

Effects of oleic (18:1n-9) and palmitic (16:0) fatty acids on the metabolic state of adipocytes

Efectos de los ácidos grasos oleico (18:1n-9) y palmítico (16:0) en el estado metabólico del adipocito

Gerardo Mantilla-Mora¹ ; Alberto Ángel-Martín¹ ; Natalia Moreno-Castellanos¹ 

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Abstract

Background: Elevated serum-free fatty acid (FFA) levels induce insulin resistance (IR) or a protective mechanism to IR development in humans; it depends on FFA type. **Objective:** This study explores the effects of oleic (OLA–unsaturated) and palmitic (PAM–saturated) fatty acids on insulin action in mature adipocytes effect. **Methods:** Cells were incubated 18 h with or without OLA and PAM at 250 µM, and 500 µM. After the culture period, were measured: adipocyte viability, size, fatty acids mobilisation, insulin signalling proteins, and glucose uptake. **Results:** Adipocytes exhibited optimal viability tolerances regardless of the kinds of fatty acids used for treatment. However, adipocytes were hypertrophic after OLA and PAM stimuli. Additionally, lipogenesis (lipid synthesis), and lipolysis (lipid breakdown) were significantly increased by treatment with OLA, or PAM (500 µM) compared to control. Moreover, OLA results showed that there was no significant reduction in signalling cascades, except for a downstream proinflammatory response. Instead, PAM hypertrophic adipocytes were insulin resistant with alteration of proinflammatory and stress markers. **Conclusions:** Current findings suggest that PAM induces insulin resistance, mitochondrial and reticulum stress on fat cells compared to those treated with OLA that, protects adipocytes to all those alterations.

Keywords: Adipocyte; Fatty Acids; Oleic Acid; Palmitic Acid; Insulin Resistance; Insulin; Lipolysis; Lipogenesis.

Resumen

Introducción: los niveles elevados de ácidos grasos libres (AGL) en suero inducen resistencia a insulina (RI) o un mecanismo de protección del desarrollo de RI en humanos, esto depende del tipo de AGL. **Objetivo:** este estudio explora los efectos de los ácidos grasos oleico (insaturados–OLA) y palmítico (saturados–PAM) sobre la insulina en adipocitos maduros. **Métodos:** las células se incubaron 18 h con o sin OLA y PAM a 250 µM y 500 µM. Después del período de cultivo, se evaluó en adipocitos: viabilidad, tamaño, movilización de ácidos grasos, proteínas de señalización de insulina y absorción de glucosa. **Resultados:** los adipocitos mostraron viabilidad óptima

1. Universidad Industrial de Santander. Bucaramanga, Colombia.

Correspondence: Natalia Moreno Castellanos. Dirección: Cra. 32 29-31 Edificio administrativo 1, Piso 1 Oficina 205. Telephone number: +57 3222458530. Email: nrmorcas@uis.edu.co

independientemente de los tipos de ácidos grasos utilizados en el tratamiento. Los adipocitos eran hipertróficos tras estímulo con OLA y PAM. La lipogénesis (síntesis de lípidos) y la lipólisis (degradación de lípidos) aumentaron significativamente con el tratamiento con OLA o PAM (500 μ M) en comparación con el control. En los resultados de OLA no se evidenció una reducción significativa en las cascadas de señalización de insulina, a excepción de una respuesta proinflamatoria posterior. En cambio, los adipocitos hipertróficos tratados con PAM presentaron resistencia a la insulina y alteración de los marcadores proinflamatorios y de estrés. **Conclusiones:** nuestros hallazgos sugieren que PAM induce resistencia a la insulina, estrés mitocondrial y del retículo en las células grasas en comparación con aquellos tratados con OLA, AGL que, en cambio, protegen a los adipocitos de todas esas alteraciones.

Palabras clave: Adipocito; Ácidos Grasos; Ácido Oleico; Ácido Palmítico; Resistencia a la Insulina; Insulina; Lipólisis; Lipogénesis.

Introduction

Obesity is associated with different metabolic disorders that often lead to insulin resistance (IR). Obesity is a pathological mechanism and major metabolic disorder developed by a problem in insulin receptor signaling in insulin target tissues such as adipose tissue, pancreas, among others^{1,2}. However, IR abnormalities are rarely in all obese people^{3,4}, there is an obesity phenotype of obese subjects called metabolically healthy obese (MHO), in contrast to the other obese phenotype, IR individuals, this group of patients shows a favourable metabolic profile, defined by high levels of insulin sensitivity, low prevalence of hypertension, and a helpful lipid and inflammation profile^{5,6}. Nevertheless, the increase of adipose tissue in both obese conditions reflected an increase in levels of circulating free fatty acids (FFA). The levels of plasma free fatty acids are released by adipose tissue, and they reflect food intake: oleic acid (OLA, 18:1) and palmitic acid (PAM, 16:0) are the most abundant in diet and plasma⁷.

According to these, cells models have been widely used to study metabolic health obese (MHO) and insulin-resistant (IR) in adipocytes phenotype called, metabolically unhealthy obese (MUHO), to simulate both physiological states. Several agents might be used to mimic obese conditions: TNF α ⁸, IL-1⁹, high insulin and high glucose¹⁰, hypoxia¹¹, among others^{12,13}.

Free unsaturated fatty acid like oleic acid (OLA) and linoleate as well as saturated fatty acids such as myristate, palmitic acid (PAM) and stearate, play the central role as obese adipocyte models due to their features associated with their chemical structure¹⁴. The saturated fatty acids are linked to IR inducing a cellular stress response related to the endoplasmic reticulum, and a chronic inflammatory state by the altered pattern of adipokines in the adipose tissue¹⁵. However, monounsaturated fatty acids might be involved as a

protective mechanism for IR, consequently of their chemical structure by being capable of altering the fluidity of the cell membrane, modulate the regulation of transcription factors and the expression of some genes related to energetic metabolism¹⁶. In this context, this work aims to visualise the response of cultured adipocytes to palmitic acid and oleic acid treatment, how these fatty acids also respond to insulin and try to simulate obese phenotypes.

Material and Methods

Cell culture

3T3-L1 [obtained from the American Type Culture Collection (Manassas, VA)] were differentiated into adipocytes, as previously described¹⁷. Briefly we seeded at 2×10^5 cell/cm² and growth in adipocyte medium [DMEM/F-12 [1:1] (Invitrogen), 17.5 mmol/l glucose, 4 mmol/l L-glutamine, 1% antibiotic-antimycotic (v/v), 1.5 g/l sodium bicarbonate] supplemented with 10% of fetal bovine serum (FBS, Gibco). After 4 days, the medium was changed to adipocyte medium supplemented with 3% FBS, 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX, Sigma), 0.1 mmol/l dexamethasone (Sigma), and 10 μ g/ml insulin (Sigma). After 3-days induction period, cells were fed every two days with the same medium, but without IBMX for the remaining eight days of adipocyte differentiation¹⁷. Media was exchanged for DMEM without insulin until day ten when all the experiments were carried out.

In vitro Models induction

Differentiated adipocytes were serum-starved for two hours and then treated with OLA (Sigma) (250 μ mol/l, and 500 μ mol/l) as previously described¹⁸, or PAM (Sigma) (250 μ mol/l, and 500 μ mol/l)^{19,20} with some protocol modifications, complexed to BSA at a 2:1 ratio for 18 hours. One sample per experiment was used to

obtain control responses in the presence of the solvent. After model induction with fatty acids, the cell models and controls were treated with insulin 100 nmol/l for 5 minutes to evaluate insulin response.

Oil Red O staining

Cells treated with fatty acids (OLA or PAM) were stained with Oil Red O to measure the diameter by optical microscopy. Briefly, cells were fixed in paraformaldehyde 4% (m/v) at room temperature in Phosphate Buffered Saline [PBS 0.01 mmol/l, pH 7.4] for 15 minutes, washed with isopropanol (Sigma) 60% (v/v), and stained with Oil Red O (Sigma) for 30 minutes. Cells were visualised by Optic Microscopy Leica DM500 (Leica).

MTT assay

Cell viability was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay. After model induction with OLA or PAM in 3T3-L1 adipocytes, cell models and controls were incubated with 0.5 g/L of MTT solution by 5 hours at 37°C and washed out with PBS and Dimethyl sulfoxide (DMSO, Sigma). Absorbance was recorded at 570 nm.

Lipolysis (Lipid Breakdown) and Lipogenesis (Lipid Synthesis)

Cellular lipolysis and lipogenesis were measured using enzymatic methods. Specifically, for lipogenesis, cells were homogenised in lysis RIPA buffer (0.1% SDS, 1% Triton-X-100, 5 mM EDTA, 1 mM Tris HCl, 150 mM NaCl and 1% deoxycholate; pH 7.4), and intracellular triglycerides were quantified by Triglycerides Liquid Stable Reagent kit (Sigma). Triglyceride levels were normalised to total protein content.

Lipolysis was also quantified in 3T3-L1 adipocyte cells models induction. Extracellular glycerol was measured by using a Free Glycerol Reagent kit (Sigma). In this case, intracellular protein content was used for normalisation. Intra- and inter-assay coefficients of variation were 3.3 and 4.2%, respectively. All assays were carried out in triplicate.

Immunoblotting

Cells were disrupted and homogenised with Triton buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton, and complete protease inhibitor) and incubated in the presence of 30 units of DNase I (Sigma) for 15 min

on ice. Protein content was quantified by the Bradford method using bovine serum albumin (Sigma) as standard as described before¹⁷. Equal amounts of protein (30 µg) were separated by 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, transferred to nitrocellulose membranes (Bio-Rad), and blocked in Tris-buffer saline with 0.05% Tween-20 (TBS-T) containing 5% dried milk for 1 h at room temperature¹⁷. Subsequently, blots were incubated overnight at 4°C with primary antibodies against Protein kinase B (Akt, Cell signalling), phospho (Thr308)-Akt (pAkt, Cell signalling), c-Jun N-terminal kinases (JNK, RD Systems), phospho (Thr183 and Thr185)-JNK (pJNK, Santa Cruz), Insulin receptor substrate 1 (IRS-1, Santa Cruz), phospho (Tyr 612) IRS-1 (pIR, Cell signalling), C/EBP homologous protein (CHOP, Santa Cruz), glutathione synthetase (GSS, Abcam), and β-actin (Sigma). Primary antibody was removed; subsequently, the membrane was incubated with secondary antibody. The antigen-antibody complexes were visualised using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG antibodies (Abcam), the enhanced chemiluminescence kit (ECL-Pls, Ge Healthcare), and autoradiography films (Sigma)¹⁷. Band intensity determined by densitometric analysis with the Gel DocTM gel documentation system-Quantity One 4.5.0 software (Bio-Rad) and normalised with β-actin density values.

Glucose Concentration by Glucose Oxidase Assay

The glucose oxidase assay was performed by using the Glucose Assay Kit (Sigma). The medium from 3T3-L1 adipocytes with or without model induction was removed, and its glucose concentration was determined as described before¹⁸. The absorbance was measured at 540 nm using a UV spectrophotometer. All assays were carried out in triplicate.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical differences between mean values were analysed one-way ANOVA with a multiple post-hoc-Newman Keuls. Comparison of two variants was made with the student's t-test. P-value <0.05 was considered statistically significant. At least three repetitions of each experiment were used. The statistical analyses were performed using the SPSS/Windows version 15.0 software (SPSS Inc. Chicago, IL, USA).

Results

Exposure to unsaturated and saturated fatty acids has been associated with a protective effect or insulin-resistant development. Therefore, we evaluated OLA and PAM model induction on mature adipocytes and their impact on adipocyte size, fatty acids mobilisation, insulin pathways responsiveness, and glucose uptake.

OLA and PAM treatments effect on viability and cell diameter of mature adipocytes.

To evaluate the effect on cell viability of adipocytes exposed to OLA and PAM as exhibit the **Figure 1a** and **1b**, respectively, we perform the MTT assay based on the activity of mitochondrial dehydrogenases. Specifically, the 250 μ M OLA and PAM concentration resulted in the maximum cell growth with 107.9% and 97.7% of viability, respectively, after applying stimuli for 18 hours. When the doses were increased to 500 μ M of

OLA or PAM stimuli, the viability of the cell decreased slightly. However, viability reductions respect to the control were not representative at both concentrations after OLA, and PAM treatment. In the present study, both concentrations 250 μ M and 500 μ M of OLA, or PAM have high viability, as exhibit the **Figure 1**. Therefore, adipocytes exhibited optimal viability tolerances regardless of the kinds of fatty acids used for treatment. Moreover, to characterise adipocytes hypertrophic, cell diameter was measured in adipocytes exposed to OLA, or PAM as show the **Figure 1c** and **1d**, respectively, which increased progressively with the concentration administered. However, the cell diameter was higher in those that were treated with 500 μ M OLA compared to OLA 250 μ M, control cells, and those treated with both PAM concentrations. In line with these results, the microscopy representative cells stained with Oil Red O after OLA as exhibit the **Figure 1e**, or PAM in the **Figure 1f**, which confirm the presence of hypertrophic adipocytes after OLA and PAM stimuli.

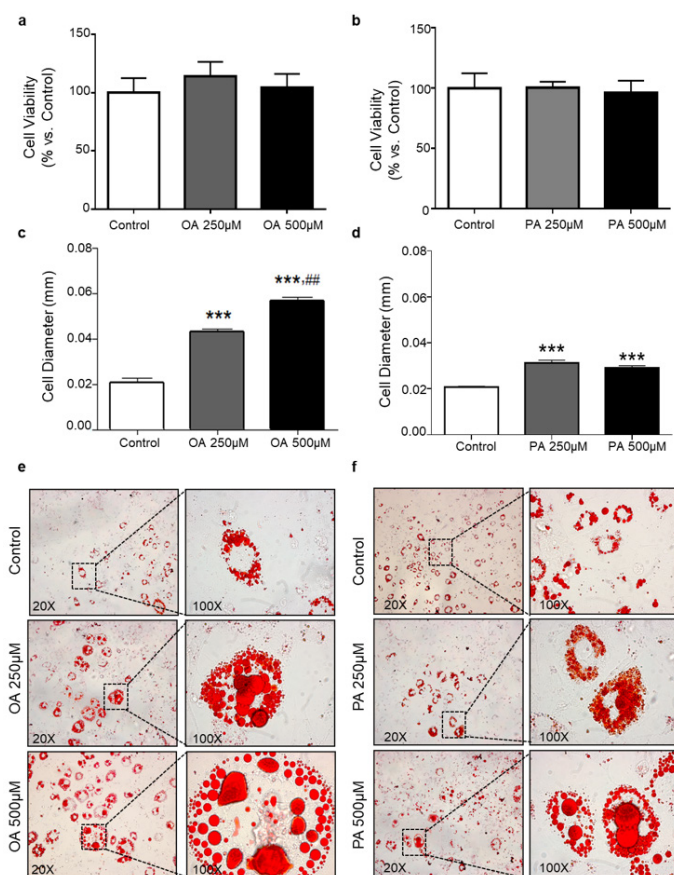


Figure 1. Effect of Oleate and Palmitate treatment on 3T3-L1 adipocytes. Cell viability graphs after (a) OLA, or (b) PAM 250 μ M, and 500 μ M treatment on adipocytes. Cell diameter graphs after (c) OLA, or (d) PAM at different concentrations. Cells morphology with oil red O staining after (e) OLA, or (f) PAM treatment. Data are represented as % cell viability vs control (100%). Bar graphs represent control cells (white), 250 μ M (grey) and 500 μ M (black). Values are the mean \pm SEM. Differences between groups were analysed by one-way ANOVA followed by Scheffe test; ***P<0.001 vs Control; ## p<0.01 vs 250 μ M OLA concentration.

OLA and PAM treatments effect on adipocytes fatty acids mobilisation

To determine OLA or PAM effect on adipocytes fatty acids mobilisation, triglycerides amount in cell lysates and the glycerol content after OLA as demonstrate the **Figure 2a** and **2b**, respectively, or PAM as exhibit

the **Figure 2c** and **2d**, respectively were measured. Both triglycerides accumulation and glycerol content were significantly increased by treatment with OLA, or PAM (500 μ M) compared to control. Furthermore, triglycerides content was higher after OLA at lower concentrations (250 μ M) respect to the control.

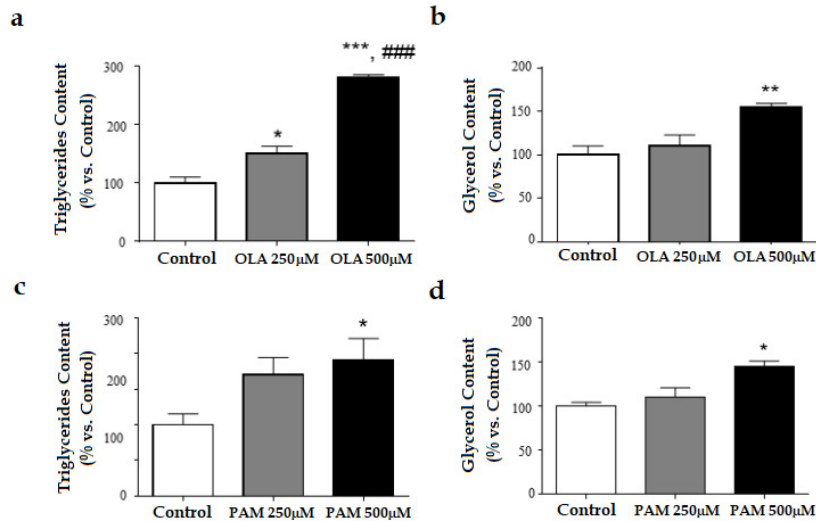


Figure 2. Fatty acids mobilisation. Intracellular triglyceride content (lipogenesis) and glycerol release (Lipolysis) graphs after OLA (a, b respectively), and PAM (c, d respectively) treatment. Data are presented as % cell viability vs control (100%). Bar graphs represent control cells (white), 250 μ M (grey), and 500 μ M (black) OLA, or PAM concentrations. Values are the mean \pm SEM. Differences between groups were analysed by one-way ANOVA followed by Scheffe test; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ vs Control; ### $p < 0.001$ vs 250 μ M OLA concentration.

Insulin pathways responsiveness of mature adipocytes to Oleate and Palmitate stimuli

To assess insulin sensitivity of hypertrophic models, we performed AKT activity measuring pAKT/AKT ratio of mature adipocytes exposed to OLA or PAM as exhibit the **Figure 3a** and **3b** at different concentrations (250 μ M and 500 μ M), and insulin stimuli (100 nM). OLA at both concentrations do not had any significant ($p > 0.05$) differences respect to control cells; however, PAM treatment showed a considerable pAkt/Akt ratio reduction respect to the control ($p < 0.05$). Considering the changes obtained with OLA, or PAM at 500 μ M concentration, the subsequent assays were performed at this dose.

Unexpectedly, in OLA-induced hypertrophic adipocytes, the downstream insulin signalling pathway, tyrosine IRS-1 (IRs), tyrosine phosphorylation IRS-1 (pIRs), oxidative (glutathione synthetase (GSH)), and reticulum (transcription factor C/EBP homologous protein (CHOP)) stress markers were not different from control cells, in contrast with the pro-inflammatory

protein kinase Jun N-terminal (JNK) which decreased respect to the control as demonstrate the **Figure 3c**. Besides, PAM stimuli signalling pathways were modified, specifically, reduced IRs level expression to control, while pIRs, GSH, CHOP, and JNK markers were increased significantly respect to control cells as exhibit the **Figure 3d**. Therefore, OLA results showed that there was no significant reduction in signalling cascades as demonstrate the **Figure 3c**, except for a downstream in the proinflammatory response. Instead, PAM hypertrophic adipocytes were insulin resistant with alteration of upstream signalling cascades.

Oleate and Palmitate treatment effect on glucose uptake of adipocyte

To assess insulin sensitivity of hypertrophic adipocyte models, we performed insulin-dependent glucose uptake assays. As shown in **Figure 4**, insulin-dependent glucose uptake was attenuated in OLA hypertrophic adipocytes. Nevertheless, PAM induces only minor impairment of insulin-stimulation of glucose uptake.

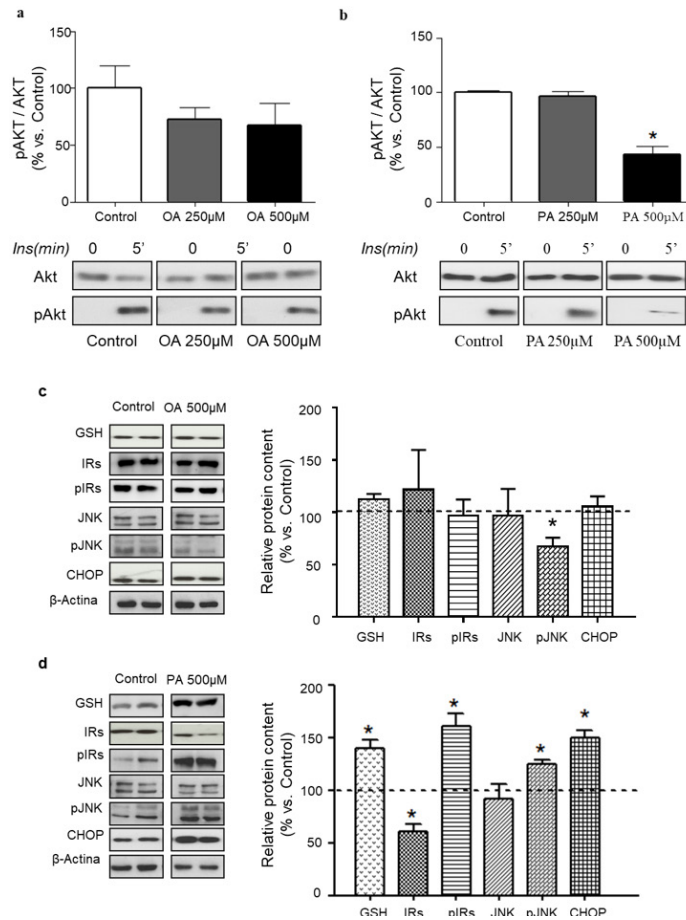


Figure 3. Effects of OLA and PAM models on 3T3-L1 adipocytes insulin signalling pathway. Insulin response (pAkt/Akt ratios) of 3T3-L1 cells exposed to (a) OLA, or (b) PAM at different concentrations (250 μ M-gray bars and 500 μ M-black bars). After treatments, control and treated cells were stimulated with insulin (100 nM) during 5 min. Cell lysates were analysed for Akt and pAkt content. Representative immunoblots of GSH, IRs, pIRs, JNK, pJNK (left panel), and quantification of protein levels (right panel) in 3T3-L1 adipocytes cultured for 18 h in the absence (Control) or presence of (c) OLA, or (d) PAM. Data were normalised to β -Actina. Data are expressed as a percentage of the values respect to the control (100%). Values are the mean \pm SEM. Differences between groups were analysed by one-way ANOVA followed by Scheffe test; * $P < 0.05$.

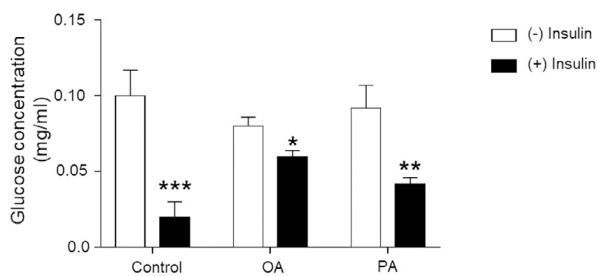


Figure 4. Glucose uptake of adipocytes exposed to OLA, or PAM. The effect of 500 μ M OLA, or PAM induction cells model on glucose oxidase assay. Bar graphs show glucose levels in OLA, or PAM/insulin presence (+), or absence (-) of six observations, expressed as glucose concentration (mg/ml). Values are the mean \pm SEM. Differences between groups were analysed by one-way ANOVA followed by Scheffe test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs Control.

Discussion

The IR, defined as a deficiency in the ability of this hormone to direct glucose into the cells and to direct its metabolism, is a metabolic state that precedes and predisposes to the development of type II diabetes. This condition eventually ends in hyperglycaemia when the pancreatic beta cells do not secrete enough insulin to cover the growing metabolic needs of this hormone. The increase of adipose tissue in obese patients is reflected in an increase in levels of circulating FFA, proinflammatory cytokines and other factors associated with IR. The plasma levels of acyl-glycerides released by the adipose tissue are a fair reflection of their dietary intake: OLA and PAM are the most abundant in the diet and plasma. This work aims to study the response of cultured adipocytes to treatment with OLA and PAM, and how these fatty acids affect the insulin response.

Herein, we demonstrate that OLA and PAM treatment stimulates in adipocytes both, hypertrophy as well as triglycerides content. Previous studies have shown the ability of adipocytes to produce growth factors and cytokines with the capacity to induce the proliferation and adipocytes hypertrophy. However, the adipocytes treatment with OLA and PAM seems to alter the pattern of cytokines, causing changes in the cell effect of the high content of OLA and PAM^{17,21,22}, mainly, as a response to the metabolic changes that cause the lipid overload in the adipocytes after both treatments.

Together with the increase in cell proliferation and hypertrophy, it became evident how the treatment with PAM and OLA encouraged the synthesis of neutral lipids. Our work shows how OLA stimulates to a greater extent, the deposit of triglycerides in the adipocyte compared to PAM. Previous studies performed skeletal muscle has allowed showing that the excess of unsaturated fatty acids promote mainly the storage of triglycerides, while saturated fatty acids tend to be deposited in the form of diacylglycerides and ceramides^{23,24}. It has also been shown that the cup of lipolysis decreases in these cells treated with PAM compared with those treated with OLA, suggesting an adaptive modification of the lipogenic profile on fat cells. All these studies carried out in different types of cells could explain our findings in mature adipocytes.

Additionally, our assays demonstrate for the first time that adipocytes treated with PAM increase the pIRS and pJNK levels compared to OLA treated cells. This suggests that PAM treatment induces in adipocytes mechanisms that could lead to IR, in this context, previous studies have shown how the increase of diacylglycerides in muscle cells, treated with PAM, activate the isoform of protein kinase C (PKC θ), which can phosphorylate JNK, activating a signalling cascade that induces the expression of proinflammatory genes²⁵. Furthermore, our results showed an increase in pJNK, greater levels in cells treated with PAM compared to those treated with OLA, a finding suggestive of a similar mechanism. The pJNK increase can lead to the activation of subunit B of the kinase I κ B (IKK β) that would induce the phosphorylation of serine residues in the insulin response substrate IRS1, explaining the increase in pIRS, found in our work and the ability to induce IR in adipocytes treated with PAM compared to those treated with OLA²³.

On the other hand, an interesting finding is the increase of glutathione synthetase (GSH) levels in PAM treated cells, which could show an increase of oxidative stress in those cells. The rise of FFA and ceramides

reduces the oxidative capacity of the mitochondria by increasing free FFA levels and triggering incomplete oxidation of fatty acids, producing reactive oxygen species linked to mitochondrial stress that could lead to cell damage and IR^{26,27}.

The OLA treated adipocytes showed lower CHOP levels than those treated with PAM. It is important to remember that PAM treatment leads to an increase in ceramides that could be responsible for endoplasmic reticulum stress, which is evident with the rise of CHOP protein in cells treated with PAM. A mechanism that could explain this could be the activation of several pro-inflammatory pathways such as Jun N-terminal kinase (JNK), as demonstrate our work. However, routes such as NF- κ B or similar receptors NOD containing residues of purine (NLRP3) could also be involved²⁸. All these variations are evident in our work when we compare levels of proinflammatory molecular markers in cells exposed to OLA and PAM. We can see how the PAM substantially modifies the expression of these markers which results in a reduced response to insulin and an increase of pro-inflammatory markers, which is like to adipocyte MUHO phenotype^{5,6,19,29}, this effect is not evident when cells were treated with OLA which is similar at least in part to the MHO adipocytes phenotype^{5,6,19,29}.

Interestingly, the adipocytes treatment with PAM increases the entry of glucose into the cell compared to the control. That result suggests that the mechanism of admission must be independent of the action of insulin if we consider the previous findings of this work. A possible mechanism could be through the epidermal growth factor (EGF) a cytokine produced by adipocytes that induce glucose transport. Despite several findings suggesting OLA with less capacity make insulin resistance than PAM, our results showed that the cells treated with PAM had better glucose transport capacity than those treated with OLA, under our conditions, which could corroborate that an insulin-independent mechanism could be activated that enhances glucose entry in cells treated with PAM^{20,30}.

Conclusions

In conclusion, our work suggest that PAM treatment induces insulin resistance, mitochondrial stress and reticulum stress on fat cells which is like MUHO adipocytes phenotype, compared to those treated with OLA, which seems similar, at least in part to MHO adipocyte phenotype, however, treatment with PAM may induces glucose transport in adipocytes by a different route from insulin-dependent.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. The ethics committee from the University Industrial de Santander approved this study.

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