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Research Paper

Combined metformin and insulin treatment reverses metabolically impaired omental adipogenesis and accumulation of 4-hydroxynonenal in obese diabetic patients

Morana Jaganjac[a], Shamma Almuraikhy[a,b], Fatima Al-Khelaifi[a], Mashael Al-Jaber[a], Moataz Bashah[c], Nayef A. Mazloum[d], Kamelija Zarkovic[e], Neven Zarkovic[f], Georg Waeg[g], Wael Kafienah[b,*], Mohamed A. Elrayess[a,*]

[a] Anti-Doping Lab Qatar, Sports City, Doha, Qatar
[b] School of Cellular and Molecular Medicine, University of Bristol, Bristol, United Kingdom
[c] Bariatric and Metabolic Surgery, Hamad Medical Corporation, Doha, Qatar
[d] Microbiology and Immunology, Weill Cornell Medicine-Qatar, Doha, Qatar
[e] Department of Pathology, Medical Faculty University of Zagreb, Clinical Hospital Centre Zagreb, Zagreb, Croatia
[f] Laboratory for Oxidative Stress, Rudjer Boskovic Institute, Zagreb, Croatia
[g] Karl Franzen's University of Graz, Institute of Molecular Biosciences, Austria

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ABSTRACT

Objective: Obesity-associated impaired fat accumulation in the visceral adipose tissue can lead to ectopic fat deposition and increased risk of insulin resistance and type 2 diabetes mellitus (T2DM). This study investigated whether impaired adipogenesis of omental (OM) adipose tissues and elevated 4-hydroxynonenal (4-HNE) accumulation contribute to this process, and if combined metformin and insulin treatment in T2DM patients could rescue this phenotype.

Methods: OM adipose tissues were obtained from forty clinically well characterized obese individuals during weight reduction surgery. Levels of 4-HNE protein adducts, adipocyte size and number of macrophages were determined within these tissues by immunohistochemistry. Adipogenic capacity and gene expression profiles were assessed in preadipocytes derived from these tissues in relation to insulin resistance and in response to 4-HNE, metformin or combined metformin and insulin treatment.

Results: Preadipocytes isolated from insulin resistant (IR) and T2DM individuals exhibited lower adipogenesis, marked by upregulation of anti-adipogenic genes, compared to preadipocytes derived from insulin sensitive (IS) individuals. Impaired adipogenesis was also associated with increased 4-HNE levels, smaller adipocytes and greater macrophage presence in the adipose tissues. Within the T2DM group, preadipocytes from combined metformin and insulin treated subset showed better *in vitro* adipogenesis compared to metformin alone, which was associated with less presence of macrophages and 4-HNE in the adipose tissues. Treatment of preadipocytes *in vitro* with 4-HNE reduced their adipogenesis and increased proliferation, even in the presence of metformin, which was partially rescued by the presence of insulin.

Conclusion: This study reveals involvement of 4-HNE in the impaired OM adipogenesis-associated with insulin resistance and T2DM and provides a proof of concept that this impairment can be reversed by the synergistic action of insulin and metformin. Further studies are needed to evaluate involvement of 4-HNE in metabolically impaired abdominal adipogenesis and to confirm benefits of combined metformin-insulin therapy in T2DM patients.

1. Introduction

Obesity increases the risk of insulin resistance and type 2 diabetes mellitus (T2DM) [1]. However, some obese individuals, often referred to as the insulin sensitive (IS) or metabolically healthy obese (MHO), exhibit a lower risk of these diseases than predicted by their obesity [2]. Understanding the mechanisms underlying the protection found in IS obesity could help individuals suffering from pathological obesity.

* Corresponding authors.
E-mail addresses: W.Z.Kafienah@bristol.ac.uk (W. Kafienah), melrayess@adlqatar.qa (M.A. Elrayess).

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Obesity is characterized by increased size of adipose tissue through hypertrophy and hyperplasia of adipocytes [3]. Preadipocytes, an abundant cell population within the adipose tissue, replenish the adipocyte pool through adipogenesis [4]. Superior adipogenesis of preadipocytes isolated from sub-cutaneous (SC) adipose tissues taken from IS obese individuals compared to insulin resistant (IR) counterparts was recently suggested to play a role in the protection process of IS obesity, which is partially mediated by lower IL-6 secretion and oxidative stress [5,6]. Obesity-associated oxidative stress leads to elevated reactive oxygen species (ROS) production causing lipid peroxidation within the adipose tissue [7] and accumulation of reactive aldehydes [8,9]. Elevated 4-hydroxynonenal (4-HNE), a bioactive lipid peroxidation product, leads to progressive impairment of cell structure and function *via* formation of stable 4-hydroxyalkenals with proteins, phospholipids and DNA [10,11]. Elevation of 4-HNE has been associated with impaired adipogenesis, insulin resistance, atherosclerosis and even obesity of apparently healthy people [6,12–15].

Metformin (dimethylbiguanidine), the most widely used drug for the treatment of T2DM [16,17], is an insulin-sensitizing agent that provides glycemic control, especially in obese individuals [18]. Metformin can reduce adipose tissue size *in vivo* [19] and *in vitro* by inhibiting adipogenesis, decreasing lipogenic gene expression and increasing AMPK activity and glucose intake [20–22]. Metformin is frequently given to T2DM in combination with insulin [23]. Previous studies have shown that intensive insulin therapy reverses the decrease in adipocyte glucose transport activity in T2DM [24] and counters inflammation by decreasing levels of IL-6 and TNFα [25]. Insulin is commonly used in *in vitro* studies to induce adipogenesis, as it triggers a series of transcription factors that drive differentiation of preadipocytes into mature adipocytes including phosphorylation and activation of CREB [26] and PI 3-kinase [27].

Characterization of adipogenesis in the omental (OM) adipose tissues, a depot that is associated with increased metabolic disease risk [28], in IS, IR and T2DM obese individuals remains to be elucidated. Additionally, the effects of metformin on human OM adipogenesis has not been tested, although metformin-mediated glucose uptake by SC and OM adipocytes was previously described [29]. Comparing adipogenesis of OM-derived preadipocytes between IS and IR individuals and the impact of local 4-HNE on this process in primary cultures would provide valuable insights into the potentially protective mechanism associated with the IS group. The aims of the study were to test three hypotheses: 1) adipogenic capacity of OM preadipocyte is impaired in IR and T2DM individuals compared to IS subjects, 2) this impairment is accompanied by elevated 4-HNE accumulation in the adipose tissue that can directly inhibit adipogenesis, and 3) a combination of metformin/insulin treatment can rescue impaired OM adipogenesis.

2. Materials and methods

2.1. Materials

IL-6 and leptin ELISAs were purchased from R&D systems (Abingdon, UK) and Insulin ELISA from Mercodia Diagnostics (Sweden). Diaminobenzidine tetrahydrochloride (DAPI) and LipidTOX™ Green Neutral Lipid were obtained from Life Technologies (Warrington, UK). RT2 Profiler human adipogenesis PCR arrays and cDNA synthesis kits were purchased from SABiosciences-Qiagen, (Hilden, Germany). Other chemicals and reagents were purchased from Sigma (Germany) unless otherwise indicated.

2.2. Cohort

Patient recruitment criteria were previously published [5]. Briefly, forty consented obese patients (20 females and 20 males, matched for age and BMI) undergoing weight reduction surgery at Hamad Medical Corporation (HMC) (Doha, Qatar) were recruited. Protocols were approved by Institutional Review Boards of HMC and ADLQ (SCH-ADL-070, SCH-JOINT-111). Blood was collected prior to operation and 1–5 g OM adipose tissues biopsies were collected during the surgery. Plasma cholesterol, fasting glucose, Hba1c and liver function enzymes were measured by COBAS INTEGRA (Roche Diagnostics, Basil). IL-6, leptin and insulin were determined using commercially available ELISA. Insulin resistance was computed by homeostatic model assessment (HOMA-IR) [30] using 30th percentile (HOMA-IR=2.4) as a threshold point. Accordingly, subjects were dichotomized into IS (HOMA-IR < 2.4, n=8) and IR (HOMA-IR > 2.4, n=32), including 11 subjects with T2DM (5 on metformin only, 5 on metformin and insulin injection and 1 diet treated).

2.3. Preadipocytes culture and differentiation

Stromal vascular fraction **(**SVF) cells were obtained by collagenase digestion of OM adipose tissues as described previously [31]. Cell pellets were re-suspended in stromal medium containing DMEM-F12 supplemented with 10% FBS and Penicillin/Streptomycin, then maintained at 37 °C with 5% CO_2 until confluence. To induce differentiation, early passaged SVF-derived preadipocytes (passages 1–3) were grown at $2\times10^4/cm^2$ in stromal medium overnight then incubated in differentiation medium (DMEM-F12, 3% FBS, 33 μM biotin, 17 μM D-pantothenate, 1 μM dexamethasone, 250 μM of methylisobutylxanthine, 0.1 μM human insulin, 5 μM of PPARγ agonist, rosiglitazone) for 7 days, followed by 12 days in maintenance medium containing same components as differentiation medium omitting methylisobutylxanthine and rosiglitazone [32]. Differentiation potential (adipogenic capacity) was determined as a percentage of lipidtox positive stained cells to total number of stained nuclei (DAPI). For experiments investigating the effect of 4-HNE, metformin, insulin and their various combinations on differentiation, cells were grown as above in the absence or presence of repeated (every 3 days) of 10 μM 4-HNE, 1 mM metformin, 0.1 μM human insulin or their combinations for the entire differentiation and maintenance periods.

2.4. Gene expression studies

RNA was extracted from differentiated OM adipocytes using Trizol following manufacturer's instructions. One microgram of RNA was used to synthetize cDNA and gene expression profiling was determined using RT2 Profiler human adipogenesis PCR arrays by assessing mRNA levels of 84 genes, including five "housekeeping genes" according to manufacturer's protocol. The list of genes included Cyclin D1 (CCND1), Fatty Acid Binding Protein 4 (FABP4), TSC22 Domain Family Member 3 (TSC22D3) and Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Beta (PPARGC1B, also known as PGC1beta). Data were normalized with the internal housekeeping genes and ΔΔCt was calculated using ΔCt from IS as the control group according to manufacturer's protocol.

2.5. Measurement of ROS production

Intracellular ROS levels were assessed using 2′,7′-dichlorofluorescein-diacetate (DCFH-DA, Fluka) probe as described previously [33]. Briefly, cells were incubated with DCFH-DA (10 μM) in the HBSS for 30 min followed by removal of the probe and treatment with 4-HNE (10 μM) in stromal medium containing 3% FBS. The fluorescence intensity (relative fluorescence units, RFU) was measured every hour for 12 h using TECAN Infinite M200 PRO plate reader equipped with gas control mode to maintain 37 °C and 5% CO_2.