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

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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 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.
Obesity is characterized by increased size of adipose tissue through hypertrophy and hyperplasia of adipocytes [3]. Preadipocytes, an early 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 (dimethylbiguanide), 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 the effects of metformin on human OM adipogenesis by assessing mRNA levels of IS as the control group according to

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 synthesize 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 (PPARγC1B, also known as PGC1beta). Data were normalized with the internal housekeeping genes and ∆ΔCt was calculated using ∆Ct 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% CO₂.
2.6. 4-HNE staining

Immunohistochemical analysis of adipose tissues biopsies was performed as previously described [6]. Briefly, formalin-fixed and paraffin-embedded sections made from paraffin blocks were stained with Haematoxylin/Eosin (HE) or with a monoclonal antibody specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates. For the immunohistochemical detection of the HNE-protein adducts the immunoperoxidase technique was used using EnVision kit (Dako, Denmark) as described previously [34]. HNE positivity was estimated using a semi-quantitative method by an experienced pathologist (−0% positive cells, + ≤ 5% positive cells, + + + 5–25% positive cells, + + + + 25–50% positive cells, + + + + + > 50% positive cells). The presence of HNE-protein adducts in connective tissue, inflammation and in blood vessels was defined as negative (−) in the absence of the HNE-protein adducts, or as low positive (+, + +) or high positive (+++, ++++) in the presence of the HNE-protein adducts [35].

2.7. Statistical analysis

Comparisons were performed using t-test, Wilcoxon–Mann–Whitney and 1-way ANOVA in IBM SPSS statistics 21. Significance was defined as $P<0.05$. Power calculations indicated that the present sample size (n=40) had 80% power to detect a minimal difference of 30% in mean differentiation capacity of IS versus IR + type 2 diabetes mellitus with 35% deviation from mean value (sigma) at a level of $\alpha=0.05$.

3. Results

3.1. Insulin resistance-associated impairment of OM adipogenesis

Forty obese and morbidly obese (BMI=43.6 ± 6.7 kg m⁻²), young (age=35.6 ± 11.9 years) patients were included in this study. General characteristics of the cohort were previously published [5] and shown for the studied group in Supplementary Table S1. Both metformin monotherapy or metformin and insulin combined therapy exhibited a comparable glycemic control over T2DM patients as indicated by the monotherapy or metformin and insulin combined therapy exhibited a comparable glycemic control over T2DM patients as indicated by the monotherapy or metformin and insulin treatment group showed a significant elevation in FPG and insulin levels in the combined treatment group. The metformin and insulin treatment group showed significant reduction in systemic IL-6 levels compared to patients treated with metformin monotherapy (Table S1).

The adipogenic capacity of SVF-derived preadipocytes was assessed in all participants. Compared to IS individuals, IR and T2DM patients exhibited reduced OM adipogenic capacity by 26.3% ($P<0.01$) (Fig. 1A, B). As the obtained data revealed that the adipogenic capacity was equally reduced in IR and T2DM groups (Fig. 1B), these two groups were further combined when conducting gene expression comparison in order to increase the power (Fig. 1C). The reduced adipogenic capacity of IR & T2DM-derived preadipocytes was associated with up-regulation of anti-adipogenic genes CCND1, FABP4 and TSC22D3 and down-regulation of the pro-adipogenic gene PPARGC1B (Fig. 1C). Interestingly, preadipocytes expanded from T2DM patients who were treated with a combination of metformin and insulin showed greater adipogenic capacity than those treated with metformin monotherapy (Fig. 1D), as well as the diet-treated individual (4%). The reversing of the adipogenic capacity in the combined treatment group (20.3%), compared to metformin monotherapy group (1.7%) (Fig. 1D), is still lower than that of the IS group (43.1%, Fig. 1B), perhaps due to the anti-adipogenic effect of metformin.

3.2. Insulin resistance-associated 4-HNE staining, adipocyte size and macrophages infiltration within OM adipose tissues

A semi-quantitative immunohistochemical analysis of adipose tissues from randomly selected samples revealed that disease progression was associated with increased 4-HNE levels in adipocytes (Fig. 2A), smaller size of the adipocytes (Fig. 2B) and reduced macrophage infiltration (Fig. 2C). However, a lower 4-HNE-immunopositivity was detected in OM tissues from metformin and insulin treated T2DM patients when compared to those treated with metfor-
Insulin resistance-associated impairment of OM adipogenesis was marked by elevated expression of the anti-adipogenic genes CCND1, FABP4 and TSC22D3, all shown previously to reduce PPARG-mediated adipogenesis [39–41], and down-regulation of the pro-adipogenic gene PPARGC1B that lays downstream of PPARG [42]. Impaired adipogenesis was also associated with higher macrophage accumulation and 4-HNE staining within the adipose tissues (Fig. 2). Indeed both macrophages and adipocytes are known to promote inflammation and alter cellular redox homeostasis. Mutual interplay between macrophage oxidative burst and ROS derived from adipocyte metabolism of excess nutrients can trigger lipid peroxidation of readily oxidizable adipocyte lipids yielding formation of 4-HNE [9]. As obesity and T2DM are associated with dyslipidemia, 4-HNE may be generated from organs or tissues other than adipose tissues and spread through the blood. This is consistent with the observations in Fig. 2D where high 4-HNE staining was primarily detected in the walls of blood vessels and in the perivascular interstitial tissues. It is likely that 4-HNE is transported while bound to proteins such as albumin since 4-HNE-protein adducts are less easily metabolized than free aldehydes that have high affinity to bind to proteins. In favor of this assumption are also findings of increased levels of 4-HNE observed by immunohistochemical analysis of the OM tissue of these patients (Fig. 2) together with enhanced proliferation and reduced differentiation (adipogenesis) of cultured pre-adipocytes treated with 4-HNE (Fig. 3). This particular product of lipid peroxidation is well known to enhance proliferation and regulate metabolic stress-response and differentiation as it acts as a growth factor for various types of cells [45–48].

Furthermore, the finding of increased ROS production in response to 4-HNE treatment in vitro indicates that 4-HNE might play crucial role in the onset of the vicious etio-pathogenic circle of obesity, inflammation, oxidative stress and T2DM, which should be further studied to better understand metabolic syndrome and develop improved preventive and therapeutic protocols.

Both metformin and insulin are prescribed therapy protocols for T2DM patients [49]. The synergistic action of insulin with metformin was shown to improve glycemic control over insulin monotherapy [23].
In this study, we hypothesized that the previously reported anti-adipogenic effect of metformin could be rescued with co-treatment with insulin. Indeed, the emerging data suggest rescued adipogenesis in the combined therapy group which is marked by lower circulating IL-6 levels and reduced 4-HNE modified proteins concentrations and macrophage infiltration within the adipose tissues. Differences in the adipogenic capacity between the metformin monotherapy and the combination therapy groups may represent a reflection of imprinted memory on the adipose tissue due to exposure to different medications and their various effects on tissue physiology [5]. The superior adipogenesis of combined treatment was further confirmed by treating cells from IR individuals with metformin in the presence of insulin. The 4-HNE treatment reduced adipogenesis, also reported previously [6,13,21], however this phenotype was rescued in the presence of insulin. Furthermore, 4-HNE and metformin significantly induced intracellular adipocyte ROS production in vitro while this effect was also partially blunted by insulin. A similar finding was previously reported in 3T3L1 mouse preadipocytes cell line where treatment with metformin increased ROS production while the combination of metformin and insulin did not [50]. The anti-inflammatory role of insulin [51] in the combined treatment group, manifested by lower circulating IL-6, reduced macrophage infiltration within the adipose tissue and the subsequent lower 4-HNE accumulation and ROS production, may have contributed to improved adipogenesis in these patients although the exact mechanism remains to be investigated. Indeed, the anti-adipogenic roles of IL-6 and 4-HNE in sub-cutaneous tissues from insulin resistance and T2DM were recently suggested [5,6] and the emerging data may suggest a similar role in OM preadipocytes. Fig. 4 represents proposed action of the combined treatment on OM adipogenesis through blunting inflammation and 4-HNE accumulation, as suggested by emerging and recently published data [6].

5. Conclusions

Our results demonstrate insulin-resistance associated impairment of OM adipogenesis and provide a proof of concept that the impaired preadipocyte differentiation and altered cellular redox homeostasis in T2DM patients may be reversible by the synergistic action of insulin and metformin, potentially allowing for a better storage of excess triacylglycerols that otherwise would be deposited ectopically. The involvement of 4-HNE as a potential pathogenic factor and a possible biomarker of impairment of OM adipogenesis in the IR and T2DM patients seems consistent with the recognized role of 4-HNE as a clinical biomarker of different diseases [52,53]. Studies are needed to evaluate these possibilities and explore the underlying pathophysiological mechanisms based on inflammation and oxidative stress.

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Duality of interest

The authors declare that there is no duality of interest associated with this work.

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Fig. 3. The effect of 4-HNE, metformin and insulin treatment on ROS production and OM adipogenesis. Preadipocytes were treated with 10 µM 4-HNE, 1 mM metformin or their combination in the presence or absence of 0.1 µM human insulin repeatedly for the entire differentiation and maintenance periods. Representative images showing impaired adipogenesis (differentiated adipocytes stained with lipidtox appear in green) in cells treated with 4-HNE and metformin and the partial rescue in the presence of insulin (×100) (A), together with ROS production (B), adipogenic capacity (C), adipocyte size (D) and cell number (E). Data are presented as Mean ± SEM (n=6). Differences in paired groups were tested by paired samples Test (UT: untreated control, Ins: Insulin, Met: Metformin). P < 0.05.

Fig. 4. A scheme representing a proposed mechanism for the anti-adipogenic effect of metformin compared to pro-adipogenic effect of combined metformin and insulin therapy highlighting the roles of oxidative stress (ROS), macrophages (MF) and inflammation.
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