynurenine at 1/400 dilution. Secondary antibody: Goat anti rabbit IgG at 1/8000 dilution. Lysate/protein 40 µg per lane. The blocking buffer: 3% non-fat dry milk in TBST. Detection with an ECL Basic Kit. Exposure time: 3 seconds: 8% SDS-PAGE GEL

PROTOCOL 2: Western blot analysis of extracts of various cell lines, using Anti-Tryptophan Hydroxylase or Anti-Tryptophan rich protein
at 1:1,000 dilution. The secondary antibody: Goat Anti-Rabbit IgG H&L Antibody (HRP) at 1:10,000 dilution. Lysates/proteins at 25µg per
lane. The blocking buffer: 3% non-fat dry milk in TBST. Detection with an ECL Basic Kit. Exposure time: 10s. 8% SDS-PAGE GEL

For POTOCOLS 1 and 2, Wester Blot: see Marucci et al. International Journal of Obesity 2019

PROTOCOL 3: ELISA (see general ELISA protocol)

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Kind regards,

Dr Stewart Newlove

Managing Director, Antibodies.com

Colorimetric sandwich ELISA protocol

This protocol represents an example of a colorimetric sandwich ELISA using indirect detection with a biotinylated antibody and streptavidin–HRP. TMB (tetramethyl benzene) is the HRP substrate used for detection.

Materials required	Suggested products	Cat. No.
Clear 96-well plate	Thermo Scientific™ Pierce™ 8-well strip polystyrene microplates	15031
Coating buffer (50 mM carbonate buffer, pH 9.4, or 10 mM phosphate buffer, pH 7.4)	Thermo Scientific™ BupH™ Carbonate-Bicarbonate Buffer Packs	28382
Blocking buffer	Thermo Scientific™ SuperBlock™ (TBS) Blocking Buffer	37535
Wash buffer (TBS or PBS with 0.05% Tween 20 detergent)	Thermo Scientific™ Pierce™ 20X TBS Tween™ 20 Buffer or Pierce™ 20X PBS Tween™ 20 Buffer	28360 or 28352
Plate sealers	Thermo Scientific™ Sealing Tape for 96-Well Plates	15036
Reagent reservoirs	Thermo Scientific™ ELISA Reagent Reservoirs	15075
Streptavidin–HRP	Thermo Scientific™ Pierce™ High Sensitivity Streptavidin–HRP	21130
TMB substrate solution	Thermo Scientific™ 1-Step™ Turbo TMB-ELISA Substrate Solution	34022
Stop solution (0.16 M sulfuric acid)	Thermo Scientific™ Stop Solution for TMB Substrates	N600
Additional materials required		
Capture (coating) antibody, biotinylated detection antibody, standards and samples, distilled or deionized water, absorbance-based microplate reader (e.g., Thermo Scientific™ Multiskan™ FC Microplate Photometer)		

Procedure

1. Prepare coating solution by diluting the capture antibody in coating buffer. Refer to “Antibody dilution recommendations” (page 5) or the manufacturer’s instructions.
2. Coat plate with 100 µL per well of coating solution. Cover plate and incubate 1 hr at room temperature or overnight (12–18 hr) at 2–8°C.
3. Aspirate contents and wash wells 1 time with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
4. Block plate with 300 µL per well of blocking buffer for 1 hr at room temperature.
5. Aspirate blocking buffer, then invert and tap plate on absorbent paper to remove excess liquid.
6. Prepare standards and sample dilutions in blocking buffer.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1–2 hr at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting the detection antibody in blocking buffer. For the recommended antibody dilution, refer to the manufacturer’s instructions.
10. Add 100 µL of the detection antibody solution to each well. Incubate for 2 hr at room temperature with gentle continual shaking (~500 rpm).
11. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
12. Make a working solution of streptavidin–HRP with blocking buffer by diluting 1:5,000. For example, to make enough for 1 plate, add 2 µL of streptavidin–HRP to 9.998 mL of blocking buffer.
13. Add 100 µL of working streptavidin–HRP solution to each well. Incubate for 1 hr at room temperature with gentle continual shaking (~500 rpm).
14. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
15. Add 100 µL of TMB substrate solution to each well. Incubate plate for 30 min at room temperature or until the desired color intensity is reached.
16. Add 100 µL of stop solution to each well.
17. Measure absorbance at 450 nm within 30 min of adding stop solution.
18. Calculate results using a log–log or 4-parameter curve fit.

Using an alkaline phosphatase system

If alkaline phosphatase (AP) is to be used instead of HRP for the enzyme conjugate, an AP-specific substrate must be used. Substitute the TMB substrate solution in step 15 with p-nitrophenyl phosphate (PNPP) (Thermo Scientific™ 1-Step™ PNPP Substrate Solution, Cat. No. 37621). Incubate at room temperature for 15–30 min. Substitute the stop solution with 50 µL 2 N NaOH to stop the reaction. Measure absorbance of each well at 405 nm.

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ARTICLE

Adipocyte and Cell Biology

GALNT2 as a novel modulator of adipogenesis and adipocyte insulin signaling

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Abstract

Background/objectives A better understanding of adipose tissue biology is crucial to tackle insulin resistance and eventually coronary heart disease and diabetes, leading causes of morbidity and mortality worldwide. GALNT2, a GalNAc-transferase, positively modulates insulin signaling in human liver cells by down-regulating ENPP1, an insulin signaling inhibitor. GALNT2 expression is increased in adipose tissue of obese as compared to that of non-obese individuals. Whether this association is secondary to a GALNT2-insulin sensitizing effect exerted also in adipocytes is unknown. We then investigated in mouse 3T3-L1 adipocytes the GALNT2 effect on adipogenesis, insulin signaling and expression levels of both *Enpp1* and 72 adipogenesis-related genes.

Methods Stable over-expressing GALNT2 and GFP preadipocytes (T_0) were generated. Adipogenesis was induced with (*R*+) or without (*R*−) rosiglitazone and investigated after 15 days (T_{15}). Lipid accumulation (by Oil Red-O staining) and intracellular triglycerides (by fluorimetric assay) were measured. Lipid droplets (LD) measures were analyzed at confocal microscope. Gene expression was assessed by RT-PCR and insulin-induced insulin receptor (IR), IRS1, JNK and AKT phosphorylation by Western blot.

Results Lipid accumulation, triglycerides and LD measures progressively increased from T_0 to $T_{15}R$- and furthermore to $T_{15}R$+. Such increases were significantly higher in GALNT2 than in GFP cells so that, as compared to $T_{15}R$+GFP, $T_{15}R$- GALNT2 cells showed similar (intracellular lipid and triglycerides accumulation) or even higher (LD measures, $p < 0.01$) values. In GALNT2 preadipocytes, insulin-induced IR, IRS1 and AKT activation was higher than that in GFP cells. GALNT2 effect was totally abolished during adipocyte maturation and completely reversed at late stage maturation. Such GALNT2 effect trajectory was paralleled by coordinated changes in the expression of *Enpp1* and adipocyte-maturation key genes.

Conclusions GALNT2 is a novel modulator of adipogenesis and related cellular phenotypes, thus becoming a potential target for tackling the obesity epidemics and its devastating sequelae.

Introduction

Obesity and adipose tissue dysfunction are major movers of insulin resistance, thus predisposing to leading causes of morbidity and mortality [1], such as coronary heart disease and type 2 diabetes [2]. A better understanding of the mechanisms controlling adipogenesis is therefore of crucial importance.

These authors contributed equally: Antonella Marucci, Alessandra Antonucci

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Insulin stimulates lipid storage in adipocytes [3]. So, it is not surprising that insulin sensitivity prospectively predicts weight gain increase [4]. Further confirming that insulin sensitivity positively controls body weight is the common observation that thiazolidinediones—insulin sensitizing molecules used as anti-hyperglycemic drugs in type 2 diabetes—increase body mass index (BMI) [5].

GALNT2, a GalNAc-transferase [6] involved in O-linked glycosylation [7], modulates the risk of atherogenic dyslipidemia [8–11], a specific hallmark of insulin resistance and acts as a positive modulator of insulin signaling in human liver cells, by down-regulating ENPP1, an inhibitor of insulin receptor (IR) [12]. Interestingly, GALNT2 expression in subcutaneous adipose tissue is increased in obese, insulin resistant individuals as compared to their non-obese counterparts [13]. Whether this association underlies a cause-effect relationship, with GALNT2 firstly predisposing to weight gain by exerting a positive modulation on adipose tissue insulin signaling and eventually concurring to develop obesity and the inevitably related insulin resistance, is a reasonable possibility that has never been addressed. In order to get deeper insights about this hypothesis, we investigated in cultured mouse adipocytes the effect of GALNT2 on adipogenesis, lipid accumulation and insulin signaling.

Materials and methods

Cell culture

Both mycoplasma-free 3T3-L1 murine fibroblast (Addex-Bio), and 293T cells (ATCC) were routinely cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (EuroClone) and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Recombinant Lentivirus production and transduction

Human ORF *GALNT2* cloned into pLX304 vector under CMV promoter (pLXGALNT2) and both dR8.91 (Packaging plasmid) and VSV-G (Envelope plasmid), used for lentiviral production, were purchased from Harvard Medical School Facilities. Plasmid pRRL-PGK-GFP was kindly provided by F. Carlotti (Nice, FR) [14].

Lentiviral particles were generated by co-transfecting either pLXGALNT2 or pRRL-PGK-GFP with both dR8.91 and VSV-G plasmids in 293T cells by calcium phosphate method [15]. Cells were then maintained in DMEM supplemented with 30% FBS for 18 h and lentiviral particles harvested, filtered through 0.45 µm filter and stored at −80 °C until used.

In order to obtain 3T3-L1 cell lines over-expressing either GFP (GFP cells) or GALNT2 (GALNT2 cells), both viral supernatants were added to fresh medium supplemented with 8 µg/ml Polybrene (Sigma-Aldrich) and 3T3-L1 cells incubated overnight. After 24 h, the medium was replaced with fresh DMEM plus 10% FBS and, then, changed every 2 days. GALNT2 transduced cells were further selected by adding 5 µg/ml of blasticidin (Thermo Fisher Scientific). GFP cells were used as control in all experiments.

3T3-L1 adipocytes differentiation

Cells were seeded either in 6-well-plates (Sigma-Aldrich) or in 18 × 18 mm sterile imaging glasses, at ~8×10^3cell/cm^2 cell density and grown in DMEM plus 10% FBS for 4 days in order to obtain 100% confluence (T_0 GFP and T_0 GALNT2 cells). Cell differentiation was induced, by using DMEM plus 1 µM dexamethasone (Calbiochem), 0.5 mM 1-methyl-3-isobutylxanthine (Calbiochem), 5 µg/ml human recombinant insulin (Gibco) and either with (*R*+) or without (*R*−) 2 µM rosiglitazone (Cayman Chemical). After 48 h, *R*+ and *R*− differentiation mediums were replaced with 10% FBS supplemented DMEM plus either 5 µg/ml insulin, with a biweekly medium replacement until day 15 (T_{15} GFP and T_{15} GALNT2 cells).

Oil Red O staining and lipid quantification

Oil Red-O (ORO) staining was performed in T_0, $T_{15}R-$ and $T_{15}R+$cells as it follows. Briefly, cells were fixed with 10% formalin for at least 1 h, rinsed with 60% isopropyl alcohol and, after complete drying, treated with ORO for 10 min. Cells were then washed 4 times with deionized H_2O and dried. Embedded ORO, an inferred measures of intracellular lipids, was eluted, by adding 100% isopropyl alcohol for 10 min, and quantified at 500 nm by Synergy-HT spectrophotometer (Bio-Tek). The resulting optical densities (OD) were expressed as percentage on either T_0 GFP or T_0 GALNT2 cells, respectively.

Intracellular triglycerides levels were measured in T_0, $T_{15}R-$ and $T_{15}R+$cells by means of the fluorimetric enzymatic Adipogenesis Assay Kit according to manufacturer's instruction (Sigma-Aldrich). The resulting fluorimetric intensities were measured at 587 nm by Synergy-HT Fluorimeter (Bio-Tek), plotted versus a triglycerides standard curve and expressed as nmol/5000 cells.

Adipo Red staining

T_0, $T_{15}R-$ and $T_{15}R+$cells were rinsed twice with phosphate-buffer saline (PBS), fixed with 4% paraformaldehyde for 15 min and then incubated with Adipo Red

stain (Lonza) for 10 min. Next, cells were washed with PBS and adipocyte lipid droplets (LD) imaged.

Imaging

ORO-stained cells were imaged in bright field by means of an inverted microscope (Axiovert 2 M, Zeiss) at ×5 magnification (Zeiss FLUAR 5 × /0.25na objective).

Images' acquisition of Adipo Red-stained adipocytes was performed by using a TCS SP8 confocal microscope (Leica) at ×40 magnification (HC PL APO CS2 40 × /1.30 OIL Leica objective). At least 30 independent micrographs were acquired for each experimental condition. LD morphology was then analyzed by using Cell Profiler software. Briefly, images were segmented to identify lipids as discrete "objects" and then area and Feret's diameter (which account for LD size, in terms of diameter for rounded objects or major axis for irregular objects) were measured.

Western blot analysis

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane Trans-Blot Turbo Transfer pack (Biorad). Blots were probed with specific antibodies, including anti-GALNT2 (ab97741, Abcam), anti β-Actin (sc-47778, Santa Cruz Biotechnology), anti-IR β-subunit (sc-711, Santa Cruz Biotechnology), anti-Phospho-IRS1^{Tyr895} (3070, Cell Signaling), anti-IRS1 (2382, Cell Signaling), anti-phospho-JNK1/JNK2$^{Thr183/Tyr185}$ (700031 Thermo Fisher Scientific), anti-JNK (9252, Cell Signaling), anti-Phospho-AKT^{Ser473} (9271, Cell Signaling) and anti-AKT (9272, Cell Signaling). Immune-complexes were then detected with the Super-Signal^{TM} West Pico (Thermo Fisher Scientific). Gel images were acquired by using Molecular Imager ChemiDoc XRS (Biorad) and band intensities quantified by Kodak Molecular Imaging Software 4.0 as OD values.

Insulin signaling

Cells were starved for 18 h with DMEM plus 0.5% FBS, stimulated with 10^{-7} M insulin for 5 min at 37 °C, and then washed with PBS and lysed. To evaluate IR β-subunit autophosphorylation, 300 µg of total lysate were immune-precipitated with anti-PY99 antibody (sc-7020, Santa Cruz Biotechnology) and analyzed by Western blot as described above. To evaluate IRS1, JNK and AKT activation, 50 µg of total lysate were subjected to SDS-Page and IRS1^{Tyr895}, JNK1/JNK2$^{Thr183/Tyr185}$ and AKT^{Ser473} phosphorylation evaluated by western blot (see above). IRS1^{Tyr895}, JNK1/JNK2$^{Thr183/Tyr185}$ and AKT^{Ser473} phosphorylation levels were normalized against IRS1, JNK and AKT content. All data were calculated as percentage of insulin-stimulated GFP adipocytes and expressed as means ± SE.

RNA extraction, cDNA synthesis, and gene expression analysis

Total RNA was isolated from mature adipocytes by using RNeasy Mini kit (Qiagen). cDNA was generated by reverse transcription with iScript™ Reverse Transcription Supermix for RT-qPCR (Biorad) according to the manufacturer's instructions and used as template for subsequent analyses. Expression levels of 72 adipogenesis-related genes (Gene Target List can be found in Supplementary Information 1) were assessed by means of pre-designed Adipogenesis M384 SYBR green panels (Biorad), on ABI-PRISM 7900 (Applied Biosystems). Among 8 reference genes (see Supplementary Information 1), *β-actin*, *Gusb* and *Hsp90ab1* were chosen as those showing the best control gene-stability measured as indicated by M value (i.e., $M < 0.5$) across samples [16]. PrimeTime qPCR Probe Assays (Integrated DNA Technologies) were used to measure expression levels of *Ennp1* (Mm.PT.58.7142160), m*Galnt2* (Mm.PT.58.5475747) and *hGALNT2* (Hs.PT.58.3633908) on ABI-PRISM 7900 (Applied Biosystems). Gene expression levels were reported as ΔΔCq, calculated as relative gene quantity normalized to the geometric mean of reference genes (PrimePCR™ Analysis Software, Biorad).

Statistical analyses

Each experiment, for each condition, has been carried out at least 3 times in duplicate. Differences between mean values were evaluated by Student's *t*-test; a two sided *p*-values < 0.05 were considered for statistical significance. Data are presented as means ± SE. Analyses were performed by using SAS Software, Release 9.4 (SAS Institute, Cary, NC, USA).

Results

Over-expression of GALNT2 in 3T3-L1 cells

Firstly, 3T3-L1 cell lines expressing either GFP (GFP cells) or GALNT2 (GALNT2 cells) were created as described in methods. As compared to GFP cells, a clear band representing human GALNT2 was detectable at ~60 KDa in GALNT2 cells at both T_0 (preadipocytes, Fig. 1a) and T_{15} (mature adipocytes, Fig. 1b).

Role of GALNT2 on mature adipocyte proportion and lipid accumulation

Adipogenesis was then induced for 15 days in T_0 GFP and T_0 GALNT2 cells. Figure 2a shows representative micrographs of undifferentiated T_0 cells (images 1 and 4) and