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New therapeutic targets for the treatment of insulin resistance based on the paracrine cross-talk between hepatocytes and Kupffer cells

Title in Spanish: *Nuevas dianas terapéuticas para el tratamiento de la resistencia a insulina basadas en la interacción entre hepatocitos y células de Kupffer*

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ABSTRACT: Chronic low-grade inflammation in adipose tissue during obesity is associated to an impairment of the insulin signaling cascade. In this study we have evaluated the impact of palmitate or oleate overload of macrophages in triggering lipoapotosis and in the cross-talk with insulin signaling in hepatocytes. Macrophages were stimulated with oleate or palmitate and levels of M1/M2 polarization markers was analyzed. Whereas proinflammatory cytokines were elevated in macrophages stimulated with palmitate, enhanced M2 markers levels was detected in macrophages stimulated with oleate. When hepatocytes were pretreated with conditioned medium from macrophages loaded with palmitate (CM-P) phosphorylation of stress kinases and endoplasmic reticulum (ER) stress signaling was increased, insulin signaling was impaired and lipoapoptosis was detected. Conversely, enhanced insulin receptor (IR)-mediated signaling and reduced levels of the phosphatase protein tyrosine phosphatase 1B (PTP1B) was found in hepatocytes treated with CM from macrophages stimulated with oleate (CM-O). In conclusion, oleate and palmitate elicit an opposite cross-talk between macrophages and hepatocytes. Whereas CM-P interferes at the early steps of insulin signaling, CM-O increases insulin sensitization by decreasing PTP1B. Therefore, targeting PTP1B is a therapeutic strategy to combat hepatic insulin resistance in obesity.

RESUMEN: La inflamación crónica de bajo grado en el tejido adiposo durante la obesidad se asocia a una disminución de la señalización de la insulina. Hemos evaluado el impacto de la estimulación de los macrófagos con oleato o palmitato y el efecto sobre la lipoapoptosis y la señalización de la insulina en los hepatocitos. Los macrófagos y las células de Kupffer se estimularon con dichos ácidos grasos para analizar los niveles de marcadores de polarización M1/M2. Los macrófagos estimulados con oleato presentaron mayores niveles de marcadores antiinflamatorios, mientras que los tratados con palmitato presentaron una elevada expresión de citoquinas proinflamatorias. Tras el tratamiento de los hepatocitos con medio condicionado de macrófagos estimulados con palmitato (CM-P) detectamos un incremento en la fosforilación de las quinasas de estrés y marcadores de estrés del retículo endoplásmico (ER), junto con un aumento en la lipoapoptosis y un bloqueo en la señalización de la insulina. Por el contrario, en hepatocitos tratados con el medio condicionado de macrófagos estimulados con oleato (CM-O) observamos un aumento en la señalización de la insulina y una reducción en los niveles de la proteína fosfatasa 1B (PTP1B). En conclusión, oleato y palmitato desencadenan respuestas opuestas entre los macrófagos y los hepatocitos. Mientras que el CM-P bloquea las primeras etapas de la señalización de la insulina, el CM-O aumenta la sensibilidad a la insulina disminuyendo la PTP1B. Por tanto, la inhibición de PTP1B es una estrategia terapéutica para combatir la resistencia a la insulina en el hígado en estados de obesidad.

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1. INTRODUCTION

Evidences from clinical and epidemiological studies have clearly established that obesity is the most common cause of insulin resistance, type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). In fact, insulin resistance in peripheral tissues such as liver and skeletal muscle is an early metabolic abnormality in the development of T2DM (1). Although the precise molecular mechanisms underlying insulin resistance associated to obesity have not been completely elucidated, one major contributor is the chronic low-grade systemic inflammation state which interferes with the early steps of the insulin signaling cascade possibly through the effects of free fatty acids (FFAs) and cytokines secreted by the overgrowing white adipose tissue (WAT) (2-4).

Proinflammatory cytokines impair insulin action by activating stress kinases such as c-jun (NH2) terminal kinase (JNK), IκB kinase (IKK) and also protein kinase C

(PKC)-mediated pathways (5, 6). In addition, cytokines down-regulate early key mediators of the insulin signaling cascade such as the insulin receptor (IR) or insulin receptor substrate 1 (IRS1) (7, 8), as well as increase the expression of negative modulators of this pathway such as the protein tyrosine phosphatase 1B (PTP1B) (9, 10). On the other hand, circulating FFAs, which are usually increased in insulin resistant states (11-13), also activate these proinflammatory pathways boosting the defects in peripheral insulin actions (14-16).

In addition to the inflammatory effects, the activation of endoplasmic reticulum (ER) stress-mediated signaling pathways by FFAs has been linked to obesity-associated immunometabolic dysregulation and insulin resistance (17, 18). ER stress is sensed by three main proteins: X-box binding protein 1 (X-BP1), PRKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6 (ATF6), which cooperate to mitigate ER stress by reducing protein translation, stabilizing proteins by chaperones and activating ER-associated protein degradation (19). Chemical chaperones (tauroursodeoxycholic acid (TUDCA) and 4-phenylbutryate) that alleviate ER stress through protein stabilization, improve systemic glucose homeostasis, increase glucose uptake in adipose and skeletal muscle and reduce hepatic glucose production (20). Moreover, a study in obese subjects has revealed that treatment with TUDCA increases insulin sensitivity in liver and skeletal muscle by approximately 30% compared to the placebo therapy (21).

Several studies have reported that besides adipocytes, adipose tissue resident macrophages, which migrate and accumulate in WAT, have a relevant role in obesity-induced chronic inflammation (22-24) through their polarization toward the M1-like state (25). Although the importance of macrophages in the molecular mechanisms triggering insulin resistance in skeletal muscle and adipose tissue has been explored (25-27), it remains unclear whether the inflammatory milieu from hepatic macrophages impact insulin signaling in hepatocytes. On that basis, in this study we have investigated for the first time the effect of Kupffer cells activated by two distinct FFAs, palmitate (saturated) and oleate (unsaturated), on stress-mediated pathways, lipoapoptosis and the insulin signaling cascade in hepatocytes.

2. MATERIALS AND METHODS

2.1. Reagents

Fetal bovine serum (FBS) (#10270) and culture media DMEM (#41966-029) were obtained from Invitrogen (Life Technologies, Gran Island, NY). TRIzol reagent (#T9424), sodium palmitate (#P9767), sodium oleate (#O7501), bovine serum albumin (BSA) (#A6003), fatty acid free-BSA endotoxin free (#A8806) and insulin (#I0516) were from Sigma Aldrich (St. Louis, MO). Bradford reagent, acrylamide, immunobloting PVDF membrane and Immobilon Western Chemiluminescent HRP Substrate were purchased from Bio-Rad (Hercules, CA).

2.2. Free fatty acid preparation

2.5 mM free fatty acid (FFA) stock solutions were prepared by modification of Spector method (28). Briefly, cold sodium palmitate or sodium oleate were dissolved in 0.1M NaOH by heating at 70°C while 0.5 mM BSA solution was prepared by dissolving fatty acid free-BSA in NaCl 0.9% by heating at 50°C (at maximum). Once BSA and FFA solutions were completely dissolved, palmitate and oleate solutions were diluted 10-times in the BSA solution and mixed by pipetting to achieve a final molar ratio of 5:1. Control BSA was prepared by adding the same amount of 0.1M NaOH into 0.5 mM BSA solution. All preparations were filtered, aliquoted and stored at –20°C.

2.3. Culture of RAW 264.7 murine macrophages

Murine RAW 264.7 macrophage cell line, kindly provided by Dr. Tarín (CNIC, Madrid, Spain), were cultured in RPMI supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Confluent macrophages were treated with BSA or FFAs solutions (750 µM conjugated oleate/BSA or 750 µM conjugated palmitate/BSA) for 24 h to obtain the corresponding conditioned media (CM) (27). CM were centrifuged to remove dead cells and directly added (without dilution) to hepatocytes for several time periods.

2.4. Isolation and culture of Kupffer cells

Kupffer cells were obtained from livers of 3 months-old male C57/BL6 mice. All animal experimentation has been conducted according to the Guide of the care and use of laboratory animals (National Academy of Sciences, USA). For Kupffer cells (KC) isolation, the supernatant from the first centrifugation of the hepatocyte isolation protocol was collected and centrifuged twice at 50 x g for 5 min to discard the pellet with the remaining hepatocytes. The latest supernatant was centrifuged at 500 x g for 5 min at 4ºC and the pellet containing the KC was resuspended in attachment media. Cells were mixed by inversion with 50% percoll and centrifuged at 1.000 x g for 30 min without acceleration or brake at room temperature. Finally, KC pellet was washed with PBS 1X, centrifuged twice at 500 x g for 10 min at 4ºC to wash out the residual percoll solution and cells were resuspended in RPMI supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Cells were then plated on 12 well-plates and maintained for 24 h before treatments. Conditioned media was prepared as described in RAW 264.7 cells.

2.5. Hepatocyte cell culture.

Human hepatocytes were isolated by the two-step collagenase procedure from non-tumor areas of liver biopsies from patients submitted to a surgical resection for liver tumours after obtaining patients' written consent (29). Primary mouse hepatocytes were isolated from non-fasting male C57BL/6 mice (10-12-week-old) by perfusion with collagenase as described (30). Cells were seeded on 6 well-plate (Corning, New York, NY) and cultured in

media containing Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) with 10% FBS, supplemented with 2 mm glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate (attachment media) and maintained for 24 h before treatments. The generation and characterization of immortalized mouse hepatocyte cell line has been previously described (31). Cells were grown in DMEM plus 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine.

2.6. Preparation of protein extracts and Western blot

To obtain total cell lysates, attached cells were scraped off and incubated for 10 min on ice with lysis buffer (25 mM HEPES, 2.5 nM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 µg/ml leupeptin). After protein content determination with Bradford reagent, total protein were boiled in Laemmli sample buffer and submitted to 8-15% SDS-PAGE. Proteins were transferred to Immunoblot PVDF membrane and, after blocking with 3% BSA or 5% non-fat dry milk, membranes were incubated overnight with several antibodies as indicated. Immunoreactive bands were visualized using the ECL Western blotting protocol. Densitometric analysis of the bands was performed using Image J software. The anti-phospho-PERK (Thr 980) (#3179), anti-phospho-eIF2α (Ser 51) (#9721), anti-phospho-JNK (#9251), anti-phospho-STAT3 (#9131) antibodies were from Cell Signaling Technology (Danvers, MA). The anti-JNK (sc-571), anti-IR β (sc-711), anti-eIF2α (sc-11386), anti-CHOP (sc-7351), anti-phospho-p38 (sc-17852), anti-p38 (sc-9212), anti-IRS1 (sc-559), anti-phospho-IR (sc-25103), anti-phospho-Akt (Thr 308) (sc-16646), anti-phospho-Akt (Ser 473) (sc-7985), anti-Akt (sc-8312) and anti-PTP1B (sc-1718) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin (T5168) was from Sigma Aldrich (St. Louis, MO).

2.7. RNA isolation and qPCR

Total RNA was isolated using Trizol reagent and was reverse transcribed using a SuperScriptTM III First-Strand Synthesis System for qPCR following manufacturer's indications. qPCR was performed with an ABI 7900 sequence detector using the SyBr Green method and d(N)6 random hexamer with primers purchased from Invitrogen. PCR thermocycling parameters were 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each sample was run in triplicate and normalized to 18s RNA. Fold changes were determined using the ΔΔCt method. Primer sequences are available upon request.

2.8. Statistical analysis

Data are presented as mean ± SEM, and were compared by using the Bonferroni ANOVA test. All statistical analyses were performed using the IBM SPSS Statistics 21.0 (SPSS Inc. IBM, Armonk, NY) software with 2-sided tests. Differences were considered statistically significant at $p<0.05$.

3. RESULTS AND DISCUSSION

RESULTS

3.1. Insulin signaling is opposite modulated in human hepatocytes pretreated with conditioned medium from macrophages stimulated with palmitate or oleate

The liver is composed primarily of hepatocytes, but also contains blood and lymph vessels, nerves and immune cells. Since all these cell types can potentially respond to a high fat-mediated inflammatory environment *in vivo*, we performed a cell culture-based approach to investigate the specific cross-talk between macrophages and hepatocytes in the context of fatty acid overload. For this goal, RAW 264.7 macrophages were treated with palmitate, a typical saturated FFA found in western diets, for 24 h. Then, culture media (conditioned medium) was removed. Conditioned medium was hereafter called CM-P (collected from RAW 264.7 cells treated with palmitate) or CM-B (from RAW 264.7 cells treated only with BSA as a control). We also obtained conditioned medium from RAW 264.7 cells treated with oleate, a well-known nontoxic monounsaturated FFA. This conditioned medium was hereafter called CM-O (collected from RAW 264.7 cells treated with oleate). Once the conditioned media were prepared, they were added for 24 h to human hepatocytes and after that these cells were stimulated with 10 nM insulin for 10 min. As depicted in Figure 1, insulin induced IR tyrosine phosphorylation and Akt phosphorylation at both residues were enhanced in hepatocytes preincubated with CM-O as compared to control hepatocytes preincubated with control CM-B. Conversely, IR tyrosine and Akt serine/threonine phosphorylations were reduced in presence of CM-P. Moreover, protein levels of IR and IRS1 decreased only in hepatocytes treated with CM-P. Altogether, these results indicate opposite effects of palmitate and oleate in triggering a cross-talk between macrophages and hepatocytes with relevant effects in insulin signaling.

3.2 Differential effects of palmitate and oleate in levels of proinflammatory cytokines expressed by macrophages

In order to confirm the differential effects of oleate and palmitate in a physiological context, we isolated and cultured Kupffer cells from C57/BL6 mice and these resident macrophages were stimulated with oleate or palmitate for 24 h. The purity of Kupffer cells was checked by the analysis of CD68 and F4/80 mRNA levels (Figure 2).

In an attempt to characterize the molecules released by macrophages to the CM, we assessed the expression of proinflammatory cytokines characteristic of M1 macrophage polarization by real-time PCR. Treatment of Kupffer cells with palmitate, but not oleate, increased IL6, IL1β, TNFα and MCP1 mRNA levels. On the other hand, treatment with oleate significantly up-regulated mRNA levels of M2 polarization markers (IL10, Mcr1, Mgl1 and arginase 1) (Figure 3).

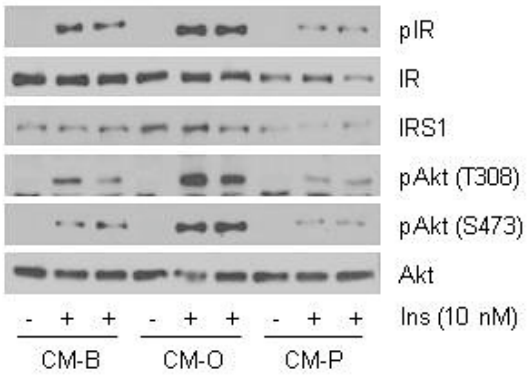

Figure 1. Insulin signaling is differentially modulated in human hepatocytes pretreated with CM-O or CM-P. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B), oleate (CM-O) or palmitate (CM-P) was added to human primary hepatocytes for 24 h. Then, cells were stimulated with 10 nM insulin for 10 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. (n=4 independent experiments performed in duplicate).

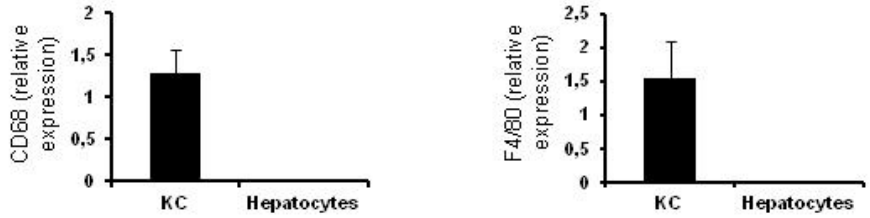

Figure 2. Purity of Kupffer cells. Kupffer cells and hepatocytes were isolated from mice as described in the Experimental Procedures and mRNA levels of CD68 and F4/80 were determined. CD68 and F4/80 were undetectable in hepatocytes (n=3 independent experiments performed in duplicate).

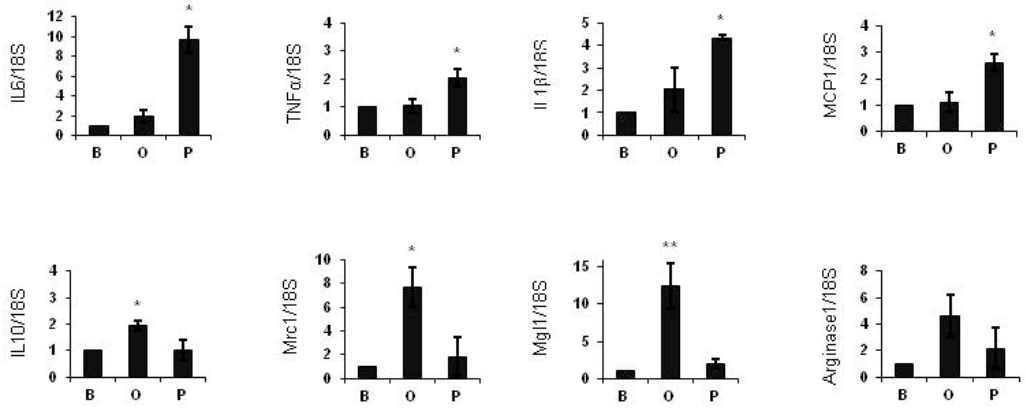

Figure 3. Expression of cytokines and markers of inflammation from Kupffer cells. Primary Kupffer cells were treated with BSA (B), oleate (O) or palmitate (P) for 24 h. TNFα, IL-6, IL-1β, MCP1, IL-10, arginase 1, Mcr1 and Mgl1 mRNA levels were analyzed by qRT-PCR. Results are expressed as fold increase relative to BSA condition (1) and are mean ± SEM (n=3), *p<0.05, **p<0.01, O or P, respectively, vs B.

3.3. Conditioned medium from Kupffer cells stimulated with palmitate activates stress-mediated signaling pathways

Next, we confirmed data on proinflammatory cytokines by analyzing the activation of stress kinases in primary mouse hepatocytes treated with CM from Kupffer macrophages stimulated with oleate or palmitate (CMK-O or CMK-P, respectively). As a control, hepatocytes were stimulated with CM from Kupffer cells loaded with BSA (CMK-B). As shown in Figure 4, phosphorylations of STAT3, p38 MAPK, JNK, PERK and eIF2α were observed exclusively in hepatocytes treated with CMK-P.

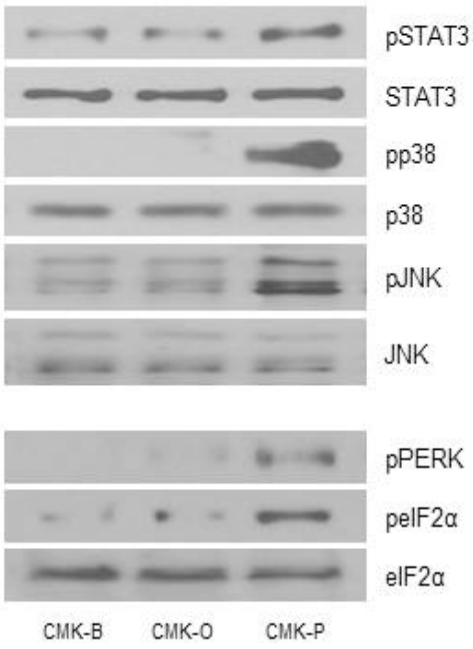

Figure 4. Paracrine effects of primary Kupffer cells stimulated with palmitate or oleate in stress pathways in primary mouse hepatocytes. Conditioned medium from primary Kupffer cells treated with BSA (CMK-B), oleate (CMK-O) or palmitate (CMK-P) was added to primary mouse hepatocytes for 30 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3 independent experiments.

In the light of these data, CHOP and the active fragment of caspase 3, indicators of apoptosis, also were detected only in hepatocytes treated with CMK-P (Figure 5).

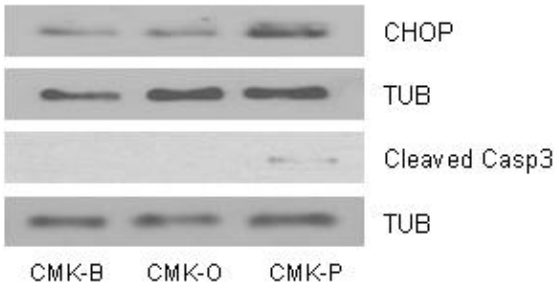

Figure 5. Paracrine effects of primary Kupffer cells stimulated with palmitate or oleate in lipoapoptotic pathways in primary mouse hepatocytes. Conditioned medium from primary Kupffer cells treated with BSA (CMK-B), oleate (CMK-O) or palmitate (CMK-P) was added to primary mouse hepatocytes for 24 h. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3 independent experiments.

3.4. CMK-P induces insulin resistance, whereas that CMK-O induces insulin hipersensitivity in primary mouse hepatocytes

Next, we analyzed the effects of Kupffer cells-derived products on insulin signaling in hepatocytes. For this goal, primary hepatocytes were treated with CMK-P or CMK-O for 24 h and subsequently stimulated with 10 nM insulin for 10 min. As depicted in Figure 6, insulin-induced tyrosine phosphorylation of the IR and Akt phosphorylation at both Ser 473 and Thr 308 residues was enhanced in hepatocytes pretreated with CMK-O whereas these responses were decreased in hepatocytes pretreated with CMK-P. These results also reflect an opposite paracrine cross-talk between hepatocytes and resident macrophages.

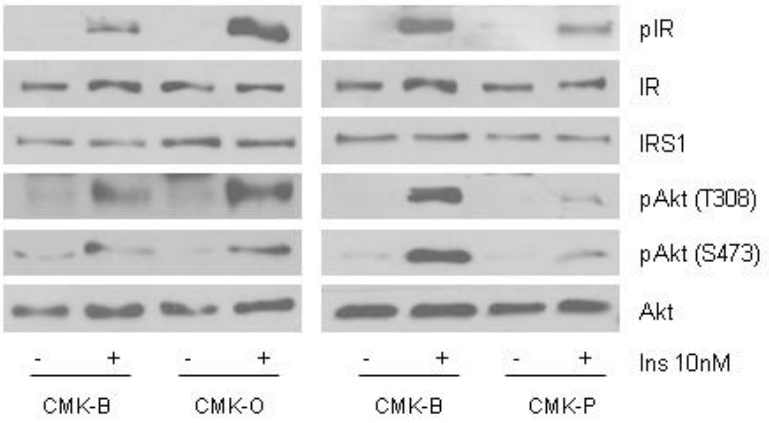

Figure 6. Paracrine effects of primary Kupffer cells stimulated with palmitate or oleate in insulin signaling in primary mouse hepatocytes. CMK was added to primary hepatocytes for 24 h. Then, cells were stimulated with 10 nM insulin for 10 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3 independent experiments.

3.5. Lower levels of PTP1B can contribute to the insulin sensitization induced by CM-O in hepatocytes.

Next, we evaluated the possibility that changes in the expression of negative modulators of the early steps of the insulin signaling could account for the insulin sensitization induced by CM-O or CMK-O in hepatocytes. Among them, PTP1B was a potential candidate given its ability to directly dephosphorylate tyrosine residues of the IR (48). Consistent with this hypothesis, we measured the expression of this phosphatase in hepatocytes incubated with CM-O. For these experiments we used immortalized neonatal hepatocytes previously generated and validated in

our laboratory (57). As depicted in Figure 7, PTP1B protein content was decreased in hepatocytes treated with CM-O.

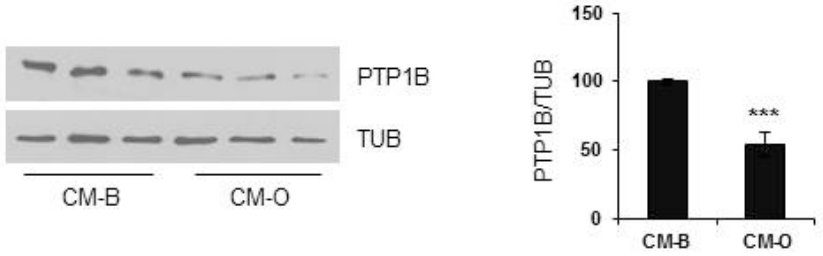

Figure 7. Lower levels of PTP1B contribute to the insulin sensitization induced by CM-O in hepatocytes. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B) or oleate (CM-O) was added to immortalized mouse hepatocytes for 24 h. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. After quantification of all blots, results are expressed as percentage of protein expression relative to CM-B condition (100%) and are mean ± SEM (n=3 independent experiments performed in duplicate), ***p<0.001 CM-O vs CM-B.

As expected, insulin signaling was enhanced in immortalized neonatal hepatocytes preincubated with CM-O whereas impaired insuling signaling was detected in cells pretreated with CM-P (Figure 8).

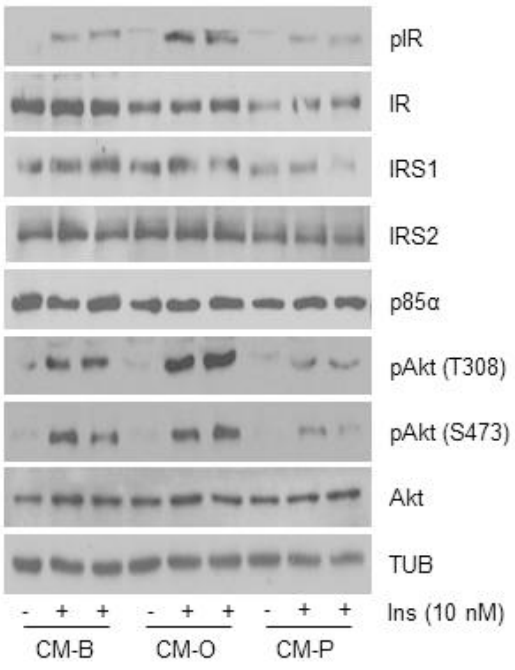

Figure 8. Insulin signaling is differentially modulated in hepatocytes pretreated with conditioned medium from RAW 264.7 macrophages stimulated with oleate or palmitate. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B), oleate (CM-O) or palmitate (CM-P) was added to immortalized mouse hepatocytes for 24 h. Then, cells were stimulated with 10 nM insulin for 10 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=4 experiments performed in duplicate).

DISCUSSION

In obesity-associated insulin resistance, M1-like macrophages polarization state has been associated with the enhancement of the proinflammatory milieu by the ability to secrete proinflammatory cytokines. This surrounding insulin resistant adipocytes that trigger proinflammatory signaling pathways in macrophages trough their binding to Toll-like receptors (TLRs) (32-34). However, we know now that beyond the interplay between adipocytes and adipose tissue resident macrophages, these inflammatory signals also dysregulate key metabolic responses in other peripheral tissues, thereby exacerbating insulin resistance (3).

The liver is a target organ of the inflammatory mediators. In obesity, the hepatic lipid accumulation (first hit) together with the proinflammatory input (second hit) trigger the necroinflammatory changes that are recognized histopahologically as steatohepatitis (NASH) (35). Of relevance, adipose tissue inflammation has been correlated with hepatic steatosis in humans (36). Furthermore, activation of Kupffer cells, the hepatic resident macrophages, to secrete proinflammatory mediators is a key event in the initiation of NAFLD, and limiting their polarization into an M1 phenotype is considered an attractive strategy against chronic liver inflammation (37-40).

In this study, we have dissected for the first time the molecular cross-talk between signals emerging from macrophage-derived products in response to fatty acid overload and insulin signaling in hepatocytes. A step further, we attempted to compare the responses of hepatocytes to macrophage-secreted cytokines derived from oleate or palmitate together with FFAs mimicking the circulating proinflammatory milieu. Interestingly, an opposite response in insulin-mediated IR tyrosine phosphorylation, the earliest event in the insulin signaling cascade, was found in both mouse and human hepatocytes

exposed to the CM from macrophages or Kuppfer cells treated with oleate or palmitate, with a significant increase or decrease, respectively, as compared to control hepatocytes (treated with CM-B). This opposite response was also evidenced in Akt phosphorylation (at both Ser 473 and Thr 308), a critical node of insulin´s metabolic actions in hepatic cells (41). Thus, these results suggested that oleate and palmitate induce different secretory responses in peripheral and liver resident macrophages and this might differentially modulate insulin signaling in liver cells. In the light of these findings, the M1 polarization state induced by palmitate, reflected by elevated TNFα, IL6, IL1β and MCP1 in agreement to Samokhvalov et al. (26), was not observed in Kupffer cells loaded with oleate. The absence of M1 polarization is critical to understand the modulation of insulin signaling by oleate in hepatocytes as will be discussed below. In fact, increased arginase 1, Mrc1, Mgl1 and IL10 levels reflects a M2 profile of macrophages after oleate challenge in agreement with recent results reported by Camell et al. on the role of dietary oleic acid in M2 macrophages polarization (42).

The ER plays a central role in the determination of cell fate under conditions of stress. Increased ER stress has been shown to contribute to the development of NAFLD (17). In this regard, CM-P-treated hepatocytes rapidly activated the PERK branch of UPR by inducing PERK, eIF2α and JNK phosphorylation, resulting in increased CHOP expression. Under these experimental conditions, STAT3 phosphorylation was also increased, this response is probably mediated by the proinflammatory cytokines IL6 and IL1β. Moreover, TNFα-mediating signaling might also boost JNK and p38 MAPK activation. The convergence of all these proinflammatory signaling cascades leads to a negative-cross talk with insulin signaling resulting in the degradation of both IR and IRS1 that agrees with results reported in hepatocytes treated with palmitate (43, 44). Therefore, lower IR and IRS1 levels detected in hepatocytes stimulated with CM-P evidence the contribution of the early activation of stress kinases in the reduced insulin-mediated Akt phosphorylation. Neither the early activation of stress kinases nor CHOP expression were detected in hepatocytes treated with CM-O, highlighting the absence of oleate-mediated proinflammatory responses in the macrophage-hepatocyte axis.

In addition to inflammation, FFAs-induced lipotoxicity contributes to the pathogenesis of NAFLD, being saturated FFAs the more toxic lipid species (45-47). Although the evaluation of the apoptotic responses under inflammatory conditions was not the major goal of this study, we detected cleavage of caspase 3 in hepatocytes treated with CM-P, suggesting that the signals derived from the macrophage M1 polarization are likely involved in lipoapoptosis. This interesting issue deserves future research.

Although PTP1B has been involved in obesity and inflammation (48-50), this is the first study showing the modulation of PTP1B protein levels by a macrophage-derived lipid product from oleate since reduced levels of LTB4 in CM-O paralleled with decreased PTP1B and enhancement of insulin-mediated IR tyrosine phosphorylation in hepatocytes. These data might be of relevance since PTP1B has emerged as a therapeutic target against obesity-mediated insulin resistance by its ability to regulate peripheral (muscle and liver) insulin sensitivity (49, 51-56) as well as the central control of appetite and energy expenditure (48, 57, 58). Based on that, our ongoing research is focused on the modulation of PTP1B expression by bioactive lipid species (eicosanoids) in hepatocytes.

In summary, we have demonstrated a paracrine cross-talk from macrophages/Kupffer cells to hepatocytes, which bears opposite differences depending on the polarization state of the macrophages and the factors secreted by these immune cells as it has been manifested upon treatment with palmitate or oleate. To our knowledge and as summarized in Figure 9, this is the first study providing data of the beneficial effects of oleate in switching macrophages polarization to increase insulin sensitization in hepatocytes through decreasing PTP1B. Therefore, targeting PTP1B is a therapeutic strategy to combat hepatic insulin resistance in obesity.

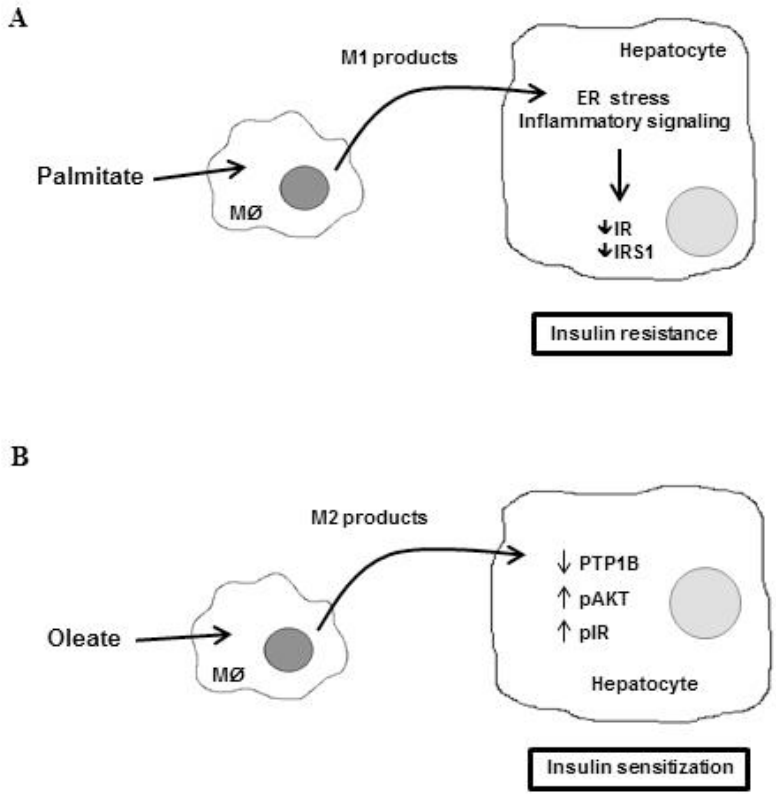

Figure 9. A. Schematic representation of palmitate-induced M1 polarization of macrophages that leads to a negative cross-talk with hepatic insulin signaling through decreasing IR and IRS1. **B.** Schematic representation of the beneficial effects of oleate in switching macrophages polarization by inducing M2 state to enhance insulin sensitivity in hepatocytes.

4. CONCLUSIONS

- Palmitate treated macrophages/Kuppfer cells have increased expression of proinflammatory cytokines.

- The CM-P/CMK-P leads to activation of stress pathways and result in insulin resistance in human and murine hepatocytes.

- Oleate treated macrophages/Kuppfer cells express markers of antiinflammatory activity.

- The CM-O/CMK-O produces a hypersensitivity to insulin in human and murine hepatocytes probably by lower levels of PTP1B.

5. ACKNOWLEDGEMENTS

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This work is dedicated in memory to Professor Margarita Lorenzo who was a member of the RANF.

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