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Macrophage-derived secretome is sufficient to confer olanzapine-mediated insulin resistance in human adipocytes

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ABSTRACT

Objective: Olanzapine and Aripiprazole are widely used second-generation antipsychotic drugs. Olanzapine, more than Aripiprazole, leads to considerable metabolic side effects including obesity and diabetes. While the underlying mechanisms are not fully understood, these side effects are likely associated with mild inflammation in the metabolic organs. An *in vitro* model that accurately recapitulates the metabolic impact of olanzapine and aripiprazole should be useful to elucidate the underlying mechanisms.
Methods: We established co-cultures of matured adipocytes derived from the human SGBS cell line and the THP-1 human monocytic cell-derived or primary macrophages to explore the effects of both drugs on the response to insulin.
Results: Olanzapine, but not aripiprazole induced insulin resistance in SGBS adipocytes only when co-cultured with THP-1 or primary macrophages, polarized either into M0, M1 or M2. Noteworthy, M2 macrophages induced olanzapine-dependent insulin resistance in the absence of induction of pro-inflammatory cytokines. Insulin resistance by olanzapine was stronger than induced by high concentration of pro-inflammatory cytokines even in combinations, suggesting the contribution of factors other than the classical inflammatory cytokines to promote insulin resistance in adipocytes by olanzapine.
Conclusion: Macrophage/adipocyte co-cultures recapitulate the features of olanzapine-induced insulin resistance and implicate the existence of yet unknown factors in mediating this effect.

1. Introduction

Schizophrenia is a mental illness which is described by the International Classification of Diseases as a disorder with fundamental, characteristic distortions of thinking and perception, and inappropriate or blunted affect in clear consciousness. This severe psychosis has unknown causes and a prevalence of more than 1 % incidence within populations across the world [27]. Schizophrenia was poorly understood and ill-defined for centuries, and treatment for the disease became available in the 1950s [26]. These early medications, however, displayed low effectiveness for schizophrenic patients with chronic illness and severe side-effects [4].

Second-generation antipsychotics (SGAs) emerged in 1980s. SGAs not only block the dopamine D2 receptors similar to the first-generation drugs, but also block the effect of the serotonin receptor 5-hydroxytryptamine-2 (5HT2) [16,27]. Olanzapine (Ola), one of the most widely used SGAs, is recognised as an effective drug against schizophrenia, but is known to cause significant side-effects in patients including sedation and weight gain [14]. This is associated with development of insulin resistance and type 2 diabetes [47]. However, pre-clinical observations have indicated that chronic Ola treatment can cause insulin resistance

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List of abbreviations

Ari	Aripiprazole
Dexa	Dexamethasone
ER	endoplasmic reticulum
Glut4	Glucose transporter 4
IRE1α	Inositol-requiring enzyme 1 alpha
IRS	Insulin receptor substrate
2-NBDG	2-(*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose
NEAA	Non-essential amino acid
NLRP3	NOD-like receptor protein 3
Ola	Olanzapine
PBMC's	Peripheral blood mononuclear cells
PGE_2	Prostaglandin E_2
SGA's	Second-generation antipsychotics
TXNIP	Thioredoxin interacting protein

without a significant weight gain [3,30]. Aripiprazole (Ari) is another approved SGA against schizophrenia. Despite being less efficacious than Ola, Ari is primarily used to prevent short-term weight gain and avoid the metabolic side effects associated with Ola [19]. While Ola is more advantageous than Ari to stabilize schizophrenia symptoms at early onset [13], its severe metabolic side effects affect compliance, particularly in women. Despite lower anti-schizophrenic efficacy, this instigates a switch to Ari in clinically stable patients, who were taking Ola and displayed metabolic syndrome symptoms [5,62]. Initially it was suggested that the diabetogenic effect of Ola is associated with its anti-muscarinic effect. However, no direct effect of Ola on insulin secretion was seen in isolated islets [24]. Thus, the mechanisms that underpin the Ola-induced insulin resistance await elucidation, which, in turn, restrict the clinical use of Ola as a treatment for severe schizophrenia.

While early evidence suggested a direct effect of Ola on metabolic cells to compromise insulin sensitivity [60], recent studies in animal models and observation in humans have proposed that insulin resistance is not direct and is a consequence of a mild inflammatory condition promoted by Ola [33,45]. Ola-mediated inflammatory conditions dissociate the insulin receptor substrate (IRS)-PI3K interaction by inhibiting IRS specific phosphorylation [33]. This prevents PI3K activation and the downstream activation of the Akt kinase [42]. Downstream to this signalling, primarily in adipocytes and muscles, the translocation of glucose transporter 4 (Glut4) to the plasma membrane is impaired and glucose uptake is inhibited [12,23,67]. Importantly, the effects of Ola on the PI3K pathway was observed at micromolar concentrations, a few orders of magnitude higher than its Ki for D2 dopamine receptor, or other GPCRs postulated to interact with Ola [46]. We therefore assume that mechanisms other than modulation of GPCR signalling are likely engaged by Ola to modulate metabolism.

Adipose tissue macrophages play essential roles in regulating obesity. Resident macrophages in lean adipose tissue are primarily of the alternative type, commonly referred to M2-like, and macrophages in obese adipose tissues are dramatically increased in number, and are predominantly, classically activated, M1-like, and promote inflammation and insulin resistance via macrophage- and fat cell-derived pro-inflammatory cytokines [20]. Female rats that received Ola displayed elevated plasma levels of IL-8, IL-6, IL-1β and increased macrophage infiltration in white and brown adipose tissue [11,69], suggesting that Ola promotes insulin resistance in an M1-dependent manner. However, the contribution of the cytokines and the different types of macrophages in adipocytes is difficult to assess *in vivo*, and a reliable *in vitro* system is needed to decipher the underlying mechanisms.

Here we aimed to develop and characterize a simple and reproducible *in vitro* model that captures the basic features of Ola-mediated insulin resistance in adipocytes in order to delineate the pharmacodynamic differences of Ola and Ari at low micromolar concentrations. We further wanted to verify that the insulin resistance is associated with an M1-induced pro-inflammatory response. To this end we set up co-cultures of mature adipocytes derived from Simpson–Golabi–Behmel syndrome (SGBS) cells and the leukemic THP-1 macrophage-like cells. The results demonstrate a lack of a direct effect of Ola on the adipocytes with a clear hierarchy for development of insulin resistance when co-cultured with M1>M2>M0 in the presence of Ola, but not Ari. Our data suggest that in addition to pro-inflammatory cytokines, additional factors shared by M1 and M2 macrophages, further potentiate the Ola-induced insulin resistance. We propose that SGBS/THP-1 co-cultures may serve as a reliable *in vitro* system to identify these factors.

2. Materials and methods

2.1. Chemicals and antibodies

Ari (129,722-12-9; Acros Organics), Ola (035M4781V; Sigma-Aldrich), Phorbol 12-myristate 13-acetate (PMA) (Sigma, P1585), Granulocyte-macrophage colony-stimulating factor (GM–CSF– Peprotech # 300–03), human transferrin (Sigma-Aldrich #T8158), human insulin (Sigma-Aldrich #91077C), cortisol (Sigma-Aldrich #H0888), triidothyronine (T3) (Sigma-Aldrich #T6397), dexamethasone (Sigma-Aldrich #D1756), IBMX (Sigma-Aldrich #I5869), rosiglitazone (Cayman #71740), biotin (Sigma-Aldrich #B4501), pantothenate (Sigma-Aldrich #21210), recombinant human IL-1 β (Peprotech # 200-01 B), recombinant human IL-6 (Peprotech # 200–06), recombinant human IL-18 (Peprotech # 200-18BP), recombinant human IL-10 (Peprotech #200–10), p-Akt (Ser473) (Cell Signalling Technology #9271 S, 1:2000), Akt (Cell Signalling Technology #9272, 1:2000), polyclonal rabbit *anti*-p97 antibody (Abcam #97302, 1:8000), horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA), 2-NBD glucose, fluorescent glucose uptake probe (Abcam #146200) and, human Glut4 Alexa Fluor 488-conjugated antibody (R&D systems # FAB86541).

2.2. Cell cultures and differentiation procedures

Human monocytic THP-1 cells (ATCC, TIB202, Manassas, Virginia) were maintained in RPMI 1640 culture medium containing 10 % heat-inactivated fetal bovine serum (FBS, Gibco, USA), 1 % sodium pyruvate (Biological Industries, Israel), 1 % penicillin/streptomycin (Biological Industries, Israel), 1 % L-glutamine (Biological Industries, Israel), 1 % non-essential amino acid (Biological Industries, Israel). The monocytes were differentiated to naïve macrophages at a density of 0.5×10^6 cells/ml by 48–72 h incubation with 150 nM phorbol 12-myristate 13-acetate (PMA) followed by 48 h incubation in RPMI medium. Naïve macrophages were polarized into M1 type by incubation with 20 ng/ml IFN-γ (Peprotech, #300–02) and 10 pg/ml LPS (Sigma, #L-2630) for 24 h. M2 macrophage polarization was obtained by incubation with 20 ng/ml IL-4 (Peprotech, #200–04) and 20 ng/ml IL-13 (Peprotech, #200–13) for 24 h according to Ref. [17].

Human pre-adipocytes SGBS cells were kindly provided by Dr. M. Wabitsch (Department of Pediatrics and Adolescent Medicine, Ulm University Medical Center, Ulm, Germany) [61]. SGBS were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma-Aldrich) with 10 % FBS, 33 μM biotin and 17 μM pantothenate. Pre-adipocytes were maintained at low density; when confluence reached 80 %, cells were split by trypsinization. Cells were not passaged more than three times. Differentiation to adipocytes was induced in a serum-free DMEM/F12 medium supplemented with 33 μM biotin, 17 μM panthotenate 10 μg/ml transferrin, 10 nM insulin, 200 pM thyroid hormone (T3), and 100 nM cortisol (referred to as

differentiation medium). During the first four days, 2 μM rosiglitazone, 250 μM isobutylmethylxanthine (IBMX), and 25 nM dexamethasone were added and then medium was changed to the differentiation medium. The medium was refreshed every four days. SGBS cultures were used for experiments after 14 days when over 85 % of cells exhibit spherical morphology determined by microscopy.

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples obtained from healthy volunteers (Blood Bank of Hadassah Medical Hospital, Jerusalem, Israel) by using Ficoll-Paque™plus (GE healthcare #10265109) density gradient centrifugation at 1200*g* for 20 min at room temperature. PBMCs were collected from the intermediate thin buffy layer of centrifuged cells and washed in PBS without $CaCl_2$ and $MgCl_2$ but with 2 % heat-inactivated FBS (Gibco, #12657029) and 1 mM EDTA. After washing, cells were centrifuged at 650 g for 5 min at room temperature. Then PBMCs were washed twice with red blood cells lysis buffer (Biological Industries, Israel), centrifuged, washed in PBS and re-suspended in complete RPMI medium (FBS, Pen/Strep, glutamine, sodium pyruvate and nonessential amino acids (NEAA).

For differentiation into macrophages isolated PBMCs were cultured in suspension at 1×10^6 cells/ml for 24 h in a complete RPMI medium and treated with 5 ng/ml human GM-CSF for 5–10 days until the cells attached to the plastic and obtain a macrophage morphology.

For SGBS co-culture experiments, THP-1 or PBMC monocytes were differentiated on Transwell inserts (Millicell standing cell culture inserts-mercury-membrane pore size of 0.4 μm, #PIHP03050) for 4–8 days and SGBS pre-adipocytes differentiated on 6 well plates for 14 days. Types of macrophages and SGBS mature cells were co-cultured (1:1) in the same DMEM/F12 medium without FBS, followed by 24 h of treatment with 5 μM of Ola or Ari. While these concentrations are much higher than the blood steady state levels, drug distribution studies in rats suggest that these are plausible concentrations for the liver and perhaps also to adipose tissue [58]. DMSO was used as control. After 24 h, the cells were washed with PBS and incubated with or without 100 nM of insulin for 15 min. After co-incubation along with treatment and insulin stimulation, adipocytes were directly collected by cell scraping in RIPA lysis buffer for protein extraction or scrape into cold PBS for flow cytometry analyses.

HepG2, Hep3B and hTERT hepatic cells were cultivated in high glucose DMEM (Sigma, USA), containing 10 % FBS, 2 mM L-glutamine, 1 % pen/strep and 1 mM sodium pyruvate as mentioned above. All cell lines were maintained at 37 °C and 5 % CO_2.

2.3. Oil red O staining

A 0.2 % stock solution of Oil red O (Sigma-Aldrich, #O-0625) was prepared in 40 % 2-propanol, and filtered before use. Staining was performed on day 14 to detect the accumulated lipid droplets in differentiated SGBS cells. Cells were washed with PBS, fixed with 4 % formaldehyde for 15 min at room temperature, then rinsed with 60 % isopropanol, and stained with Oil Red O solution at room temperature for 20 min. After removing the staining solution, the cells were washed at least once with 60 % isopropanol and 3 times with distilled water and dried. The stained lipid droplets were visualized by light microscopy. In addition, to obtain a quantitative evaluation of adipocyte differentiation, stained lipid droplets were solubilized in 100 % isopropanol and optical density (OD) was measured at 520 nm.

2.4. Western blotting analyses

SGBS mature adipocytes, HepG2, Hep3B, hTERT were washed in cold PBS, scraped in RIPA lysis buffer supplemented with complete protease inhibitor cocktail (Millipore Sigma # 12352200). Tubes were shaken vigorously at 4 °C for 10 min and lysates were cleared by centrifugation at 12,000 g, 20 min, 4 °C. A sample was taken for protein quantification and a reducing 5X Laemmli buffer was added, and the sample boiled for 5 min at 95 °C. Fifty micrograms of protein samples were subjected to SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The primary antibodies used were total-Akt, phospho-Akt and polyclonal rabbit *anti*-p97. Horseradish peroxidase-conjugated secondary anti-rabbit antibody was used. Detection was performed with Immobilon crescendo chemiluminescent HRP substrate with Bio-Rad ChemiDoc™ XR, and quantified by Image Lab™ software.

2.5. RT-qPCR

Total RNA was extracted using Bio triRNA (Bio-lab ltd) and DNase protocol. cDNA was prepared from 1 μg total RNA with iScript™ cDNA synthesis kit (Bio-Rad). The amplification reaction assays contained SYBRGreen PCR Master Mix (Bio-Rad). Actin was used for normalization. Specific primers are listed in Table S1.

2.6. Analysis of glucose uptake

SGBS adipocytes were incubated with/without 100 nM insulin for 15 min after 24 h treatment. Cells were washed twice with Krebs-Ringer Phosphate (KRP) buffer (135 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, and 1.4 mM $MgSO_4$, 10 mM sodium pyrophosphate, pH 7.4) and incubated with KRP buffer for 10 min, before insulin was added again and incubated for 15 min. Glucose uptake was initiated by addition of 5 μM of 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) for additional 15 min with or without insulin. Cells were washed with ice-cold PBS and collected for flow cytometry. Analysis was performed using Cytoflex FACS and CytExpert software for data processing.

2.7. Surface translocation of Glut4

SGBS adipocytes were washed and incubated for 20 min with 1X PBS containing 0.2 % BSA. Subsequently, 100 nM insulin was added and incubated for 15 min followed by addition of 0.3 μg anti-Glut4 antibody for 30 min. Cells were washed with ice cold PBS and analyzed by flow cytometry.

2.8. Software and statistical analyses

GraphPad Prism 5, CytExpert, and ImageJ software were used to create figures and to analyse the data. The data were analyzed with repeated measure two-way ANOVA test including Bonferroni post-test or one-way ANOVA Tukey test comparing each drug treatment group with control. Treated groups were also analyzed with paired *t*-test calculator against a control group. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Ola does not induce insulin resistance in SGBS adipocytes when added directly or in the presence of macrophage polarizing cytokines

To study the effect of Ola on adipocytes we used the pre-adipocyte SGBS cell line. Following differentiation into mature adipocytes, and in accordance with literature [59,66], mature SGBS cells stop dividing and change their morphology from a flat fibroblast-like appearance to spherical cells filled with lipid droplets. An approximately six-fold increase in lipid content was observed, assessed by oil red O staining (Suppl. Fig. 1). All experiments hereafter were performed with mature SGBS cells. At first we analyzed the direct effect of Ari and Ola on SGBS adipocytes. Since polarized macrophages were later used in co-culture settings, we also assessed the effect of the cytokines used for macrophage polarization. See the schematic of the experiments in Fig. 1. Responsiveness to insulin was assessed by the phosphorylation of Akt at Ser473 following a short incubation with insulin. Incubation with 5 μM

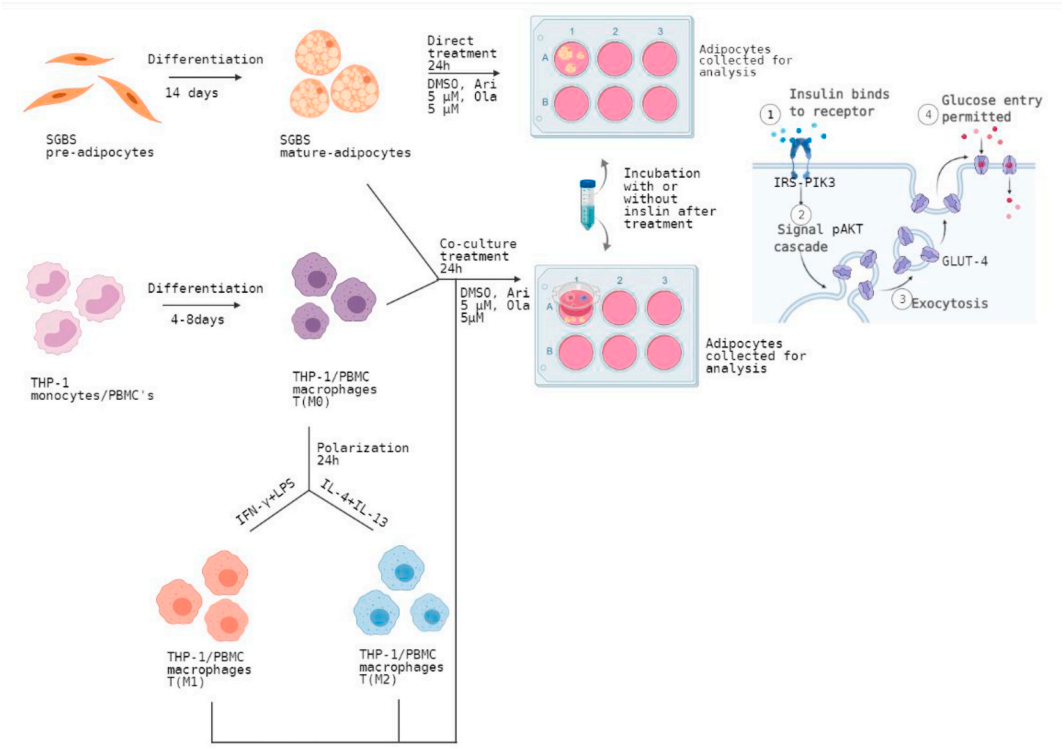
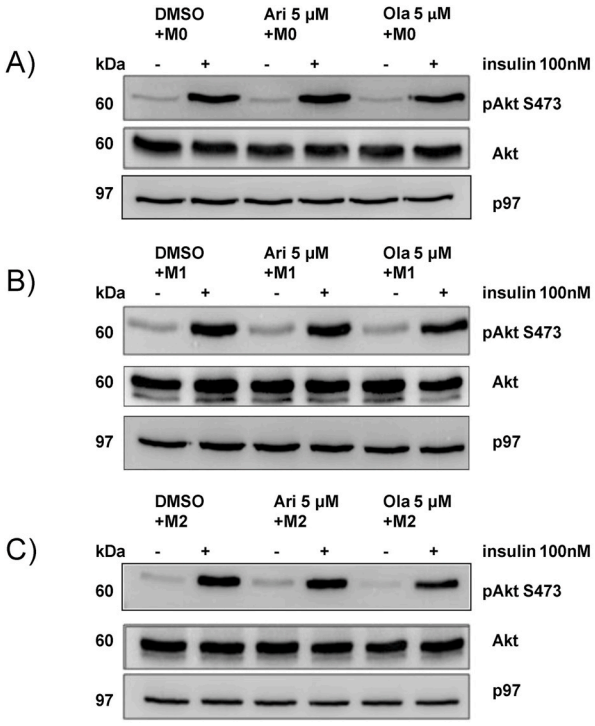


Fig. 1. Schematic representation of direct or co-culture experimental setup of SGBS mature adipocytes and THP-1/PBMC macrophages along with Ari and Ola treatment. SGBS pre-adipocytes differentiated in mature adipocytes. Cells were used for direct and co-culture treatment. THP-1/PBMC monocytes differentiated into M0 (naïve) and further polarized into M1 (classically activated) and M2 (alternatively activated) macrophages followed by 5 µM of Ari and Ola treatment. 100 nM of Insulin was added for 15 min. Adipocytes were collected to analyse insulin regulating pathway.



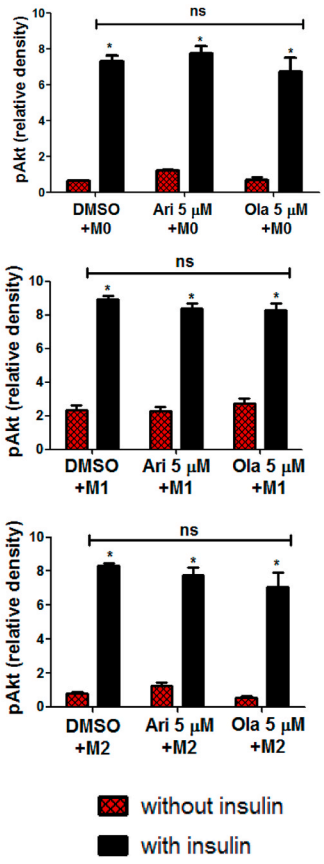


Fig. 2. Neither Ari nor Ola directly affect insulin signalling in SGBS mature adipocytes. Western blot analyses of pAkt expression in treated SGBS mature adipocytes. A) Without cytokines, only treatment (simulating M0), B & C) M1-and M2-promoting cytokines along with 5 µM of Ari and Ola. Responses were quantified by densitometry and normalized to the expression of total Akt. P97 was included as loading control. Densitometry data are shown as mean ± SD of three independent experiments (*p < 0.05, ns: non-significant).

Ari and Ola for 24 h, either without cytokines or in the presence of M1 (IFN-γ + LPS) or M2 (IL-4 + IL-13) polarizing cytokines, did not affect the response to 100 nM insulin (Fig. 2A–C, respectively). This confirms that neither Ola nor Ari directly affects the response to insulin in SGBS cells.

3.2. Pro-inflammation cytokines, even at supra-physiological amounts, mildly affect insulin signalling in SGBS adipocytes

The classical pro-inflammatory cytokines, IL-1β, IL-18, IL-6 and TNFα, have been implicated to induce insulin resistance [33]. As Ola, more than Ari, induced *IL-1B* and *IL-6*, while *TNFA* mRNA was hardly affected [43]. IL-18 shares a similar inflammasome-dependent secretion mechanism as IL-1β [56], and was moderately induced by Ola relative to Ari at the mRNA level (Suppl. Fig. 4B). Thus, we decided to test whether incubation of SGBS directly with IL-1β, IL-18 and IL-6 individually, and in combination, will confer a reduction in insulin responsiveness. A minimum concentration of 20 ng/ml from each cytokine was needed to confer insulin resistance (Fig. 3). Higher concentrations did not progressively induce a higher insulin resistance, suggesting saturation. At saturation, insulin responsiveness was improved by approximately 45 % at concentration of 100 ng/ml of each cytokine cocktail (Fig. 3D). The combination of cytokines at 50 ng/ml showed a significant reduction in insulin signalling. Importantly, the suppression effect of Ola in the context of THP-1/SGBS was stronger than the effect of the cytokine combination (Figs. 4 and 5 B, C). These data suggest that while pro-inflammatory cytokines are able to mediate insulin resistance, they most likely do not underlie the entire effect in the context of Ola seen *in vitro*. While not tested directly, we consider it unlikely that IL-17 is involved in Ola-mediated insulin resistance in our model, since Ola did not affect its transcript levels. We also analyzed the effect of Ola on anti-inflammatory cytokine expression in M2 polarized THP-1 as well as PBMC cells. IL-10, the typical marker of M2, was not altered by Ola or Ari, and *IL-10* itself did not affect insulin responsiveness in SGBS (Fig. 3E). Thus, the Ola-mediated effect in the M2 macrophages is most likely not related to *IL-10* modulation.

3.3. Ola but not ari reduces insulin signalling in SGBS cells in the presence of THP-1-derived macrophages

Previous data implicate tissue resident macrophages as the source of cytokines in response to Ola [33,36]. We then examined the effects of macrophages on SGBS adipocytes in the presence of the drugs. As a source of macrophages, we decided to use the well-characterized leukemic monocyte cell line THP-1, which has been widely used as model for primary human macrophages to study polarization and function, also in diabetes [1,7]. THP-1 cells generate macrophages upon incubation with PMA, coined as naïve macrophages (M0). M0 are then polarized *in vitro* into classically activated (M1) in the presence of IFN-γ + LPS, and alternatively activated (M2) in the presence of IL-4 + IL-13. We seeded transwell chambers with THP-1 macrophages and SGBS adipocytes. Since SGBS cells are differentiated over 14 days, they were plated on the plastic, while the THP-1 macrophages were generated on the filters with 0.4 μm pore size. In this configuration the two cell types are physically separated, allowing soluble mediators to diffuse freely between the cells.

When co-cultured with THP-1 macrophages, a reduced response to insulin was observed in SGBS cells in the presence of Ola. The addition of Ola to M0 macrophages, reduced the response of SGBS cells to insulin by 30 % relative to Ari, a response level similar to the non-treated DMSO control (Fig. 4A). Strikingly, in the presence of M1 macrophages, Ola treatment almost obliterated the response to insulin while a milder effect was observed when adipocytes were co-culture with M2 macrophages (Fig. 4B and C). These results show that Ola, and not Ari, confers a reduction in the responsiveness to insulin in SGBS cells in the presence of macrophages. The effect is stronger for M1 polarized cells, but it is

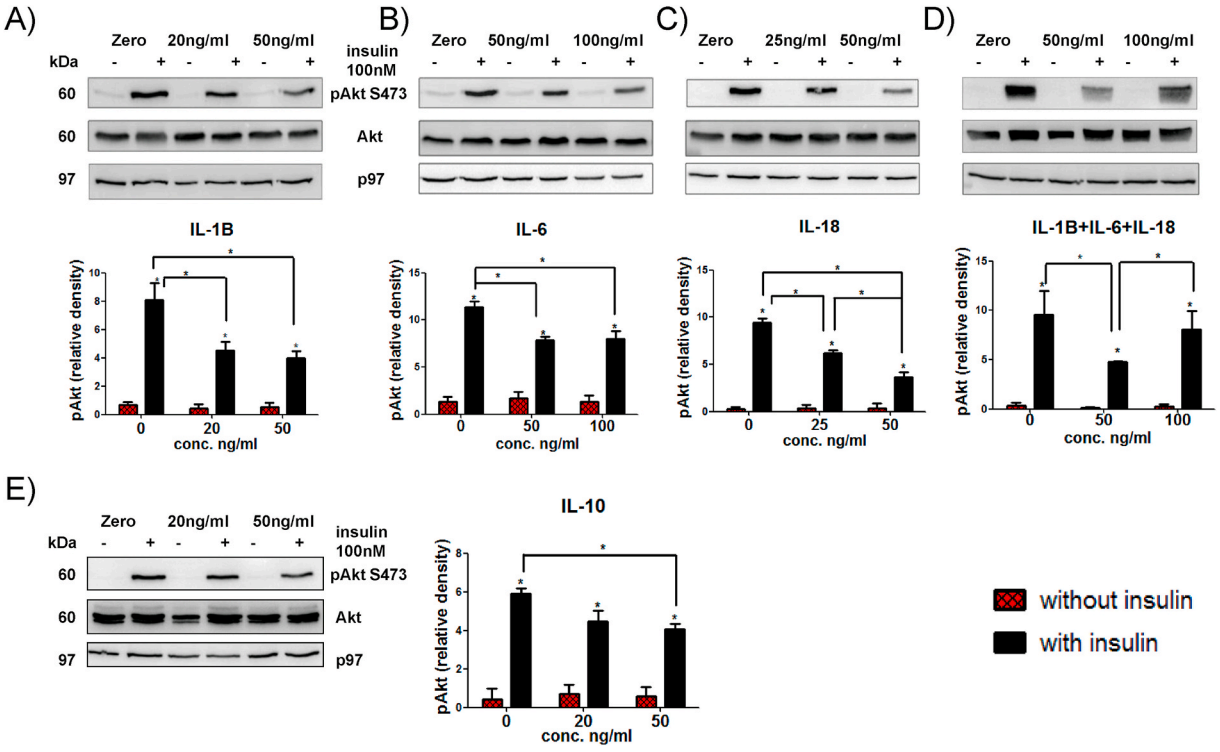


Fig. 3. A mild effect of pro-inflammatory (M1) and IL-10 (M2) cytokines on insulin signalling in SGBS adipocytes. Western blot analysis of the expression of pAkt in SGBS stimulated by cytokines. The indicated concentrations of cytokines were added for 24 h prior to stimulation. (A) IL-1B. (B) IL-6. (C) IL-18. (D) IL-1+IL-6+IL-18 (combination of pro-inflammatory cytokines), (E) IL-10. Response was quantified by densitometry. Shown as the mean ± SD of three independent experiments (*$p <$ 0.05, ns: not significant).

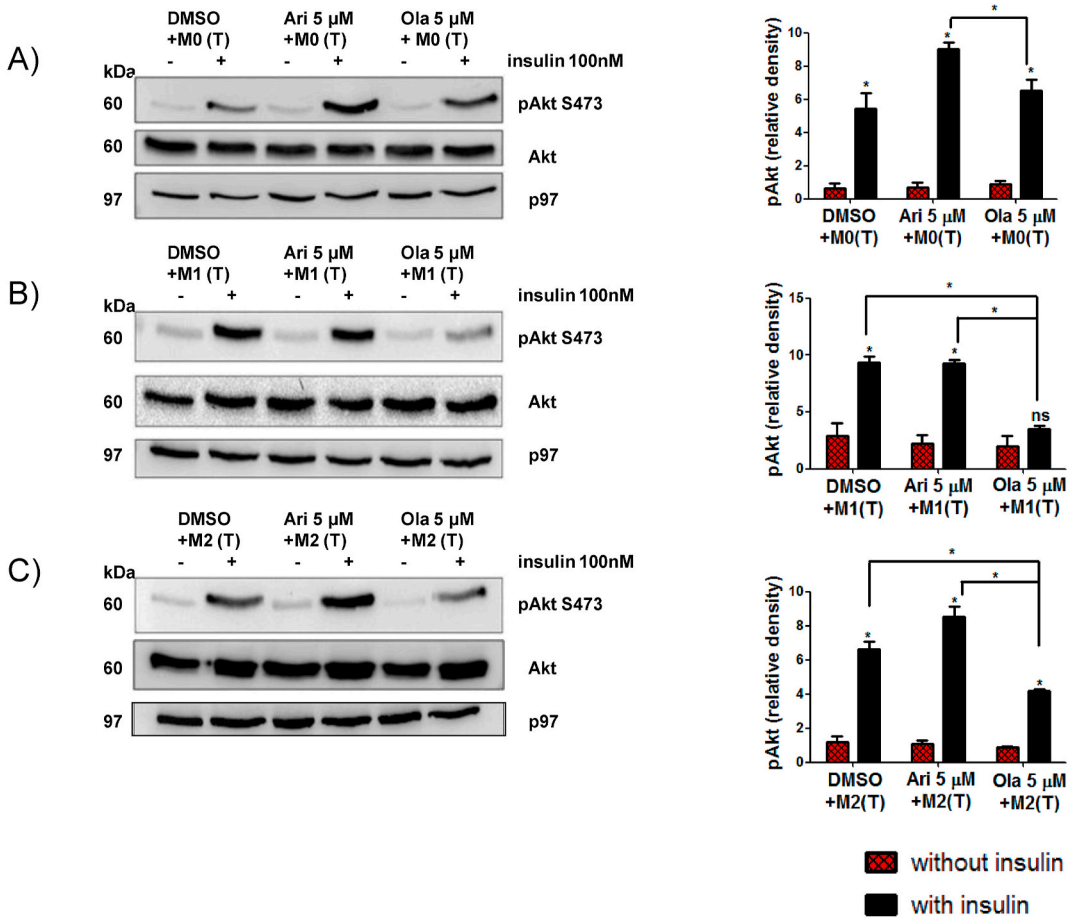


Fig. 4. Reduced response to insulin in SGBS adipocytes when Ola is added in the presence of THP-1 macrophages. Western blot analyses of pAkt in the treated SGBS mature adipocytes co-cultured with THP-1 macrophages. (A) M0 (T) (naïve THP1 macrophages), (B) M1(T) (M1 polarized THP-1 macrophages), (C) M2(T) (M2 polarized THP-1 macrophages). Responses were quantified by densitometry and normalized to the expression of total Akt. Densitometry data are shown as mean ± SD of three independent experiments (*$p < 0.05$, ns: non-significant).

evident also for M2 and even in the presence of naïve M0 macrophages (Suppl. Fig. 2A). This recapitulates the inflammatory model proposed for the clinical effect of Ola [9], but also suggest that macrophages in general are responding to Ola in a manner that affects insulin signalling in adipocytes. This could be either by secretion of pro-inflammatory cytokines also from M2 macrophages, or due to the secretion of yet to be determined factors. These data may explain the metabolic abnormalities and insulin resistance often accompanied with weight gains observed in individuals receiving Ola treatment [8], which could be mediated by an M2 state *in vivo*.

3.4. *Ola promotes insulin resistance in SGBS in the presence of PBMC-derived macrophages*

It was important to validate the considerable effect of Ola on the insulin response using primary macrophages. To this end PBMCs were used. Differentiation into macrophages and the polarization *in vitro* was monitored by levels of CD68, a marker of mature macrophages. The transcript levels of *CD68* were induced by 3 to 4-fold following 24 h of differentiation and continued to be higher after 48 h for all three macrophage subtypes (M0, M1 and M2) compared to the monocytes (Mϕ) from the corresponding culture (Suppl. Fig. 3A). To confirm the polarization, specific classically activated pro-inflammatory M1 and alternatively activated M2 macrophage markers expression were measured by RT-qPCR. M1 polarized macrophages presented increased mRNA levels of the M1 pro-inflammatory cytokine genes *IL-1β*, *TNFα, IL-6* and *CD80* at 24 and 48 h (Suppl. Fig. 3B), and low expression of *IL-10*, compared to M0 and M2 macrophages. M2 macrophages showed higher expression of the M2 markers *IL-10, CCL-18, CD206* (Suppl. Fig. 3C) compared to M0 and M1 macrophages, respectively. Thus, as expected, the macrophage subtypes were successfully established from PBMCs *in vitro*.

In our recent studies, treatment of THP-1 macrophages with low micromolar concentrations of Ola and Ari during their polarization enhanced M1 phenotype [43]. We therefore wanted to examine whether this occurs for PBMC-derived macrophages. PBMCs were isolated in biological triplicates from three different batches of blood samples. GM-CSF was used to differentiate the monocytes into primary macrophages M0 (termed PB-M0) which were further polarized into M1 (PB-M1) and M2 (PB-M2) for 24 h in the absence and presence of Ari, Ola and dexamethasone (Dexa, a steroidal anti-inflammatory agent). The effect of drugs was assessed by mRNA expression of each inflammatory cytokine. In PB-M0 macrophages, Ari and Ola did not elevate the mRNA levels of pro-inflammatory cytokines; instead, Ari at 5 µM decreased the expression of *IL-1*β and *IL-6* (Fig. 7A and B). We noticed same effects by 10 µM of Ari for *IL-6* at supra-therapeutic concentration in THP-1 macrophages [43]. A significant decrease in the expression of *IL-1*β, *IL-6*, *TNFα* and *CD*-80 by Dexa was noticed (Fig. 7A, B, C, F). This shows the expected effect of Dexa on the expression of pro-inflammatory cytokines [6,18].

The pro-inflammatory cytokine IL-17 was reported to be elevated in the serum of schizophrenic patients [10], without a connection to therapy. *IL*-17 mRNA levels were significantly increased by Ari, Ola, and unexpectedly, also by Dexa in PB-M2 macrophages. In PB-M1

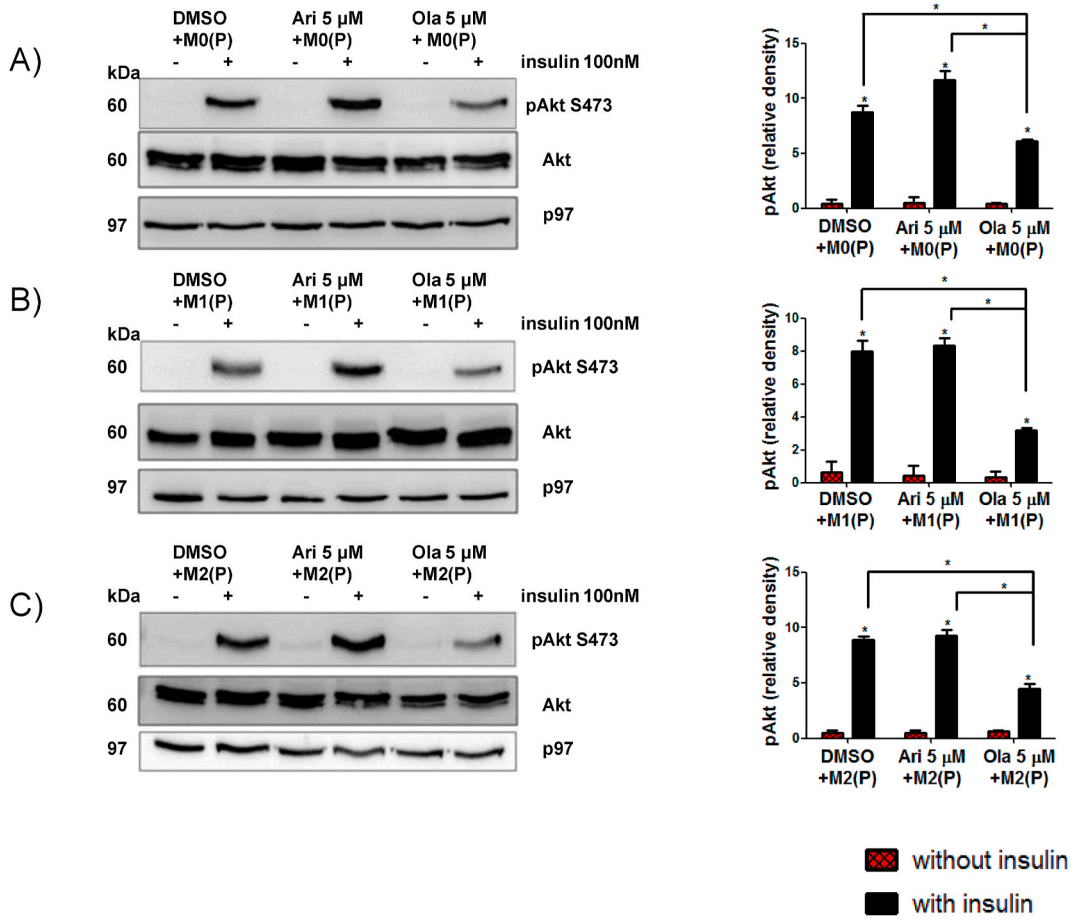


Fig. 5. A reduced response to insulin in SGBS adipocytes when Ola is added in the presence of PBMC macrophages. Western blot analysis of pAkt in the treated SGBS mature adipocytes co-cultured with PBMC macrophages. (A) M0 (PB) (naïve PBMC macrophages), (B) M1(PB) (M1 polarized PBMC macrophages), M2(PB) (M2 polarized PBMC macrophages). Responses were quantified by densitometry and normalized to the expression of total Akt. Densitometry data are shown as mean ± SD of three independent experiments (*$p < 0.05$, ns: non-significant).

macrophages Ola more than Ari elevated the mRNA expression of pro-inflammatory cytokines by 40–60 % ($p < 0.001$), except for *IL-17* and *IL-18*. Dexa showed a significant reduction of the pro-inflammatory cytokines IL-1β and TNF-α (Fig. 7A, C) and consistent induction of anti-inflammatory cytokines *IL-10* and *CCL18* (Fig. 7G and H), supporting the generation of completely polarized M1 cells. Dexa also showed induction of *IL-18* in both PB-M1 and PB-M2 macrophages. This suggests that the *in vitro* differentiated PB-M2 and THP-1-derived M2 cells are different (Fig. 7E). Using these cells, Ola and Ari did not affect the cytokine expression of PB-M2 macrophages. Ari showed a mild reduction in the expression of pro-inflammatory cytokines *IL-1β*, *IL-6* and *TNFα* in PB-M0 and PB-M2 macrophages and a slight induction of CCL-18 in PB-M1 cells, suggesting an anti-inflammatory phenotype of these cells. Ola more than Ari, induced the expression of *IL-17* in PB-M2 cells (Fig. 7D). We did not observe an effect on *IL-6* and *TNFα*. We conclude that in the presence of Ola, primary macrophages generated *in vivo* exert a stronger pro-inflammatory cytokine signature. This conclusion is consistent with the effect of Ola on THP-1 macrophages [43].

We then studied the effect of Ari and Ola on insulin signalling in SGBS cells in the presence of the primary macrophages. The different PBMC macrophages were co-cultured with SGBS adipocytes in transwells and the insulin response was measured by Akt phosphorylation. Ola at 5 μM consistently led to a 60–70 % reduction of insulin responsiveness in the adipocytes relative to DMSO and Ari, in all three macrophages subtypes PB-M0, PB-M1 and PB-M2 (Fig. 5 A-C), consistent with the Ola-mediated effect on Akt phosphorylation seen with THP-1 macrophages. Thus, Ola is operating in a macrophage-dependent manner and similar to what was observed for THP-1 macrophages, primary macrophages in all three configurations confer insulin resistance in adipocytes in the presence of Ola (Suppl. Fig. 2B).

3.5. Ola does not affect insulin signalling in hepatocytes cell lines in the presence of THP-1 macrophages

Inflammation is a key component in the pathogenesis of insulin resistance also in tissues other than adipose, such as in the skeletal muscles and liver. Reportedly, Ola induces hepatic insulin resistance [37]. Whether this is a direct effect or related to inflammatory conditions in the liver has not been fully determined. We then wanted to examine the cross talk with macrophages using the *in vitro* co-culture model with hepatic cell lines. Human hepatoma cell lines Hep3B, HepG2 and the telomerase immortalized human hepatocyte hTERT [63] were used and co-cultured with THP-1-derived macrophages in transwells. Cells were treated with the two anti-schizophrenic drugs followed by insulin stimulation using identical conditions as for the SGBS-THP-1 co-cultures. For all three hepatic cell lines, Ola and Ari treatments, in the presence of THP-1 M1 macrophages did not significantly affect Akt phosphorylation (Fig. 6). It should be noted that when compared to SGBS, hepatocytes generally responded less strongly to insulin and the responses were less consistent, maybe owing to the fact that these cells are constantly proliferating, while mature SGBS don't. Similar data were seen in the presence of the two other THP-1-derived (M0 and M2) macrophages under transwell configuration (data not shown).

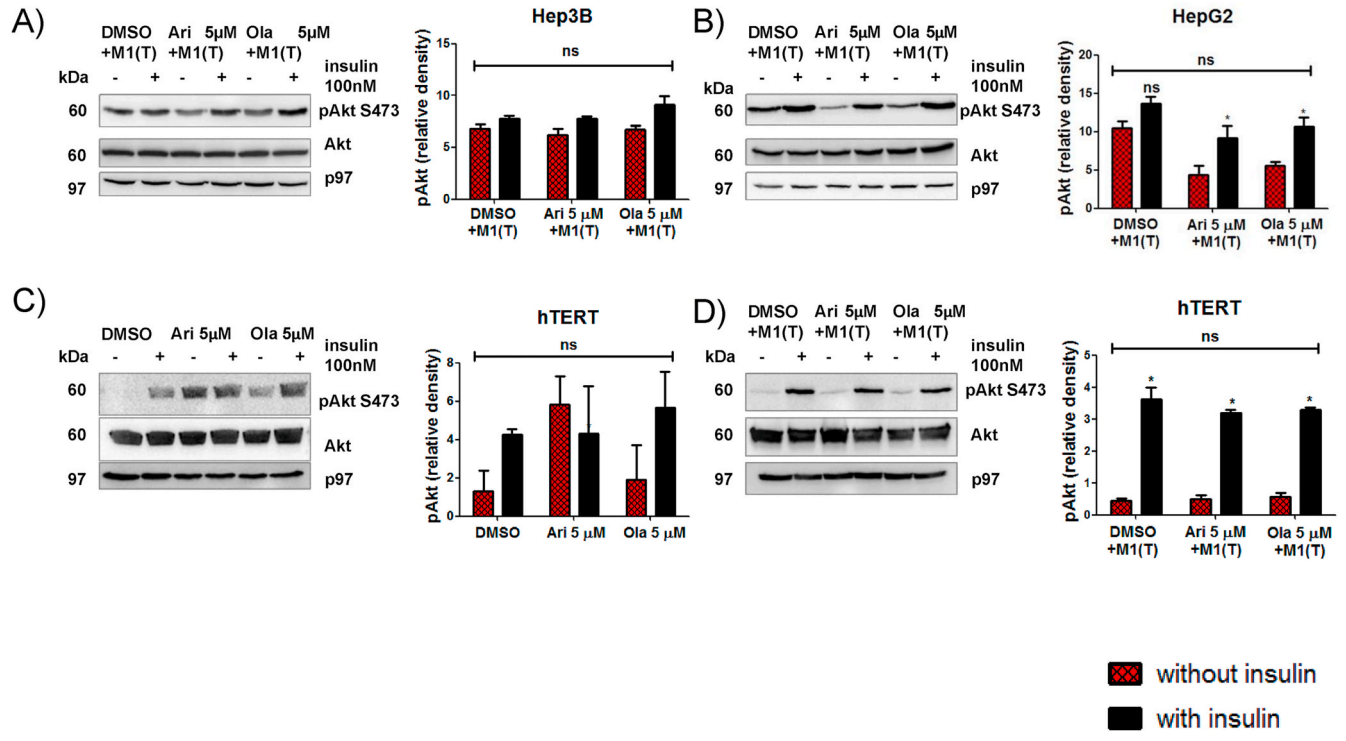


Fig. 6. Ola does not affect insulin signalling in hepatocytes directly and in presence of THP-1 macrophages. Western blot analysis of the expression of pAkt in the treated hepatic cell lines co-cultured with THP-1 macrophages. (A) Hep3B cells, (B) HepG2 cells, (C) hTERT cells (direct treatment), (D) hTERT cells (co-cultured). Responses were quantified by densitometry and normalized to the expression of total Akt. Densitometry data are shown as mean ± SD of three independent experiments (*$p < 0.05$, ns: non-significant).

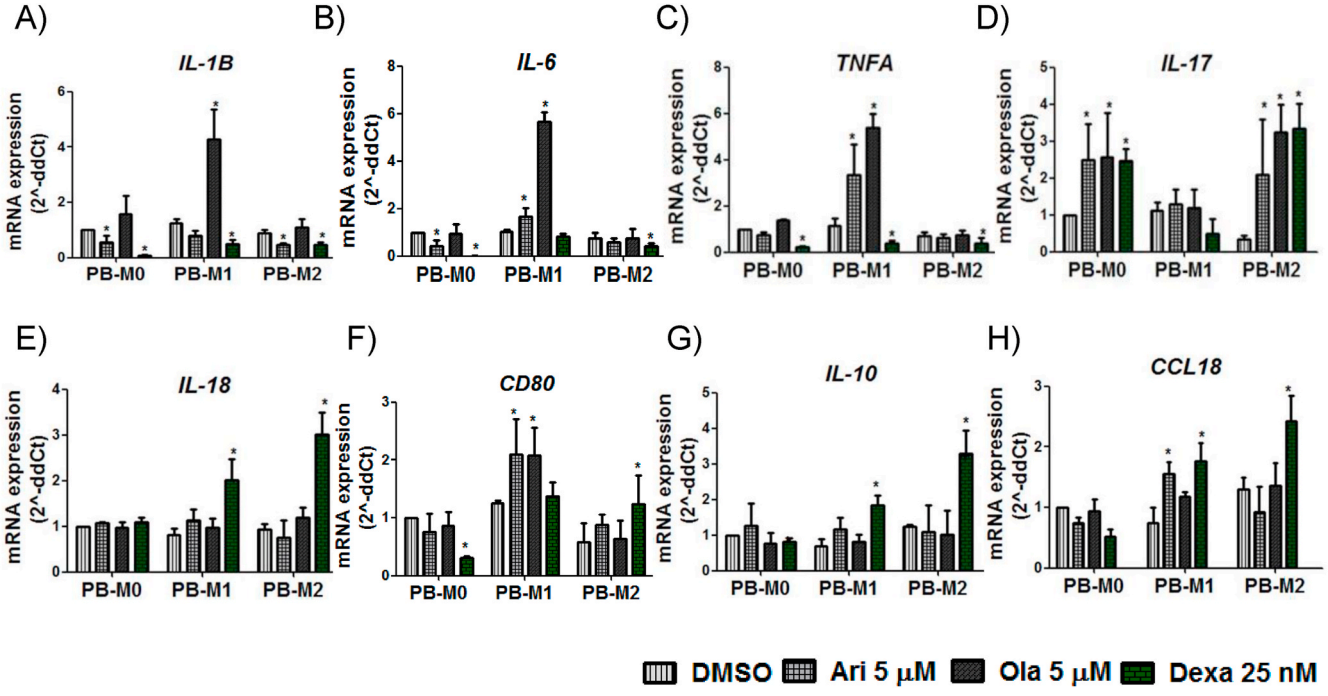


Fig. 7. Ola more than Ari induces pro-inflammatory cytokines in PBMC derived M1 polarized macrophages. Human PBMCs were differentiated into macrophages M0 and polarized into two different states M1 and M2 along with 24 h of drug treatment. The mRNA expressions of different cytokines were studied by RT-qPCR. The vehicle controls of each setting (M0, M1 or M2) were used for assessing the drug effects. All data points are expressed as mean ± SD of four independent experiments. Significance was determined by paired *t*-test and two-way ANOVA including Bonferroni post-test (*$p < 0.05$, ns: not significant).

3.6. The reduction in insulin signalling is correlated with reduction in cell surface expression of Glut4 and reduced glucose uptake

To ensure that the increase in insulin resistance conferred by Ola is also associated with reduced uptake of glucose, we performed a direct glucose uptake assay using the fluorescent analogue of glucose, 2-NBDG. Firstly, we established the gating strategy of mature SGBS adipocytes to quantify the uptake by flow cytometry in the presence and absence of insulin. SGBS adipocytes were starved overnight and then incubated with and without insulin. 2-NBDG was added together with the insulin. When analyzed by flow cytometry, only a small fraction of the cells was positively labelled with 2-NBDG. We observed that a significantly higher population stained positive in the presence of insulin (Suppl. Fig. 5A). We thus quantified the response to insulin based on the percentage of cells that incorporated 2-NBDG.

We then wanted to analyse the direct effect of Ari and Ola on glucose uptake in SGBS adipocytes. We did not expect drug-induced glucose uptake, since Ola and Ari did not affect Akt phosphorylation upon insulin addition. SGBS adipocytes were treated for 24 h with the antischizophrenic drugs and later incubated with 2-NBDG with or without insulin. Cells were collected for flow cytometry and analyzed. The results demonstrated that 5 μM Ari and Ola both in the absence of any cytokines and in the presence of M1 and M2 polarization cytokines responded to insulin as demonstrated by a 40–70 % increase in the number of 2-NBDG positive cells compared to non-stimulated cells (Suppl. Fig. 6 A-D). This shows that in accordance to AKT phosphorylation, Ari or Ola in the presence and absence of the polarized cytokines did not affect glucose uptake in adipocytes with response to insulin when added directly.

Next, we tested the effect of both drugs on glucose uptake by SGBS

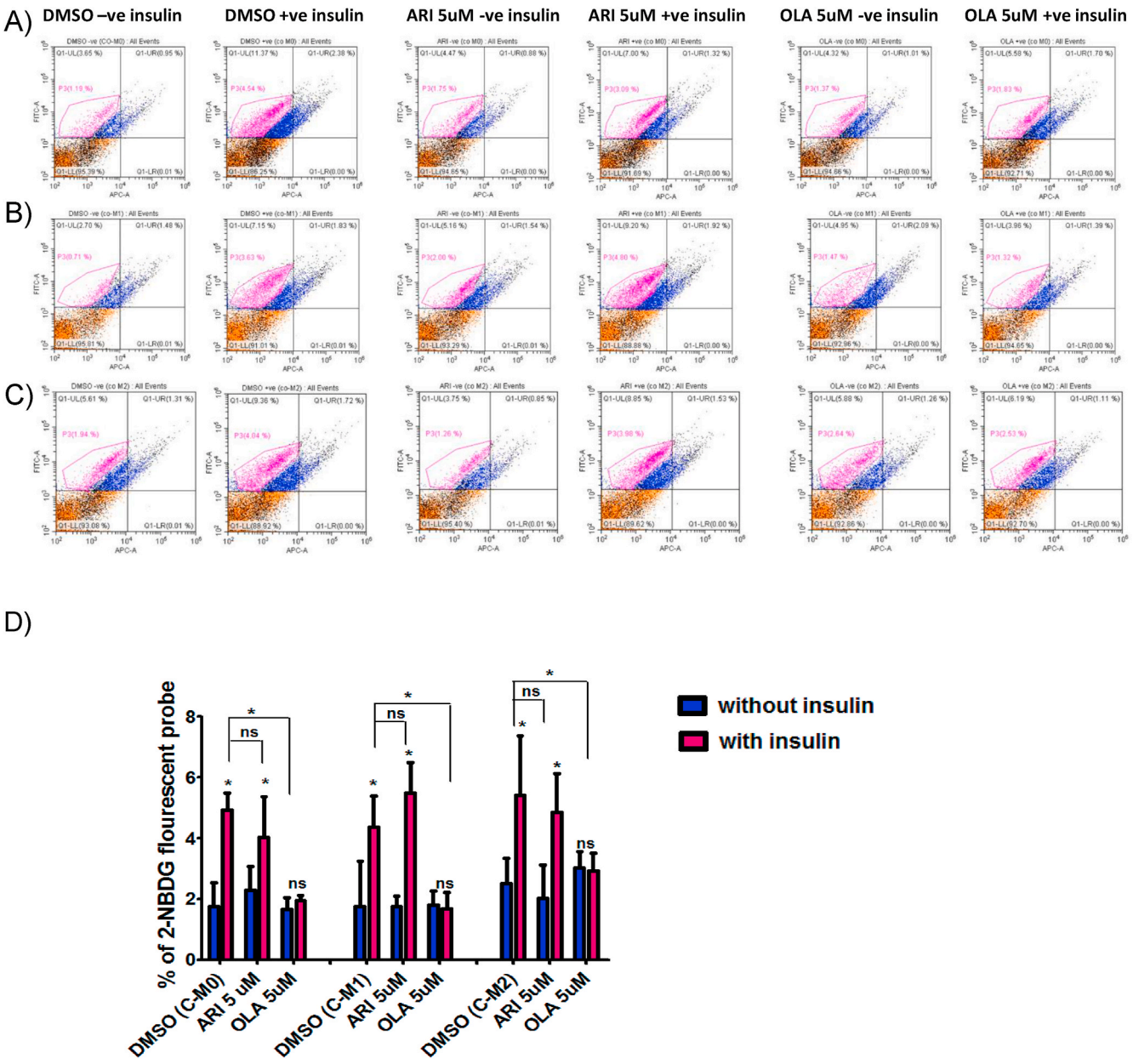


Fig. 8. Ola decreases glucose uptake in SGBS adipocytes in the presence of THP-1 macrophages. Cells were treated and co-cultured with THP-1 macrophages. Adipocytes were collected and analyzed by flow cytometry. (A) M0(T), (B) M1(T), (C) M2(T). Plotting of FITC versus APC-A channels and a gate of cells that facilitate glucose uptake (above diagonal). The proportion of the stained cells was analyzed by two-way ANOVA and paired *t*-test as mean ± SD of three independent experiments ($*p < 0.05$, ns: not significant).

adipocytes in the presence of THP-1 macrophages. Ola treated adipocytes that were co-cultured with M0, M1 and, M2 THP-1 macrophages showed a significant 40–60 % reduction in the number of cells positive for 2-NBDG relative to DMSO (Fig. 8A–D). The proportion of 2-NBDG positive cells reduced more for M1 macrophages than for M0 and M2 in the presence of Ola. These functional data of SGBS are consistent with the reduction in insulin signalling.

Insulin regulates glucose uptake by promoting the translocation of Glut4 from endosomal membranes to the cell surface in adipocytes [22,28,35]. Thus, we examined whether levels of surface Glut4 correlate with the glucose uptake data. First, we wanted to analyse whether the cells respond to insulin under normal condition. After overnight starvation, SGBS adipocytes were incubated along with Glut4 antibody and insulin. Cells were analyzed by flow cytometry. Again, not all cells were positively stained with Glut4. We therefore quantified this fraction in the presence and absence of insulin. A 60 % increase in the number of the Glut4 positive cells was recorded when insulin was added (Suppl. Fig. 5B). Subsequently, we examined the direct effect of drugs on Glut4 expression at the cell surface. The number of SGBS cells which translocated Glut4 to the surface was similarly increased by insulin in the presence of Ari and Ola or the M1 and M2 polarized cytokines (Suppl. Fig. 7A–D). However, upon co-culturing with THP-1 macrophages, Ola significantly reduced the proportion of surface Glut4 positive cells by 50–60 % relative to DMSO and Ari. M0 macrophages did not exhibit a consistent effect (Fig. 9A–D). This suggests that Ola in a macrophage-dependent manner attenuates surface translocation of Glut4 in adipocytes in congruence with its effect on insulin signalling. In conclusion, our SGBS/THP-1 system indicates that Ola promotes insulin resistance and shows that both M1 and M2 macrophages are able to confer this phenotype *in vitro*.

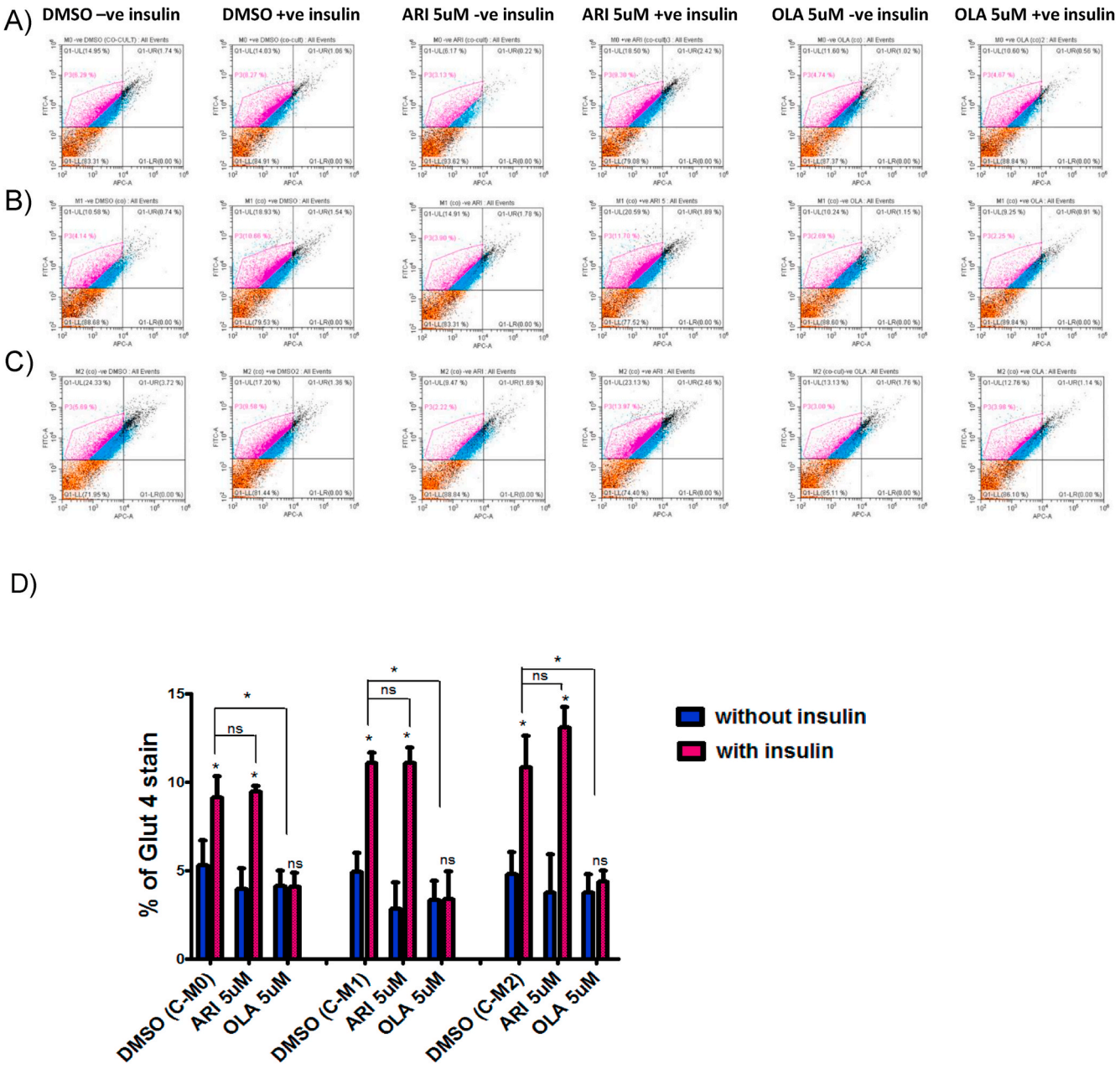


Fig. 9. Ola treatment reduces the surface translocation of Glut4 in SGBS adipocytes in the presence of THP1 macrophages: Cells were treated and co-cultured with THP-1 macrophages. Adipocytes were collected and analyzed by flow cytometry. A) M0(T), B) M1(T), C) M2(T). FITC versus APC-A plot were made with a gate around cells that were translocated by Glut4 transporter. The proportion of the stained cells was analyzed by two-way ANOVA and paired *t*-test as mean ± SD of three independent experiments (*$p < 0.05$, ns: not significant).

4. Discussion

Ola has a broad anti-schizophrenic activity and has been endorsed for cases of severe schizophrenia. However, the metabolic effects of Ola, primarily increasing the risk for developing type 2 diabetes [29,33]), has reduced its use for patients prone to develop diabetes, such those with a high BMI. Of note, not all patients develop insulin resistance and type 2-diabetes upon Ola treatment [21]. In that study, the adverse metabolic effects of Ola treated patients seemed not to be dose-dependent and only susceptible individuals were found to be prone to develop these symptoms.

The prevailing paradigm for the causes of the peripheral activities of Ola suggests effects unrelated to its activity on the dopamine and serotonin receptors, and concentrations higher than what penetrate the blood brain barrier. In our hands, Ola does not directly affect the primary metabolic cells. Concentrations up to 50 μM Ola did not induce endoplasmic reticulum (ER stress) in hepatocytes [15], and did not alter lipolysis in human adipocytes [44]. From these observations, it may be inferred that the mechanisms by which Ola confers insulin resistance are indirect. However, the lack of a validated and reproducible *in vitro* model that enumerates these activities has not been reported. Here, we recapitulated these effects in a simple co-culture system of the SGBS and THP-1 cell lines (Fig. 1) and showed that Ola only impacts the insulin responsiveness of adipocytes in the presence of macrophages. In our study, we used low micromolar concentrations of Ari and Ola that are higher than the nanomolar bloodstream concentration of Ola. However, these seemingly supra-pharmacological concentrations may develop locally in the adipose tissue owing to the lipid solubility of the drug, as was reported post mortem in human and in animal studies [2,58]. We further showed that while Ola promotes the expression of pro-inflammatory cytokines, as previously reported, the insulin resistance effect of Ola exceeds the effect of the cytokines even when added at high supra-physiological concentrations. This suggests that factors other than the inflammatory cytokines play a role.

The inter-connectivity of low and chronic conditions of inflammation that is induced by activated macrophages is thought to underlie the pathogenic progression of insulin resistance [9,49]. As insulin dysfunction persists, infiltration of macrophages is promoted into adipose tissues ([31,52]. This perturbation of homeostasis, probably inflicted by Ola, creates a positive feedback loop that over time may result in pathology. Our results indicate that the effect of Ola, at low micromolar concentrations, is rapid and sensed by adipocytes to alter the proximal signalling events of insulin and glucose uptake.

One possible effect of Ola on the macrophages can be the induction of a low-grade stress conditions, such as oxidative stress or endoplasmic reticulum (ER) stress. Indeed, *in vitro* studies showed that patients with insulin resistance and type 2-diabetes have increased expression of thioredoxin interacting protein (TXNIP) and markers of the unfolded protein response in the peripheral macrophages, indicative of oxidative and ER stress conditions, respectively [53]. More importantly, Ola primarily promotes the secretion of pro-inflammatory cytokines in M1 macrophages such IL-1β, IL-6, TNF-α and also reduces the response to insulin in adipocytes, which may occur by activation of the inflammasome, downstream to the stress conditions. The activation of the inflammasome subunit NLRP3 receptor has also been implicated by TXNIP in the presence of ER stress [38,64], where IRE1α a sensor of unfolded proteins in the ER, guides adipose tissue macrophages towards M1 polarization [48]. We assume that the primary targets of Ola in its peripheral activity are molecular stress modules in the macrophages. On the other hand, anti-inflammatory effect of SGA has also been demonstrated. Schizophrenia patients showed increased pro-inflammatory cytokine plasma levels [32] that seemed reduced after administration of anti-psychotic treatment despite weight gain [39]. In accord with this, treatment of monocytes such as THP-1 and PBMC with 10 μM Ari and 100 nM Ola showed a significant reduction in the expression of pro-inflammatory cytokines such as IL6, IL1β, and TNFα [51], whereas, tissue macrophage induced the level of pro-inflammatory cytokines [69]. This shows macrophages in different microenvironments respond differently to SGAs.

We found that naïve and even M2 type macrophages also impart inhibition of insulin signalling in adipocytes by Ola, albeit to lower levels than observed for M1 macrophages. This may be related to the fact that Ola treatment causes metabolic dysfunctions and insulin resistance also without weight gain [8]. Importantly, whereas the M1 state is associated with hindrance of mitochondrial oxidative phosphorylation and inhibition of nitric oxide production, which prevent repolarization of M1 to M2; M2 macrophages, on the other side, are more plastic and can be polarized into M1 type *in situ* [57]. Thus, it is possible that Ola can induce a pathological cascade also when the M1/M2 balance is in favour of M2 and gradually shifts the balance towards the M1 subtype.

Our data suggest the cooperation of multiple factors secreted by macrophages to drive insulin resistance in adipocytes by Ola and not Ari treatment. Inflammatory cytokines are probably the most important players driving this response; additionally, other secreting factors may contribute to the cellular dysfunction. Adipose tissue macrophages in obese mice and humans have a mixed M1/M2 phenotype, suggesting complex states *in vivo* [50,68]. A proteomic study showed that adipose tissue macrophages from obese humans do not significantly express M1 markers. However, when exposed to conditions of high glucose, insulin and palmitate, which are elevated in plasma of type 2 diabetic patients, macrophages induce multiple factors and favour an M1 phenotype [40, 54]. While we have not combined Ola and additional stress promoting factors in our study, it is plausible that once mild diabetes develops, the presence of Ola facilitates the secretion of those factors to further promote the metabolic dysfunction. This is in agreement with the fact that schizophrenic patients already mildly obese at the initiation of therapy, are more prone to gain weight and develop a full-blown metabolic syndrome upon Ola treatment.

Though implicated by our data, additional factors, other than the typical pro-inflammatory cytokines, generated in an Ola-dependent manner from macrophages have not been identified. It has been reported that Ola affects the expression and activity of enzymes that are involved in protein and lipid biosynthesis [25,34]. In addition, antipsychotics may affect enzymes that enhance post translational modifications and by that generate secreted molecules with altered biological activities. For instance, Telford et al. showed that patients undergoing Ola treatment display an altered protein *N*-glycosylation repertoire; most prominently, the serum of patients displayed an aberrant distribution of multiple sialylated bi- and tri-antennary glycans and an altered abundance of several serum proteins carrying *N*-glycosylation including alpha-1 acid glycoprotein [55]. Ola may also alter the release of non-protein mediators. For instance, prostaglandin E_2 (PGE_2), has been shown to alter metabolic functions. The PGE_2 receptor EP4, which is abundantly expressed in macrophages, accumulates in adipose tissues and plays crucial roles in the pathogenesis of insulin resistance [65]. A recent study suggested that an increase in prostaglandin secretion in plasma may activate the inflammatory pathway in patients undergoing Ola treatment [41]. Whether prostaglandins and other bioactive lipids play a role in Ola-promoted metabolic dysfunction needs further investigation. We suggest the THP-1 secretome for further analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cpnec.2021.100073.

Author contribution

P.D and B.T designed the experiment. P. D, M.S, F·F performed experiment. A.S, J.W.E, M.J.P, X. A, M.W. assisted with adipocyte studies and exporting cells, B.T, M.T-A, P. D worked on manuscript.

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