UNIVERSIDAD AUTÓNOMA DE GUERRERO
FACULTAD DE CIENCIAS QUÍMICO-BIOLÓGICAS
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA
MAESTRÍA EN CIENCIAS BIOMÉDICAS

Efecto de la metformina sobre marcadores bioquímicos-moleculares y secreción de exosomas en células HUH7 con resistencia a insulina

T E S I S

QUE PARA OBTENER EL TÍTULO DE:

MAESTRO EN CIENCIAS BIOMÉDICAS

Presenta

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Chilpancingo de los Bravo, Gro., Enero, 2020

UNIVERSIDAD AUTÓNOMA DE GUERRERO
FACULTAD DE CIENCIAS QUÍMICO BIOLÓGICAS
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Este trabajo se realizó en el Laboratorio de Investigación en Epidemiología Clínica y Molecular de la Facultad de Ciencias Químico-Biológicas de la Universidad Autónoma de Guerrero en Chilpancingo, Guerrero, México.

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Durante el período en que curso la Maestría en Ciencias Biomédicas, el Q.F.B. José Manuel Villalva Pérez recibió beca para estudios de maestría del CONACYT con número 627608 y CVU 857635.

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Characterization of Huh7 cells after the induction of insulin resistance and post-treatment with metformin

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Abstract

Liver-specific insulin resistance is associated with the development of the main challenges in metabolism resulting in dyslipidemia, hyperinsulinemia and hyperglycemia. *In vitro* models developed for researching the hepatic insulin resistance are limited and employed cell lines without similar characteristics to primary human hepatocytes. Huh7 cell line has been established as a model with similar characteristics to primary human hepatocytes. In addition, it has been identified in the Huh7 cell line that infection with the hepatitis C virus induces insulin resistance. Therefore, we analyzed the induction of insulin resistance (IR) in the Huh7 cell line using an overdosage of insulin and treatment with metformin for its reversal, with the purpose of establishing an insulin resistance model useful for metabolic and pharmacological studies. Insulin-resistant Huh7 (Huh7-IR) showed a reduction in glycogen levels, glucose uptake stimulated by insulin or tyrosine phosphorylation from β-fraction of insulin receptor post insulin stimulation; with an increase of lipid intracellular content, these biomarkers are frequently observed in insulin resistant hepatic cells. Moreover, the treatment of Huh7-IR with 0.5, 1 or 2 mM of metformin by24 h decreased the biomarkers associated with an insulin resistance state. These results suggest that Huh7-IR could be used as an *in vitro* system to research the hepatic insulin resistance in metabolic and pharmacological studies.

Keywords Insulin resistance; Huh7 cells; metformin; insulin resistance biomarkers

Introduction

Insulin resistance is considered as the incapacity of tissues to respond to the biological action of insulin; this condition is associated with the development of several chronic diseases, such as diabetes mellitus type 2, cancer, hypertension, stroke, and coronary heart disease (Facchini et al. 2001). Patients diagnosed with insulin resistance frequently show metabolic disorders, such as hyperinsulinemia, hyperglycemia, and fatty liver; using animal models focused on the research of tissue specific insulin resistance, it has been shown that only the hepatic insulin resistance can result in similar conditions observed in the patients (Michael et al. 2000). The hepatogenic insulin resistance approach suggests that the development of insulin resistance in muscle or adipose tissue is the consequence of hepatic insulin resistance (Brown and Goldstein 2008). Epidemiological studies have confirmed the pertinence of the proposed approach (Kumashiro et al. 2011). For this reason, protocols focused on the development of specific insulin resistance models are necessary.

Primary human hepatocytes are the gold standard for *in vitro* hepatic research. However, the use of this model is limited due to the difficulty of maintaining and lowering proliferative capacity. Consequently, cancer cell lines derived from liver have been widely used for research in xenobiotic metabolism and hepatic metabolic diseases; nevertheless, this cell frequently shows a reduction in the expression of CYP450s in comparison with primary human hepatocytes (Bale et al. 2016). Few hepatic cell lines present a similar expression in CYP450s compared with primary human hepatocytes. For example, HepaRG shows a similar expression, but its actual use is limited. Huh7 is another hepatocyte-derived cellular carcinoma cell line that shows a similar expression

in CYP450s (Choi et al. 2009; Guo et al. 2011). Moreover, in vitro hepatic insulin resistance models have been underdeveloped. In this regard, HepG2 is the cell line most used for researching hepatic resistance induced by short free fatty acids, high glucose or insulin exposure (Zang et al. 2004; Cordero-Herrera et al. 2014). However, HepG2 cells shows a fetal phenotype characterized by the high expression of an insulin-like growth factor-1 (IGF-1) that leads in the overactivation of molecular targets associated with the insulin pathway. Unlike HepG2 cells, Huh7 cells does not present the overexpression of IGF-1 (Gunn et al. 2017). Furthermore, insulin resistance has only been established by way of infection with the hepatitis C virus (Hsieh et al. 2012; Parvaiz et al. 2015; Liu et al. 2016). Therefore, the aim of this study was to evaluate the induction of insulin resistance in Huh7 by an overdosage of insulin and treatment with metformin for its reversal.

Materials and methods

Cell culture

The hepatocellular carcinoma Huh7 cell line was obtained from ATCC (Manassas, VA, USA). The cells were cultured at 37 °C and 5% CO_2 in Williams' Medium E (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 10% of fetal bovine serum (FBS) (ByProducts, Guadalajara, JAL, MEX), and 1% antibiotic/antimycotic (100 penicillin units, streptomycin 0.1 mg, 0.25µ amphotericin B; Invitrogen Inc., Grand Island, NY, USA). Culture medium was replaced every 48 h.

Insulin resistance induction

Before inducting the insulin resistance, the medium was removed, and the cells were washed twice using PBS (Dulbecco´s Phosphate Buffered Saline; pH 7.4, NaCl 0.138 M, KCl 0.003 M). Next, Huh7 cells were seeded at 2 x 10^5 cells/mL in Williams Medium E without FBS. Then cells were exposed to 1000 nM of human insulin (Sigma-Aldrich, St. Louis, MO, USA) by 24 h; cells derived from this experiment were called Huh7-IR. Control cells were only exposed to Williams Medium E without FBS by 24 h. Afterwards, cells were washed twice using PBS and stabilized by 24 h in supplemented Williams Medium E.

Metformin treatment

In order to investigate whether the insulin resistance could be reversed, the Huh7-IR cells were exposed at 0.5, 1 y 2 mM of metformin (CAS#: 657-24-9; purity ≥97.9%, Sigma-Aldrich, St. Louis, MO, USA) by 24 h, noting that the final metformin content was <1% in the cell cultures.

Cytotoxicity assay

Cell viability was measured using the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) considering the method provided by (Mosmann 1983). Briefly, the cells were suspended at 5 x 10^5 cells/mL. They were exposed to metformin at concentrations of 0.5, 1, or 2 mM for 24 hr. The medium was then removed and 50 µL of MTT (0.5 mg mL^{-1} in DMEM without phenol red) was added and incubated for 4 h. Finally, 150 µL of DMSO was added to each well. The absorbance

at 545 nm was measured after 30 min using a Statfax 2100 plate reader (Awareness Technology, Palm City, FL, USA). Three independent experiments were performed in triplicate for each condition.

Glucose uptake assay

Glucose uptake by insulin stimulation was performed using the Glucose Uptake Assay Kit (Abcam, Cambridge, MA, USA), according to the manufacturer's instructions. In brief, 5 x 10^5 cells were seeded in a 96-well plate. After, the treatment the cells were washed twice with PBS and incubated with Krebs-Ringer-Phosphate-Hepes buffer (20 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, pH 7.4)/2% of BSA. Next, cells were stimulated with 100 nM of insulin for 20 min, then 10 µM 2-Deoxyglucose was added to the cells for 20 min. The 2-Deoxyglucose was metabolized to 2-Deoxyglucose-6-phosphate (2-DG6P), and the accumulated 2-DG6P was oxidized to generate NADPH. The levels of NADPH are indirectly associated with glucose uptake. Changes in absorbance were determined at 412 nm using a kinetic plates reader (Molecular Devices, San Jose, CA, USA).

Glycogen content assay

The glycogen content was measured using the reactive Anthrone (Sigma-Aldrich, Toluca, MEX, MEX), as previously described by (Yan et al. 2016), with minor modifications. For this assay, 11 x 10^6 cells were used. In brief, controls; Huh7-IR, and Huh7-IR-metformin treated, were washed using PBS. The cells were recovered in KOH at 30% (w/v in distilled water) and boiled for 20 min (at 100 °C). Afterwards, the cells were centrifugated by 15 min at 12000 g. The pellet was dissolved in 1.5 mL of ethyl alcohol (purity ≥99.8%); the mixture was centrifugated at 12000 g, supernatant was discarded, and the pellet was incubated at 55 °C by 12 h. Finally, the pellet was dissolved in water and mixed with 1 mL of anthrone reactive (0.2% w/v in 99% H_2SO_4) and boiled for 20 min. The absorbance was measured at 620 nm using Nanodrop 2000c spectrophotometer. The concentration of glycogen was determined from a standard curve and normalize by protein content.

Determination of intracellular lipid content

For evaluating the levels of intracellular, the Oil Red O stain (Sigma-Aldrich, Toluca, MEX, MEX) was used following the manufacturer protocols. The control cells, Huh7-IR, and Huh7-IR-metformin treated were washed and fixed using 4% of paraformaldehyde solution for 30 min. Next, cells were stained with Oil Red O solution for 1 h, the dyed was removed, and the cells were rinsed four times. Then dye was extracted using 2-isopropanol (purity 99.9%), and the absorbance was measured at 510 nm. To normalize cell confluency, Oil Red O staining cell monolayers were stained with a crystal violet stain solution (0.5 g of crystal violet powder dissolved in 80 mL of distillated H_2O and 20 mL of absolute methanol) by 5 min. Next, excess dye was removed by washing the cells with distillated water, then the cells were dried. Crystal violet retained in the cells was extracted using 0.5 mL of 33% acetic acid per well, and the absorbance was measured at 600 nm. Data are expressed as the ratio of Oil Red O absorbance to crystal violet absorbance.

Analysis of phosphorylation of tyrosine 1885 in the β subfraction of insulin receptor

Western Blot and imagen analyses were performed considering the previous reports (Taylor and Posch 2014). In brief, after insulin stimulation, the cells from the different experiment groups were lysed with RIPA Lysis Buffer (containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% Trition X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 µg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride.) suppled with 1X halt protease phosphatase inhibitor Cock Tail (Sigma-Aldrich, St. Louis, MO, USA), cell proteins were adjusted to 40 µg, and separated by SDS-PAGE and transferred to nitrocellulose membranes. Next, membranes were incubated with the corresponding primary antibodies. We used anti-phospho tyrosine (phospho Y1185; Abcam, ab62321) specific to β-fraction of insulin receptor. The normalization of Western Blots was performed using an anti-total insulin receptor and tubulin levels. Afterwards, the incubation with primary antibodies was followed by the incubation with peroxide-conjugated anti-rabbit or anti mouse immunoglobulin. The immunoreactive bands were finally detected by chemiluminescence, and the densitometry analysis was performed in ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The statistical analyses were performed using the statistical package STATA version 15.0 (College Station, TX). All data are reported as mean ± standard error. Analysis of variance (ANOVA), and Dunnet post hoc test were performed. P-values <0.05 were considered statistically significant. The figures were prepared using the GraphPad v.7 software (La Jolla, CA, USA).

Results

Cell viability

The induction of insulin resistance by an overdosage of insulin showed a metabolic cell increase in comparison with controls cells. However, this increase was not significant. As shown in Figure 1, no significant difference between cells exposed to metformin (MF) and HUh7-IR was observed.

Glucose uptake by insulin stimulation

Insulin increases the glucose uptake in control cells by 12.7 pM in comparison with unstimulated control cells. Huh7-IR cells showed a reduction in glucose uptake (18.6 pM) induced by insulin stimulation comparing with insulin stimulated control cells. The exposure to 2 mM of MF increases the glucose uptake induced by insulin (35.2 pM) in comparison with untreated Huh7-IR (Figure 2).

Glycogen content

The glycogen content in Huh7-IR was decreased for 32 µg in comparison with control cells. In comparison, with Huh7-IR, the cell exposed to 0.5, 1 or 2 mM of MF increases the glycogen concentrations for 8 µg, 12 µg or 25 µg, respectively (Figure 3).

Intracellular lipid content

Huh7-IR significantly increased (0.28-fold) the lipid intracellular content in comparison with control cells; the exposure of Huh7-IR cells to 1 or 2 mM of MF decreased the lipid intracellular content for 0.25-fold or 0.28-fold, respectively (Figure 4).

Activation of insulin receptor by insulin stimulation

The phosphorylation of tyrosine 1885 (pY1885) in the β subfraction of insulin receptor after of insulin stimulation is considered as a molecular biomarker of sensibility to insulin. A reduction of 0.35-fold of pY1885 after insulin stimulation was observed in Huh7-IR in comparison with control cells. Treatment Huh7-IR for 24 h with all MF concentrations increases the pY1885 in Huh7-IR cells. However, those increases were not significant (Figure 5).

Discussion

Liver is an organ involved in the metabolism of lipids, proteins, and carbohydrates. Moreover, liver to perform the xenobiotics metabolism. Metabolic alterations in the liver such as insulin resistance have been linked to the development of dyslipidemia or hyperglycemia (Samuel and Shulman 2016). It is therefore necessary that the development of new models focus on researching liver-specific insulin resistance. In the present study, we investigate whether insulin overdosage can induce insulin resistance in Huh7 cells. Results derived from this study shows both that induction of insulin resistance state in Huh7 cells exposed to insulin overdosage and that metformin treatment significantly decreases this resistance state observed in the model. The data obtained could be relevant for researching liver-specific insulin resistance using a hepatic cell considered as an efficient model for studying xenobiotic metabolism.

We identify that the induction of insulin resistance in Huh7 cells by overdosage of insulin was not associated with changes in cell viability. In addition, the exposure to 0.5, 1, or 2 mM of MF by 24 h does not change the cell viability in Huh7-IR or Huh7-control cells (data not shown). The cytotoxic effect of MF on Huh7 cells has been associated with an increase in the apoptosis through the hyperactivation of the transcription factor CCAAT/enhancer-binding protein delta (CEBPD)-induced during the autophagie. However, this process was observed only when the cells were exposed to 5 mM of MF by 24 h or 48 h.

It is known that 50% of insulin released by β-pancreatic cells is taken up by the liver through the hepatic portal vein; a final event of insulin metabolic-pathway activation in the liver is the glucose uptake; reduction in glucose uptake post insulin stimulation is considered a good biomarker of insulin resistance (Satake et al. 2002). Huh7-IR showed a reduction in glucose uptake post insulin stimulation. To date, no induction of insulin resistance by overdosage of insulin in Huh7 cells has been reported. However, the infection of Huh7 by the hepatitis C virus induces insulin resistance, with subsequent reduction in glucose uptake post insulin stimulation (Hsieh et al. 2012). Reduction in glucose uptake induced by insulin stimulation suggested a negative regulation in the PI3K-Akt pathway activated by insulin (White 2003).

Hyperglycemia, hypertriglyceridemia, and hyperinsulinemia are states observed during the hepatic insulin resistance. These conditions stem from (1) the incapacity of insulin to abolish the hepatic glycogenolysis and gluconeogenesis, the (2) lipogenesis *de novo* stimulated by insulin in the liver, and (3) the reduction in the

insulin uptake by the liver (Brown and Goldstein 2008). Animal models with liver-specific insulin resistance shows a reduction of hepatic glycogen and an increase in hepatic lipid contents (Michael et al. 2000). In this regard, the results obtained from Huh7-IR showed a reduction in glycogen content with an increase in the intracellular lipid levels. The hepatic paradox insulin establishes that during an insulin resistance state, the insulin loses the capacity for decreasing the transcription of phosphoenolpyruvate kinase and glucose 6-phosphatase (enzymes implicated in the glycogenolysis and gluconeogenesis). This incapacity results in the reduction of glycogen content. In addition, insulin stimulates the synthesis *de novo* of triglycerides by increasing the activity and transcription of acetyl-coenzime A carboxylase (ACC) and fatty acid synthase (Brown and Goldstein 2008).

During insulin resistance a reduction of auto tyrosine phosphorylation in β subfraction from insulin receptor post insulin stimulation has been observed (Pessin and Saltiel 2000). Huh7-IR cells shows a reduction in the phosphorylation in tyrosine 1885 (pY1885) in the β subfraction from insulin receptor after insulin stimulation. Increases in the activity of negative insulin pathway regulators are linked with reduction in molecular targets activated by insulin. PKC (a serine/threonine kinase) can decrease the auto tyrosine phosphorylation in β subfraction from the insulin receptor and decrease the activity in the insulin receptor substrate class (IRS1 and IRS2), leadings to an insulin resistance state. PKC is activated due to the increase in the cytoplasmatic levels of diacylglycerol (Schmitz-Peiffer and Biden 2008). Results of studies derived from human liver biopsies showed a high correlation among diacylglycerol content, the activity of PKCε and hepatic insulin resistance (Kumashiro et al. 2011). Based on the previous information, we hypothesized that the increase in lipid intracellular content leads to activation of PKCε, which activity results in the reduction of both auto tyrosine phosphorylation on the insulin receptor and IRS. More studies are necessary to evaluate the main molecular or cellular targets altered in Huh7-IR.

Finally, we evaluated the reversion of the insulin resistance state observed in Huh7-IR using metformin (MF is a synthetic biaguanide). At present, MF has been one of the most popular medications used in the treatment of insulin resistance. In addition, MF is taken up into hepatocyte through organic cation transporter 1 (OCT1) (Giannarelli et al. 2003). MF is accumulated in a mitochondria matrix leading to the inhibition of complex I that results in decreases in the synthesis of ATP (by the reduction in NADH oxidation) and an increase of AMP levels. This pathway promotes the activation of 5´-adenosine monophosphate kinase (AMPK). AMPK induces inactivation of ACC by phosphorylation, thereby reducing the *de novo* lipogenesis. Furthermore, AMPK decreases the levels of lipogenic transcriptional factor SREBP-1 (sterol regulatory element binding protein 1), resulting in a decrease of activity and transcription of fatty acid synthetase (FAS), conditions that leads to the reduction of lipid content in the liver (Zhou et al. 2001).This study showed that MF induces a reduction of lipid content in Huh7-RI cells. The lipid-lowering effect of MF has been reported in insulin resistant HepG2 cells treated with 0.5, 1, or 2 mM of MF for 24 h. The author suggested that activation of AMPK results in the inactivation of ACC, resulting in a reduction of the lipid content, which leads to improvement of the insulin sensibility (Zang et al. 2004). Furthermore, HepG2 exposed for 24 h to high glucose (30mM) and high insulin (100 nM) with 2mM of MF showed a reduction in the transcription of Stearyl-coenzyme A desaturase 1 (SCD1) and FAS; these event were associated with a reduction of lipid content into

cells (Zhu et al. 2018). More studies are necessary for identifying the mechanism lowering lipid effects in Huh7-IR in response to MF.

On the other hand, we observed an increase of glucose uptake and glycogen levels in Huh7-IR exposed to MF. Similar results were found in H4IIE cells exposed to MF and insulin. The data showed that the increased glucose uptake stimulated by insulin was associated with the increase in glycogen synthesis in the MF treated cells (Purrello et al. 1988). Increase in glycogen synthesis and a reduction in the glycogenolysis are mechanism through which MF improve the glycogen levels. AMPK activated by MF can phosphorylate the CREB-regulated transcription coactivator 2 (CRTC2) that results in the inhibition of its association with CREP and in a reduction of transcription of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). Reduction of PGC-1α leads to a reduction on PEPCK and G6Pase enzymes implicated in glycogenolysis (Viollet et al. 2012).

Conclusions

In summary the data obtained in the present study, shows the induction of an insulin resistance state in Huh7 cells exposed to overdosage of insulin. The data indicates that treatment with metformin may decrease the insulin resistance state observed in the model. The novel model could be relevant for researching the liver-specific insulin resistance using a hepatic cell considered as an efficient model to study xenobiotic metabolism.

Acknowledgments This work was supported by CONACYT grants (Project with code number 283801). During the study José Manuel Villalva-Pérez and Jesús Isimar Serafín-Fabían were grant recipients of the Consejo Nacional de Ciencia y Tecnología (CONACyT) of Mexico.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ramírez-Vargas MA, Villalva-Pérez JM and Serafín-Fabian JI. The first draft of the manuscript was written by Ramírez-Vargas MA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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Figure captions

Fig. 1 Effect of overdosage of insulin and MF on Huh7 viability. Huh7 were incubated with 1000 nM of insulin with or without various concentrations of MF for 24 h, and the cell viability (%) was determined using the MTT assay. The data represents the mean and standard error of three independent experiments

Fig. 2 Glucose uptake stimulated by insulin. The glucose uptake post insulin stimulation was analyzed. Huh7-IR showed a reduction in glucose uptake. Huh7-IR-metformin treated showed an increase in the glucose uptake compared with Huh7-IR. *** $p < 0.001$, ****$p < 0.0001$

Fig. 3 Intracellular lipid content. The lipid levels were measured using oil red o stain; Huh7-IR showed an increase intracellular lipid level; a decrease in the intracellular lipid levels was observed in Huh7-IR-metformin treated. **$p < 0.01$

Fig. 4 Glycogen cell concentration. The glycogen concentration was determined in Huh7-IR cells. A significant decrease in the glycogen levels was observed in Huh7-IR cells compared with control cells. Huh-7 cells treated with metformin increased the glycogen levels in comparison with untreated Huh7-IR. *$p < 0.05$, **$p < 0.01$, *** $p<0.001$, ****$p < 0.0001$

Fig. 5 Activation of β-fraction of insulin receptor by insulin. The pY-1185 levels were determined after stimulation with 100 nM of insulin. Huh7-IR cells showed a reduction in the activation of the β-fraction of the insulin receptor (IR) in comparison with control cells. Huh7-IR treated with 2 mM of metformin showed an increase of 0.2-fold activation in the β-fraction of the insulin receptor compared with untreated Huh-7

Fig. 1

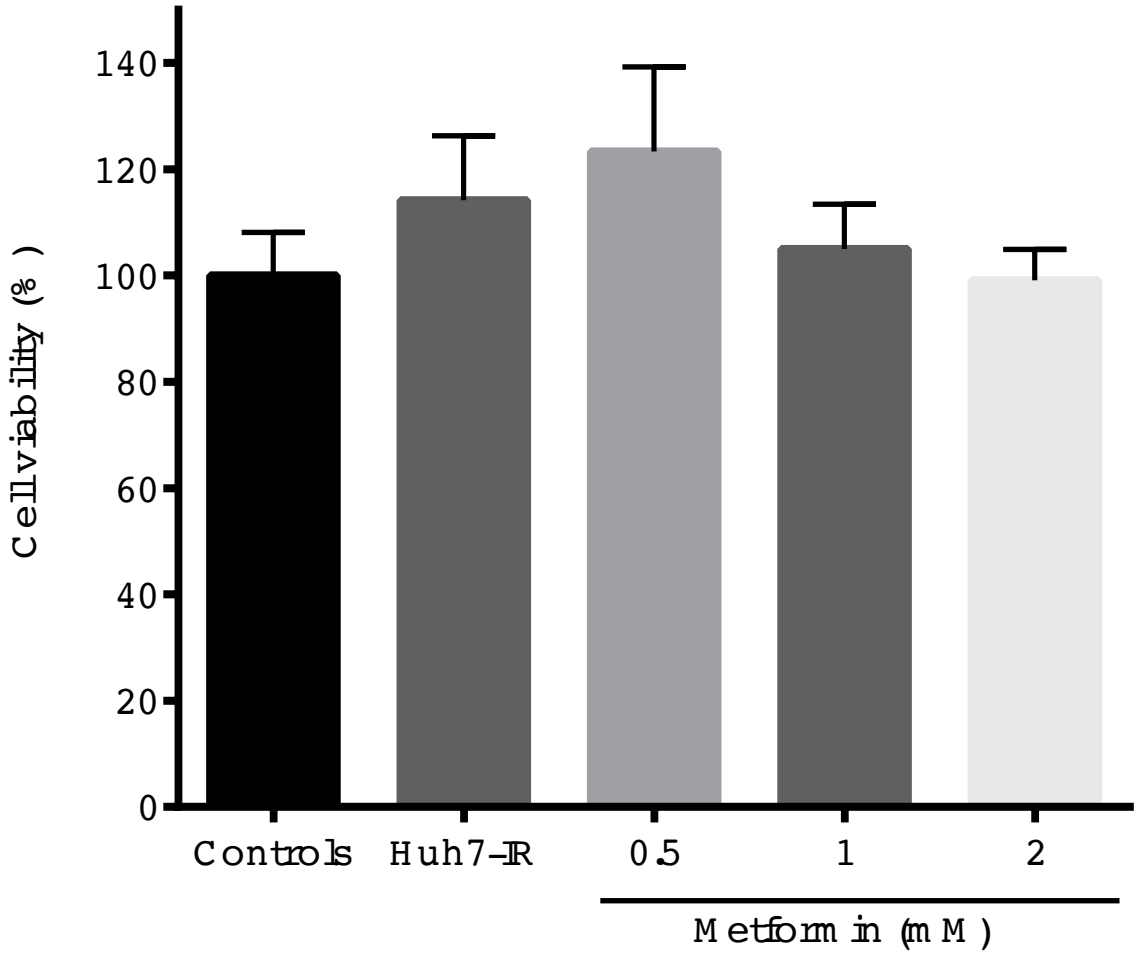

Cell viability (%)
140
120
100
80
60
40
20
0
Controls
Huh7-IR
0.5
1
2
Metformin (mM)

Fig. 2

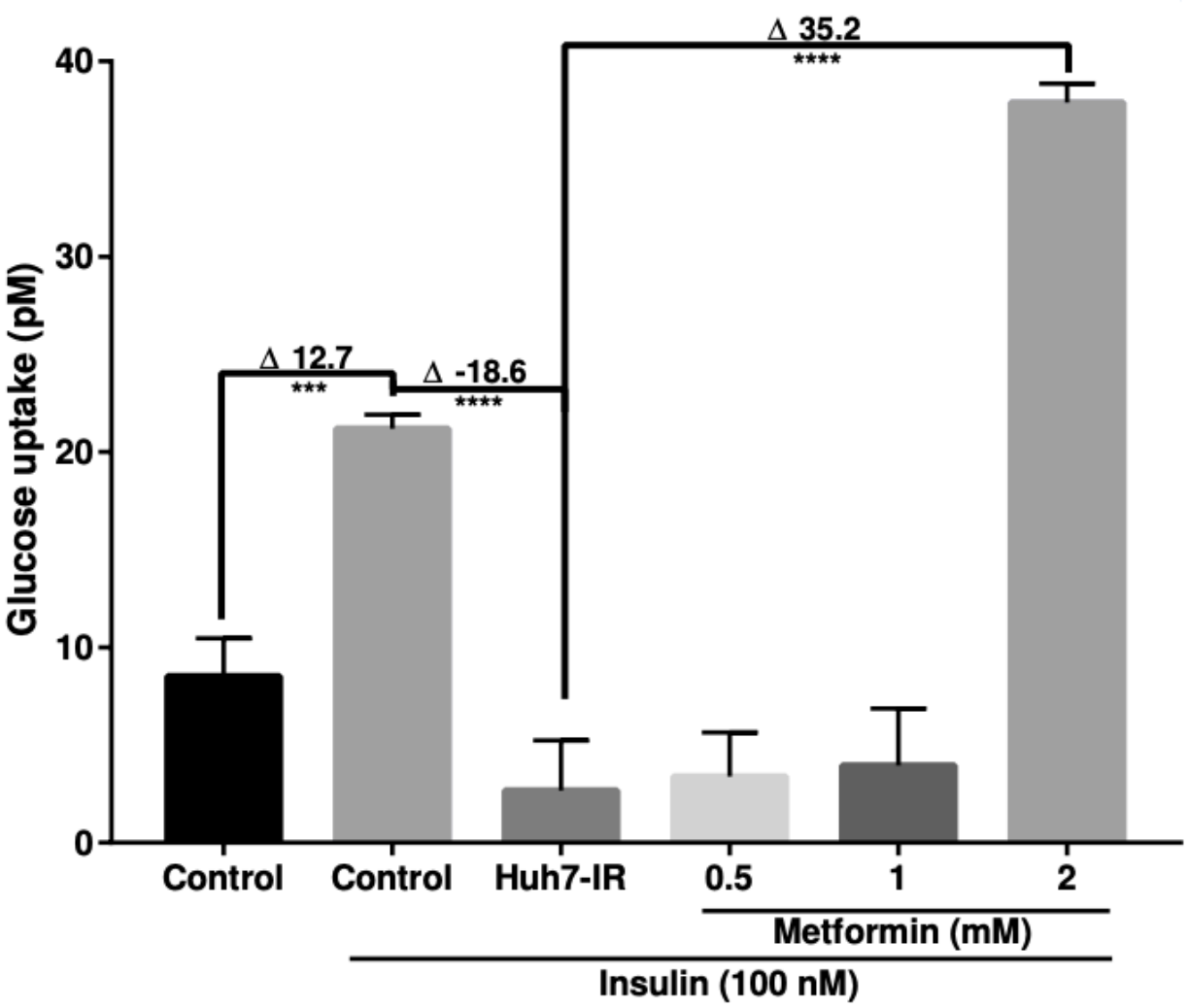

Δ 35.2
****
Δ 12.7
***
Δ -18.6
****
Glucose uptake (pM)
40
30
20
10
0
Control
Control
Huh7-IR
0.5
1
2
Metformin (mM)
Insulin (100 nM)

Fig. 3

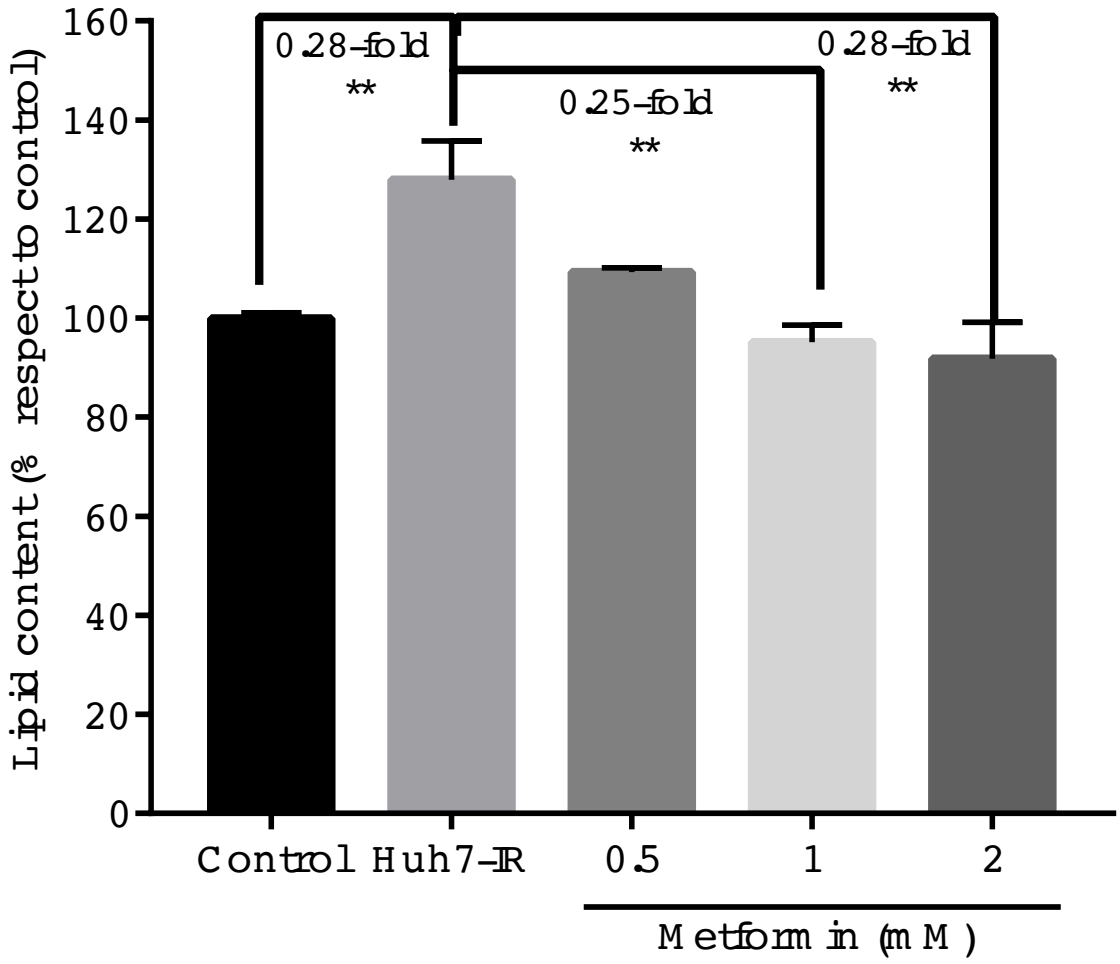

Lipid content (% respect to control)
160
140
120
100
80
60
40
20
0
0.28-fold
**
0.25-fold
**
0.28-fold
**
Control Huh7-IR
0.5
1
2
Metformin (mM)

Fig. 4

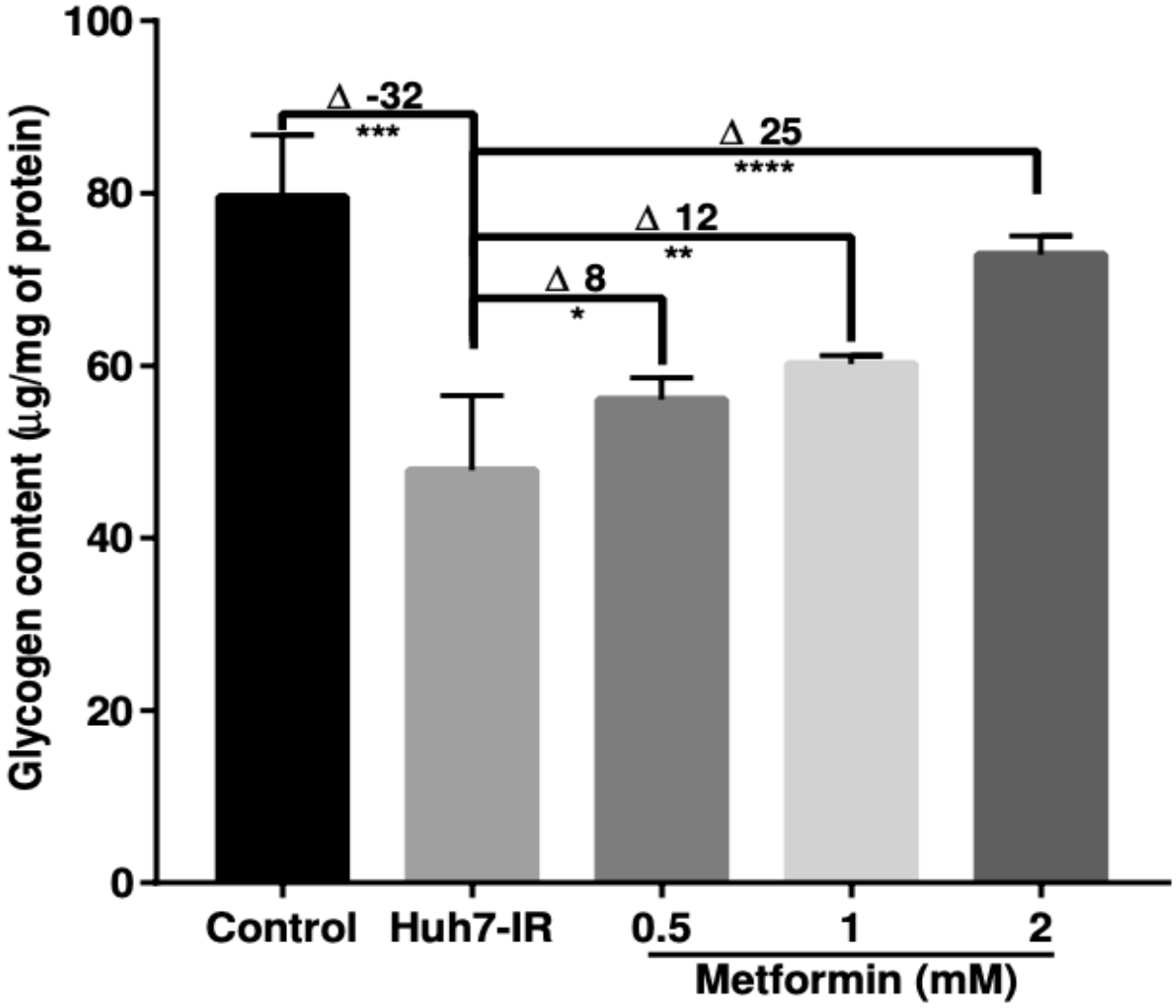
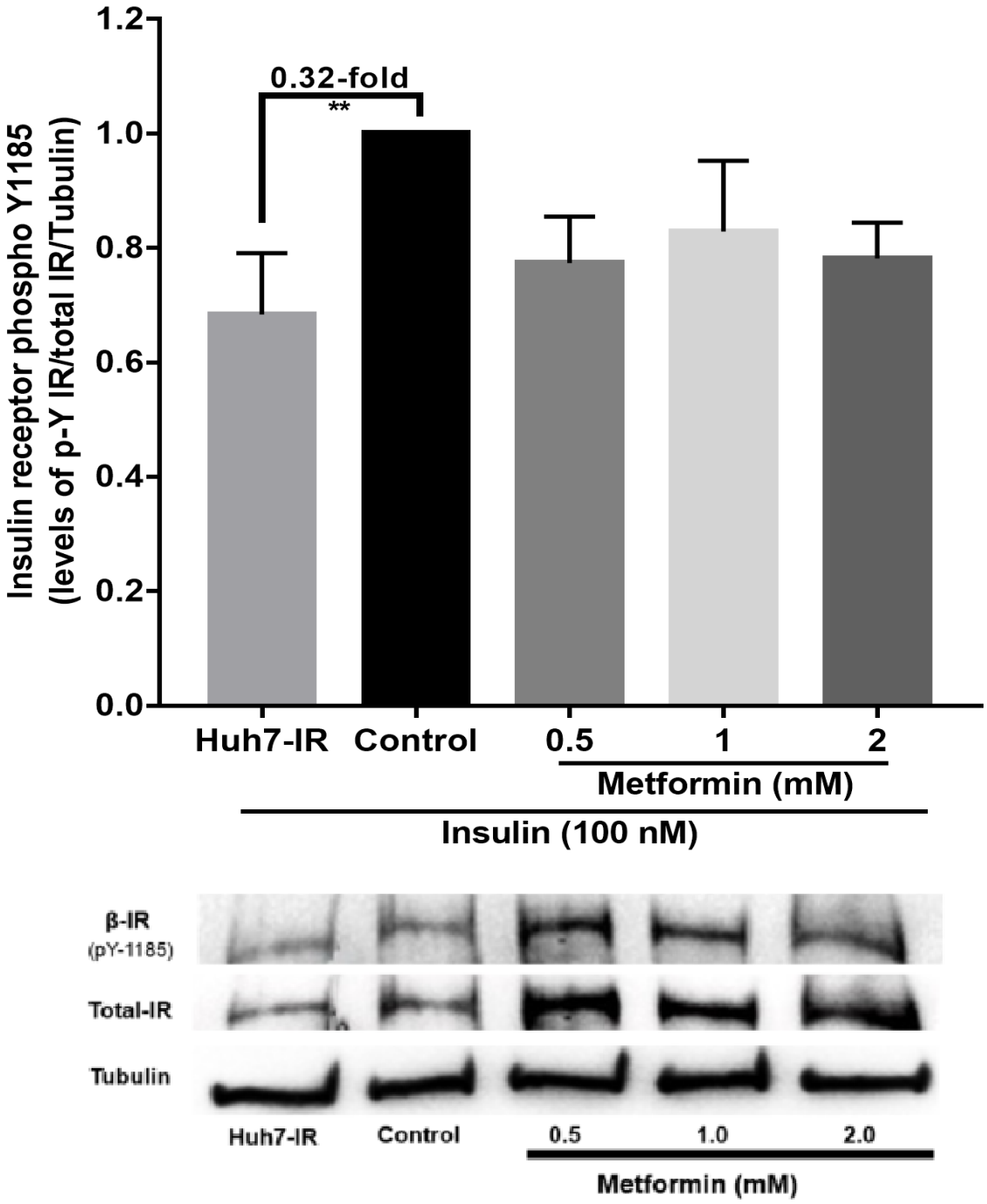

100
80
60
40
20
0
Glycogen content (µg/mg of protein)
Δ -32
***
Δ 25
****
Δ 12
**
Δ 8
*
Control
Huh7-IR
0.5
1
2
Metformin (mM)

Fig. 5


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Insulin receptor phospho Y1185
(levels of p-Y IR/total IR/Tubulin)
0.32-fold
**
Huh7-IR
Control
0.5
1
2
Metformin (mM)
Insulin (100 nM)
β-IR
(pY-1185)
Total-IR
Tubulin
Huh7-IR
Control
0.5
1.0
2.0
Metformin (mM)

ANEXO 1

Resultados complementarios

Exosomas y el efecto de la metformina en células Huh7-RI

Se identifico la presencia de exosomas mediante la detección de CD9 un marcador de exosomas, como se observa en la figura 6 las células Huh7-RI muestran una mayor producción de exosomas liberados en el sobrenadante en comparación con el grupo control; por otra parte las Huh7-RI tratadas con 2 mMol de metformina por 24 h mostraron una disminución en el marcador CD9, sin embargo estos cambios no son significativos.

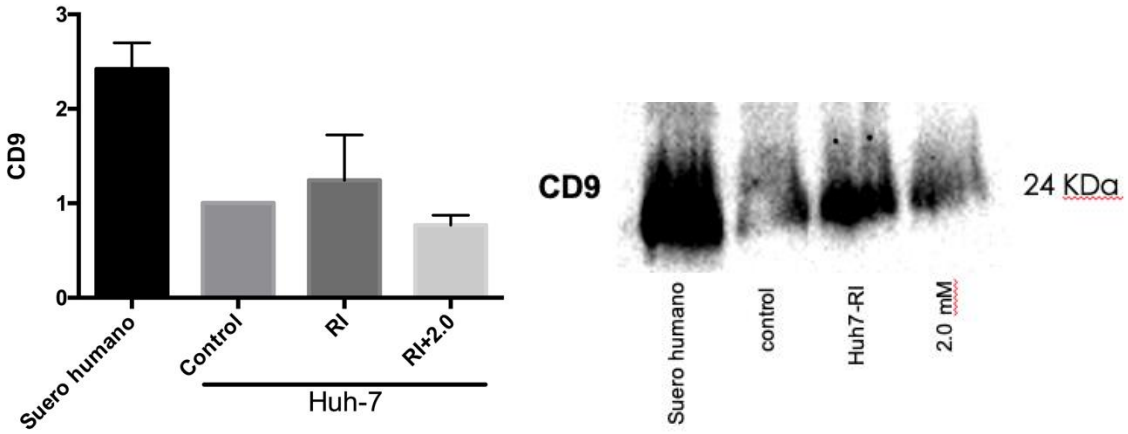


Figura 6. Detección de exosomas en células Huh7. Se detecto la presencia de exosomas utilizando CD9 como marcador. Se utilizado como marcador positivo de exosomas a suero humano. El western blot realizado se corrió en condiciones no desnaturalizantes. No se observaron diferencias estadísticamente significativas.