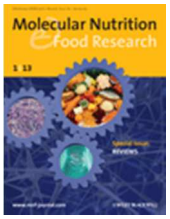

Extracellular Vesicles from Hypoxic Adipocytes and obese subjects reduce Insulin-Stimulated Glucose Uptake.

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Extracellular Vesicles from Hypoxic Adipocytes and obese subjects reduce Insulin-Stimulated Glucose Uptake.

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Keywords: adipocyte, exosome, glucose transport, insulin.

Abbreviations: EVs: extracellular vesicles; ERK: Extracellular Regulated Kinase; PI3-Kinase:phosphatidylinositol 3-kinase; MCM: macrophage conditioned media.

Abstract

Scope: We investigated the effects of extracellular vesicles (EVs) obtained from *in vitro* adipocyte cell models and from obese subjects on glucose transport and insulin responsiveness.

Methods and results: EVs were isolated from the culture supernatant of adipocytes cultured under normoxia, hypoxia (1% oxygen) or exposed to macrophage conditioned media (15%v/v). EVs were isolated from the plasma of lean individuals and subjects with obesity. Cultured adipocytes were incubated with EVs and activation of insulin signalling cascades and insulin-stimulated glucose transport were measured.

EVs released from hypoxic adipocytes impaired insulin-stimulated 2-deoxyglucose uptake and reduced insulin mediate phosphorylation of AKT. Insulin-mediated phosphorylation of extracellular regulated kinases (ERK1/2) was not affected. EVs from individuals with obesity decreased insulin stimulated 2-deoxyglucose uptake in adipocytes (p=0.0159).

Conclusion: Extracellular vesicles released by stressed adipocytes impair insulin action in neighbouring adipocytes.

Introduction

White adipose tissue (WAT) regulates glucose and lipid homeostasis and functions as an endocrine tissue releasing adipokines that contribute to regulate insulin sensitivity. White adipocytes respond to insulin by activating glucose transport through the translocation and activation of the glucose transporter GLUT4 [1]. Excess glucose is converted to glycerol for esterification of fatty acids into triglycerides, which are later hydrolysed to provide fatty acids during fasting. In obesity adipose cells fail to respond to insulin and glucose transport and lipid regulation are impaired. During WAT expansion cells are exposed to local hypoxia which contributes to the dysregulation of production and secretion of adipokines [2]. Adipocytes are also exposed to pro-inflammatory cytokines following the recruitment, infiltration and activation of macrophages within WAT, which in turn further impair insulin action.

In recent years the importance of a novel mechanism for intercellular communication has emerged based on the release of extracellular vesicles (EVs) [3]. EVs of various sizes, namely exosomes (with sizes between 30-150 nm), microvesicles (ranging from 100-1000 nm) or apoptotic bodies (ranging 150-5000nm) are released by eukaryotic cells. EVs contain protein and RNA species that regulate physiological functions and gene expression in target cells [4] eliciting both autocrine and paracrine effect in various cell types. EV composition and release are modified in pathological conditions, with EVs serving as biomarkers for human disease [5, 6].

Adipocytes have been shown to produce EVs of heterogeneous sizes [7-10] which contribute to regulate lipid deposition [11, 12] and angiogenesis [13], reviewed in [14]. Deng et al., [15] were the first to report that EVs from adipose tissue explants from obese (*ob/ob*) mice induced insulin resistance *in vivo* when injected into wild type mice [15]. Despite the available data obtained in animal and *in vitro* studies, adipocyte-derived EVs remain poorly

characterized and limited information is available on their effects in normal and pathophysiological conditions.

This study aimed at determining the impact of adipose-released EVs on insulin action. Specifically, we tested the hypothesis that EVs released by stressed adipocytes and those found circulating in human obesity impact on insulin-stimulated glucose uptake.

Material and Methods

Materials reagents and antibodies: Tissue culture media and reagents were from Sigma-Aldrich (UK). B-tubulin antibody was from Sigma-Aldrich, Phospho-Ser473 AKT, total AKT, phospho44p42, total ERK (p44/p42) antibodies were from Cell Signalling. Anti-CD81 antibody was from AD Serotec(UK) anti-TSG101 (Ab83), and anti-MHCI (clone 1158Y) anti-beta actin (Ab6276), anti-catalase (Ab15834) and anti-GAPDH (Ab9484) antibodies were from Abcam (UK). Anti-VEGF (VG-1) antibody was from Santa Cruz Biotechnology.

Cell culture: Human Simpson–Golabi–Behmel syndrome (SGBS) cells were grown and differentiated as described [16]. 3T3-L1 cells were cultured and differentiated as described [17]. Prior to the isolation of EVs from the conditioned media, cells were washed in PBS and cultured in Dulbecco´s Modified Eagle Medium (DMEM) without any serum supplementation for 24hr. For the macrophage conditioned media treated group, DMEM was supplemented with 15% v/v of media from Bone Marrow Derived Macrophages (BMDM) cells. Isolation of BMDM cells was carried out as described [18]. This media contained on average 195 ng/ml of IL6 and 150ng/ml of TNFα quantified by ELISA. For the hypoxia experiments, 3T3-L1 adipocytes were incubated in normoxia (95% air, 5% CO_2) or inside an hypoxic chamber (hypoxystation1% O_2, 5% CO_2 and 95% N_2) for 24 hrs. EV treatments were carried out in DMEM without supplementation.

Subjects: Plasma wasobtained from n=8 lean and from n=9 women with obesity recruited to the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain), and

were used for EVs isolation. Exclusion criteria were: abnormal blood counts and liver, kidney or thyroid dysfunction, evidence of chronic illness other than obesity, chronic use of medication, acute illness or signs of infection in the month preceding enrolment. The study protocol was approved by the Ethics Committee and the Committee for Clinical investigation (CEIC) of the Hospital Universitari Dr. Josep Trueta. All subjects gave their written informed consent. Plasma samples kept at -80ºC until the isolation of EVs. Fasting glucose levels were measured by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Brea, CA). Plasma insulin was determined by ELISA using a commercial kit (Catalog E6000-K, Millipore, USA) following the manufacturer's instructions.

Isolation of EVs: Isolation of EVs was carried out as described [19, 20]. Briefly, cells were grown and differentiated as indicated above in media containing exosome-depleted FBS. Collection of conditioned media was done following a 24h incubation period in DMEM at either normoxic or hypoxic conditions or DMEM containing MCM. Media was centrifuged at 2,000xg,10min and filtered (0.22µm) to remove dead cells and cellular debris. The supernatant was centrifuged at 17,000×*g* 30min. The supernatant was centrifuged at 100,000xg 4ºC for 75 minutes. The pellet resuspended in sterile phosphate buffered saline (PBS) and centrifuged again at 100,000×*g* 4°C for 75 min. The final pellet containing small EVs was resuspended in PBS, aliquoted and stored at -80ºC until use. The same procedure was used for the isolation of plasma EVs, with a minor modification. The plasma was diluted 1:3 volume in PBS prior to the initial centrifugation. EV size distribution and concentrations were determined by nanoparticle tracking analysis (NTA) using a Malvern Nanosight NS300 instrument. EV preparations were aliquoted and stored at -80ºC.

Cryo-electron microscopy: EVs were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL, Germany) and processed as described [21]. Images were obtained in a JEM-2200FS/CR transmission cryo-electron microscope (JEOL, Japan).

Insulin-stimulated 2-deoxyglucose uptake. Cells were washed in Krebs Ringer Hepes buffer (pH=7.4) twice and incubated in this buffer for 45 minutes. Insulin was added at 100 nM for

15min (SGBS cells) or 30 min (3T3L1) prior to the assay. 2-deoxyglucose uptake media was added containing 2µCi/ml of ^{3}H-2deoxyglucose and unlabelled substrate at 0.1mM final concentration. Uptake was allowed to proceed for 10 min (3T3L1 cells) or 15min (SGBS) and stopped with 4 washes in ice cold PBS containing 50 mM glucose. Cells were lysed in 0.1N NaOH and a fraction of lysate counted in scintillation cocktail. An aliquot of lysate was used for protein determination. Measurements were normalized to cellular protein content.

Cellular lysates and Western blotting were carried out as previously described [22].

Extraction of RNA and qPCR analysis was carried out as previously described [22]. Primer sequences used:*Glut1*: Forward (5'-3'):GCTGTGCTTATGGGCTTCTC; reverse(5'-3'): CACATACATGGGCACAAAGC; and *Actb*: forward(5'-3'): CCTGTGCTGCTCACCGAGGC, reverse(5'-3'): GACCCCGTCTCTCCGGAGTCCATC was used as normalizing gene.

Statistical analysis*:* T-test or ANOVA as indicated in the figure legends, were carried out using GraphPad Prism6 with a confidence interval of 95% and statistical significance was considered if $p < 0.05$.

Results

EVs from hypoxic adipocytes impair insulin-stimulated glucose uptake and AKT activation in adipocytes.

In obesity, adipose tissue expansion results in local tissue hypoxia and inflammation. These two mechanisms cause cellular stress and thus, could contribute to the generation of EVs by adipose cells. To evaluate the role of the EVs released by adipocytes under these conditions independently, we generated two *in vitro* models using the well stablished 3T3L1 mouse adipose cell line (Supl.fig1). For this, cells were either incubated under hypoxia (1%O_2) or normoxia (21%O_2, control cells) or in DMEM media alone or supplemented with macrophage conditioned media (MCM, 15%v/v) enriched in pro-inflammatory cytokines. Both conditions

caused the adipocytes to become insulin resistant as seen by a decrease in the activation of AKT following insulin stimulation (Suppl. Fig1).

We next isolated EVs released by the cells into the conditioned media and examined EV preparations by cryoelectron microscopy (Fig.1A) and immunoblotting (Fig.1C) to confirm purity of the preparations. EV size and concentration was assessed by NTA (Fig1.B). EV size distribution was consistent among experimental groups and consistent with that of exosomes (Fig 1B). EV preparations were enriched in CD81, a marker of exosomes, and markedly devoid of a mitochondrial (prohibitin-1) and endoplasmic reticulum (GRP78) markers (Fig 1C).

To determine the effects of EVs on insulin sensitivity, 3T3-L1 adipocyte cells were either left untreated or treated with equal amounts of EVs isolated from either normoxic, hypoxic or cells exposed to MCM. Insulin-stimulated glucose uptake was measured after 24hr. In untreated control cells insulin increased 2-deoxyglucose uptake by 3-fold over basal (Fig.2A, B and C). Cells treated with EVs from control adipocytes exhibited a similar response to untreated cells. However, cells treated with EVs from hypoxic adipocytes displayed a 25% decrease in the insulin-stimulated response, with no changes in the basal glucose transport (Fig.2A and 2C). Uptake in cells treated with EVs from cells exposed to MCM was no different than that of untreated cells or treated with EVs from control cells (Fig.2B). Cellular viability was not affected by the EVs treatment (not shown). Heating the hypoxic EV preparations to 40ºC for 30 minutes prior to the treatment of cells restored insulin-stimulated glucose uptake (Fig.2C).

To explore the molecular mechanisms, we determined the expression of GLUT4 the main glucose carrier involved in the insulin-mediated glucose transport, insulin receptor and activation of the proteins involved in insulin signalling. No differences were observed in the expression of Glut4 or the insulin receptor between cell groups (Fig 3A). We determined the activation of the insulin signalling pathway by monitoring phosphorylation of Ser473 in AKT and of Thr201/Tyr204 on extracellular regulated kinases (ERK) p44/p42 in response to

insulin following the treatment with EVs. In a dose dependent manner, we detected a small but significant decline in AKT S473 phosphorylation in cells treated with EVs from hypoxic cells compared to untreated or cells treated with EVs from control adipocytes (Fig.3B and C). We did not detect any difference in the activation of the ERK proteins in any of the experimental groups (Fig. 3B and C). No differences in the expression levels of AKT or ERK proteins were seen.

EVs from the plasma of subjects with obesity impair insulin-mediated 2-deoxyglucose uptake.

To confirm the above data in the context of human obesity, we next isolated EVs from plasma obtained from lean and obese women (suppl. Table1) using previously established ultracentrifugation protocols [19]. EV samples were analysed by western blotting to confirm the presence of EV markers (CD81, MHCI) and the absence of other cellular membrane markers (insulin receptor, GRP78) and were compared to EVs released by 3T3-L1 adipocytes (Fig. 4A). Plasma EVs contained exosomal markers such as CD81 and MHCI, but were devoid of TSG101 compared to EVs released by 3T3-L1 adipocytes (Fig.4A). EV particle distribution was similar to the cell models and consistent with that of exosomes, with a peak around 100-150nm. (Fig.4B)

To preserve any species compatibility that could affect the entrance of EVs into cells, we next examined the effect of these EVs on insulin action in the SGBS human adipocyte cell line. Following differentiation of cells into adipocytes, cells were incubated in DMEM for 24hr to remove hormones and subsequently treated with EVs from either lean women or women with obesity at equal concentration (2 10^5 EV/ml) as determined by NTA, and we assayed 2-deoxyglucose uptake in response to insulin as described previously [16]. Transport was normalized to cellular protein content. Insulin-stimulated glucose transport was decreased in cells treated with EVs obtained from women with obesity (Fig.4C).

Discussion

Numerous stimuli induce the production of EVs in distinct cellular types. Here, we sought to investigate the hypothesis that EVs released by stressed adipocytes and those circulating in obese subjects could affect insulin action.

The size distribution of our EV preparations was consistent with those found in other systems including the Otsuka Long-Evans Tokushima Fatty rats [23] and adipose tissue derived mesenchymal stem cells [24].

Previous reports found that obesity leads to an increase in EV release in rodent animal models [15, 25]. However, we did not detect any differences in the concentration of circulating EVs found in subjects with obesity (not shown). However, in agreement with [26] the model of hypoxic 3T3-L1 adipocytes produced more EVs compared to normoxic cells, a phenomena seen in other hypoxic cell types. Hu et al.,[27] found hypoxia upregulated the expression of FIP4 a regulatory protein of the small GTP binding protein rab11, which is present in adipocytes and has been involved in exosomal release [28]. Thus, expression of genes regulated by HIF1α and increased rab11-mediated trafficking could facilitate EV release in adipocytes, however more experiments are needed to confirm this.

EVs from hypoxic adipocytes and also from subjects with obesity impaired insulin-stimulated glucose uptake *in vitro* in cultured adipocytes. Our findings agree with those by Kranendonk et al. [29] who found adipose tissue-derived EVs caused insulin resistance in hepatocytes inhibiting insulin mediated AKT phosphorylation, with concomitant decrease in the expression of gluconeogenic genes.

Proteomic profiling studies of EVs released by adipocyte cell models have been documented [8] including that of hypoxic 3T3-L1 cells [26] and of animal models of obesity and diabetes [23] but to date not much information is available for circulating EVs in human obesity. Available literature from *in vitro* systems suggests that EVs from adipocytes contain protein and RNA species involved in regulating lipid metabolism and adipokine production [8, 9, 11, 26, 30-32] .

While the precise molecular mechanisms are at present unknown, we provide evidence that EV action implicates at least in part the AKT pathway. The effect of hypoxic EVs on glucose transport was thermolabile, suggesting an enzymatic activity may be responsible for this effect, We found that PTEN, a protein phosphatase that reduces phosphatidylinositol 3,4,5-triphosphate levels was present in EVs from hypoxic 3T3L1 adipocytes (not shown). Thus, it is plausible that PTEN exported in EVs may be active in recipient cells. Further experiments are necessary to confirm this.

Finally, since adipocytes produce adipokines that modulate insulin action, it is possible that EVs could affect insulin signalling indirectly through an adipokine-mediated mechanism. This remains to be explored.

To sum up, our study provides evidence that EVs released by hypoxic adipocytes and in human obesity negatively impact on insulin-stimulated glucose uptake, in part by inhibiting AKT phosphorylation. The precise molecular entities need to be fully elucidated and warrant further investigation.

References

Figure legends:

Figure 1. Characterization of EVs released by 3T3-L1 adipocytes. A) Cryo-electron microscopy from Control (untreated), hypoxic or cells exposed to macrophage media (MCM). Scale bar: 100 nm. B) NTA of EVs from normoxic/ hypoxic cells and control/MCM-exposed 3T3L1 adipocytes. C) Immunoblot of cellular lysates (L) or EVs from control, hypoxic or MCM exposed cells. Equal amounts of lysate or EVs (1µg) were separated by SDS-PAGE and immunoblotted with specific antibodies as indicated.

Figure 2. EVs from hypoxic adipocytes inhibit insulin-stimulated glucose uptake. 2-deoxyglucose uptake in 3T3L1 adipocytes that were either left untreated (none) or treated for 24hr with 10µg of EVs from control or hypoxic adipocytes (A) or with EVs from either control or MCM-exposed adipocytes (B). The graphs show mean+SEM of a representative experiment and n=3-4 biological replicates. Data is representative of 3 independent experiments.*indicates $p<0.05$, One way ANOVA. C) Heat treatment of hypoxic EVs restores insulin stimulated glucose uptake. Adipocytes were left untreated or treated for 24hr with EVs from control or hypoxic cells or EVs from hypoxic cells that were heated 40ºC for 30 mins. Following the treatment 2- deoxyglucose uptake was determined. The graph show mean+SEM * indicates $p<0.05$, One way ANOVA (Tukey's test).

Figure 3. EVs from hypoxic adipocytes inhibit insulin signalling. A) Immunoblot of cellular lysates from adipocytes treated for 24hr with 10 µg/ml of EVs from control, hypoxic or MCM-exposed cells. Equal amounts of lysates were immunoblotted with antibodies as indicated. B) 3T3L1 adipocytes were left untreated or treated with 5-10 µg/ml EVs for 24hr (C: control, H: Hypoxia), and stimulated with insulin (100nM, 30 min). Lysates were obtained and immunoblotted with the indicated antibodies. C) Quantification of AKT and ERK

phosphorylation.Graphs show mean±SDEV of n=4 experiments for AKT and n=2 experiments phospho-ERK, *** indicates p<0.01. One Way ANOVA.

Figure 4. A) EVs from plasma (EV blood), EVs from human adipocytes (EV adipo) and adipocyte cellular lysates were separated by SDS-PAGE and immunoblotted with antibodies for EV markers as indicated. B) Size distribution of plasma EVs by NTA . C) Insulin-stimulated 2-deoxyglucose uptake in SGBS cells treated with 2 10^5 EV/ml of EVs obtained from lean (n=8) or obese subjects (n=9). Data shows mean±SDEV, each EV was tested in triplicate in two independent experiments, p=0.0153 (t-test).

Declaration of Interest. No conflict of interest.

Author contributions : JM, FJO,SM, data acquisition and analysis; JM, FJO, JMFP, MW,JMFR, SM writing of manuscript.

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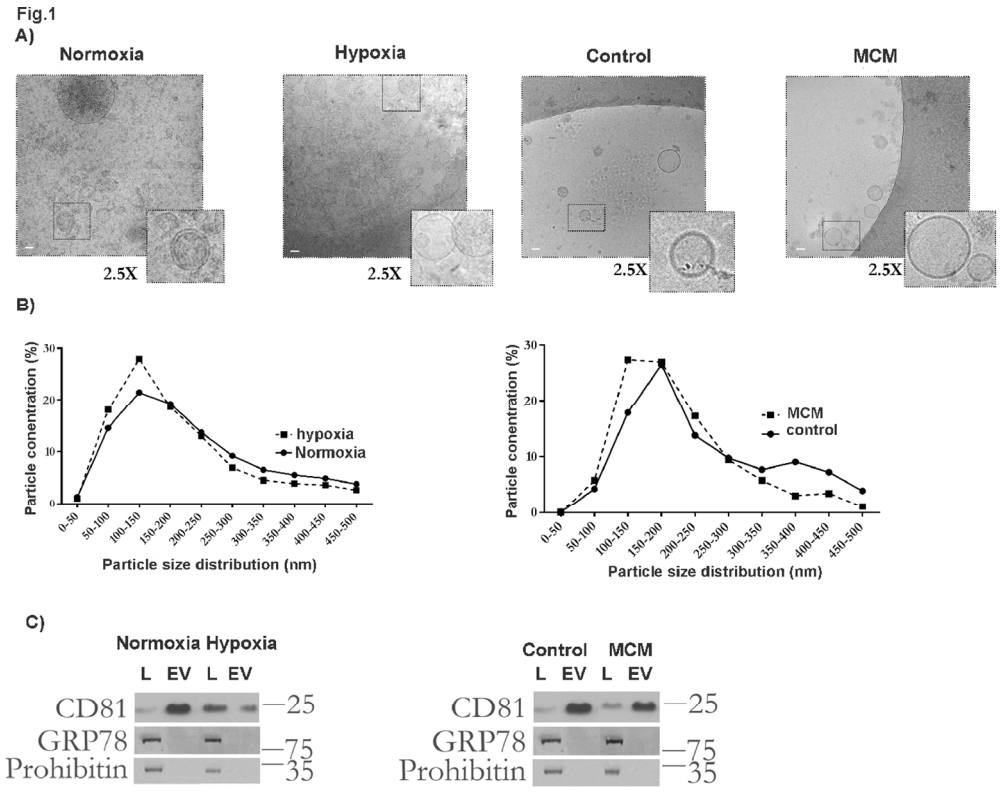

Figure 1

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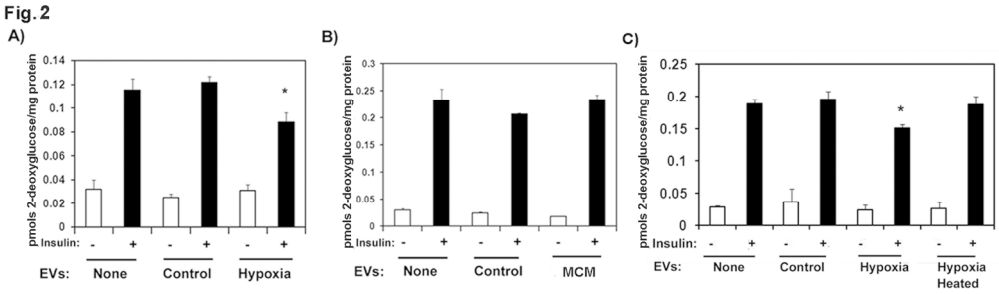

Figure 2

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Fig. 3

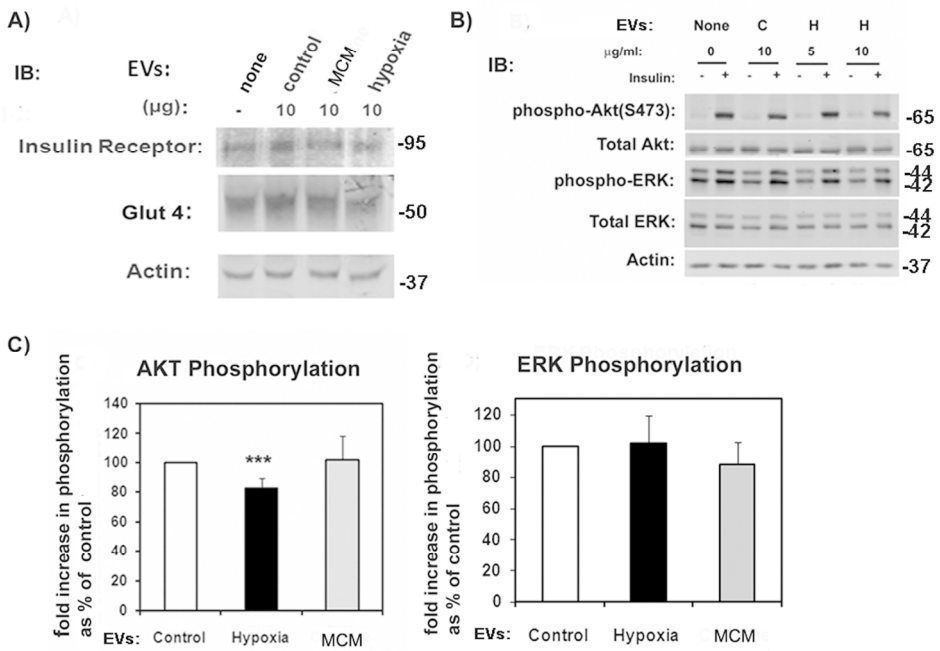


Figure 3

150x105mm (300 x 300 DPI)

Fig.4

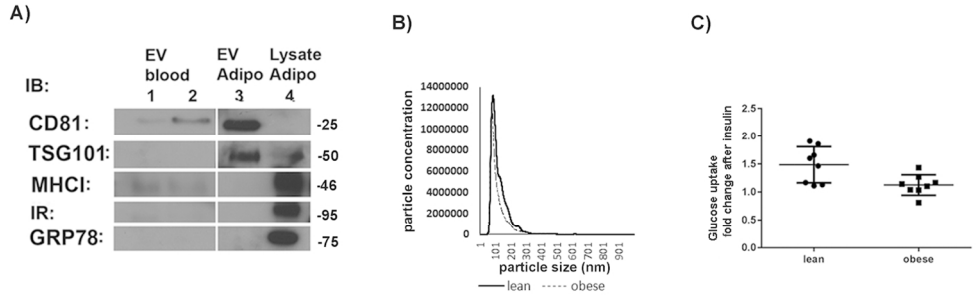
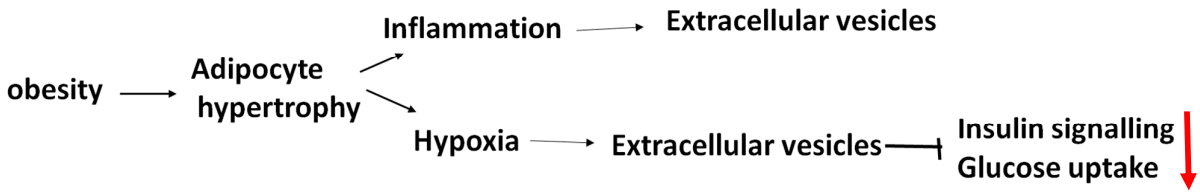


Figure 4

119x43mm (300 x 300 DPI)



Graphical Abstract-text:
Caloric excess leads to adipose tissue expansion, with the development of local hypoxia (low oxygen) and inflammation. Hypoxic adipocytes release extracellular vesicles which impair insulin signalling and glucose uptake contributing to adipose tissue insulin resistance.