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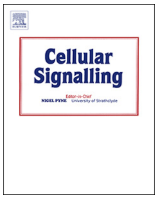

Isomers of conjugated linoleic acid induce insulin resistance through a mechanism involving activation of protein kinase Cε in liver cells

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ABSTRACT

Conjugated linoleic acid (CLA) constitutes a group of isomers derived from linoleic acid. Diverse studies have suggested that these unsaturated fatty acids have beneficial effects on human health. However, it has also been reported that their consumption can generate alterations in hepatic tissue. Thus, in the present study, we evaluated the effect of two of the major isomers of CLA, cis-9, trans-11-CLA and trans-10, cis-12-CLA, in the regulation of insulin signaling in a hepatic cell model, clone 9 (C9). We found that the two isomers decrease insulin-stimulated phosphorylation of the main proteins involved in insulin signaling, such as Akt at Ser^{473} and Thr^{308}, the insulin receptor at Tyr^{1158}, IRS-1 at Tyr^{632}, and GSK-3 at Ser$^{9/21}$. Protein expression, however, was unaffected. Interestingly, both isomers of CLA promoted phosphorylation and activation of PKCε. Inhibition of PKCε activity by a dominant-negative form or knockdown of endogenous PKCε prevented the adverse effects of CLA isomers on insulin-induced Akt phosphorylation. Additionally, we also found that both isomers of CLA increase phosphorylation of IRS-1 at Ser^{612}, a mechanism that probably underlies the inhibition of IRS-1 signaling by PKCε. Using confocal microscopy, we found that both isomers of CLA induced lipid accumulation in C9 cells with the presence of spherical cytosolic vesicles, suggesting their identity as neutral lipid droplets. These findings indicate that cis-9, trans-11-CLA and trans-10, cis-12-CLA isomers could have a significant role in the development of insulin resistance in hepatic C9 cells through IRS-1 serine phosphorylation, PKCε activation, and hepatic lipid accumulation.

1. Introduction

Metabolic syndrome includes a group of abnormalities, such as hypertension, central obesity, insulin resistance, and hypertriglyceridemia. It is associated with an increased risk of developing type-2 diabetes mellitus (DM2), probably due to low-grade inflammation associated with this condition [1]. Alterations in the composition of fatty acids in the diet may contribute to these states; diets that are rich in unsaturated fatty acids counteract this effect [2]. Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids (PUFA) derived from linoleic acid (LA, C18:2 n-6), with a conjugated double bond in different cis- and trans-arrangements and synthesized by rumen bacteria or by enzymatic isomerization of LA [3–5]. To date, twenty-eight isomers of CLA have been identified, with the predominant isomers being cis-9, trans-11-CLA (c9,t11-CLA), which represents up to 90% of total CLA in food, and trans-10, cis-12-CLA (t10,c12-CLA), present in a smaller quantity [6,7].

It has been shown that the CLA-mix, with approximately 50% of each isomer, both from synthetic and natural sources, has beneficial effects in a variety of inflammatory conditions including, colitis,

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Abbreviations: CARS, coherent anti-stokes raman scattering; CLA, conjugated linoleic acid; c9t11-CLA, *cis*9, *trans*11 of conjugated linoleic acid; t10, c12-CLA, trans10, cis12 of conjugated linoleic acid; DAG, diacylglycerol; DM2, type-2 diabetes mellitus; FFA, free fatty acid; HFD, high-fat diet; IR, insulin receptor; IRS-1, insulin receptor substrate-1; LA, linoleic acid; PUFA, polyunsaturated fatty acids; Ser, serine; TEM, transmission electron microscopy; Thr, threonine; Tyr, tyrosine

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atherosclerosis, metabolic syndrome and rheumatoid arthritis [4]. The anti-inflammatory role of CLA isomers makes CLA a primary candidate for dietary treatment in several states of insulin resistance. Nonetheless, it has been proposed that anti-inflammatory and antidiabetic effects of CLA are isomer-specific [4]. The c9,t11-CLA isomer is responsible for reducing levels of pro-inflammatory cytokines and insulin resistance, promoting insulin sensitivity in myotubes and adipose tissue cells [8,9]. On the other hand, the t10,c12-CLA isomer induces insulin resistance, reduces body fat accumulation [10], and increases inflammatory interleukins and macrophage infiltration in white adipose tissue [11,12], effects that are contradictory between both isomers.

In the liver, insulin resistance constitutes a crucial factor in the development of DM2, mainly because normal insulin actions are defective, increasing hepatic glucose production and decreasing glucose uptake and its storage as glycogen. It has been described that PKCε is the predominant PKC isoform activated in the liver following fat feeding, and implicated in hepatic insulin resistance and steatosis [13]. This effect is associated with decreased insulin-stimulated insulin receptor substrate (IRS) tyrosine phosphorylation by the insulin receptor (IR), leading to the inability of insulin to activate hepatic glycogen synthesis and to suppress hepatic glucose production. Additionally, some reports have shown that IR expression is inversely correlated with PKCε activation, a possible mechanism that could explain the impaired insulin signaling in diabetic obese rats [14] and the human HepG2 cell line [15]. Moreover, lipid-induced hepatic insulin resistance could be prevented by knocking down PKCε [16].

Studies in animal models have shown that dietary supplementation with CLA, particularly of t10,c12-CLA isomer, promotes severe fat accumulation in the liver, lipotoxicity, and insulin resistance associated with a decreased activity of Akt [17,18]. It has been proposed that dietary CLA increases hepatic DAG, membrane-associated PKCε, and promotes the pathophysiology responsible for the development of hepatic insulin resistance [19]. However, the molecular mechanisms of PKCε activation in hepatic insulin resistance induced by CLA are still unknown, being relevant to investigate the link between the effects of these fatty acids on insulin signaling pathway and the role of PKCε in hepatic cells.

2. Materials and methods

2.1. Materials

Ham's F12 K nutrient mixture, Kaighn's modification (F12 K medium), c9,t11- and t10,c12-CLA isomers (≥96% pure), linoleic acid (≥99% pure), palmitate (≥99% pure), insulin, Oil Red O dye and fatty acid-free bovine serum albumin (FAF-BSA) were from Sigma-Aldrich (St. Louis, MO). Antibiotic-Antimycotic 100× (penicillin, streptomycin, amphotericin B) (Gibco, Carlsbad, CA) and LipofectAMINE 2000 (Invitrogen, Waltham, MA) were from Thermo Fischer Scientific. Fetal Bovine Serum (FBS) was from BYproductos (Guadalajara, Jal). Trypsin-EDTA 0.25% was from Thermo Fischer Scientific (Waltham, MA). ECL (Enhanced Chemiluminescence) reagent was from Immobilon™ Millipore Corporation (Billerica, MA). Polyclonal and monoclonal primary and secondary antibodies used in the present study are listed in Supplementary Table 1.

2.2. Cell culture

Clone 9 (C9) rat liver epithelial cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in F12 K medium supplemented with 10% (*v*/v) FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B at 37 °C in a humidified O_2 (95%)/CO_2 (5%) atmosphere [20]. For all studies, cells were used between passages 2 and 15 when they have a clear response to insulin stimulation [20]. For experiments, cells were reseeded on six-well plates until cells have reached 80% confluence and then cultured with F12 K medium supplemented with 1% FBS and 1% FAF-BSA, before free fatty acid (FFA) treatments.

2.3. Fatty acids treatments

Stock solutions of CLA isomers, linoleic acid (LA) or palmitate were prepared in DMSO and conjugated to BSA by diluting them with F12 K medium containing 1% FAF-BSA for a final concentration of 50 μmol/L of CLA isomers or LA or the indicated concentrations of palmitate. C9 cells were then individually stimulated with CLA isomers, LA or palmitate for the indicated times and concentrations, followed by the addition of vehicle or 10 nM insulin for 10 min. After treatment, cells were placed on ice, media was removed by aspiration, and the cells were washed twice with cold PBS and lysed in 100 μl Laemmli sample buffer 1×. Cell lysates were frozen at −20 °C for Western blot analysis. For each experimental design, we performed between three to five independent experiments, as indicated in each figure legend.

2.4. Immunoblot analysis

For Western blot determinations, cell lysates were thawed at room temperature, briefly sonicated, heated at 99 °C, and centrifuged for 5 min at 12,000 rpm. The supernatant was electrophoresed on SDS-PAGE (6%, 8 or 10%) gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Blocking was carried out by incubation for 1 h with 5% Blotto, non-fat dry milk (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBS/0.1% Tween (TBST) at room temperature. Blots were incubated overnight at 4 °C with the specified primary antibodies, at the dilutions indicated in Supplementary Table 1, and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibody binding was visualized with ECL reagent and quantified with ImageJ software (National Institutes of Health). To ensure equal loading of proteins, the blots were reprobed with antibodies against the corresponding total protein, at the dilutions indicated in Supplementary Table 1.

2.5. Transfection assay

C9 cells were grown in F12 K medium supplemented with 10% FBS, re-seeded on six-well plates and transiently transfected with the dominant-negative (DN) form of PKCε. The PKCε-DN construct contains the full-length open reading frame of PKC with a K → R point mutation that abolishes the ATP binding ability of the enzyme [21]. To silence the expression of PKCε, cells were transiently transfected with pSuper-PKCε RNAi (Addgene plasmid # 10798) that was donated by Dr. Alex Toker to Addgene. The effectiveness and specificity of this plasmid were previously well established [22]. In all cases, C9 cells were transfected using Lipofectamine 2000, according to the manufacturer's instructions. After 6 h of transfection, cells were fed with fresh medium (F12 K with 15% FBS) for 16 h. The CLA treatment was done as previously described.

2.6. Oil red O staining

C9 cells were plated onto glass coverslips and cultured for 2 days before treatments. The cells were stimulated with CLA isomers for 48 h. Cells were fixed with 3.7% paraformaldehyde and washed three times with PBS, followed by staining with Oil Red O dye (Sigma-Aldrich) in the dark for 5 min at room temperature, then they were washed 5 times with PBS and visualized with a Zeiss LMS-700 confocal microscope (Jena, Germany). Lipid accumulation in cells was determined using the free-ImageJ Software (v1.48, NIH), as previously described [23]. Briefly, an outline was drawn around each cell to measure the area and the mean fluorescence, along with several adjacent background readings. Then, the Corrected Total Cell Fluorescence (CTCF) was calculated as follows: CTCF = Integrated density – (area of selected cell × mean fluorescence of background readings). Statistical analysis was performed using GraphPad PRISM, version 7.0b (La Jolla, CA). Comparisons between groups were performed by one-way ANOVA test and followed by the Bonferroni's post hoc test. Values of $p < 0.05$ were considered statistically significant.

2.7. Statistical analysis

Average intensities from Western blot films were analyzed, and comparisons among all groups were performed with one-way analysis of variance (ANOVA) when appropriate. If statistical significance was found, the Dunnett's (comparison to control) or Bonferroni (comparison between all groups) post hoc test was performed. Data comparison between two groups was performed by two-tailed *t*-test. GraphPad PRISM version 7.0b. was used for all statistical analysis. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. CLA isomers induce insulin resistance in C9 cells

To investigate the ability of CLA isomers to induce insulin resistance in hepatic cells, C9 cells were exposed to 50 μM c9,t11- or 50 μM t10,c12-CLA for different times (24–60 h). Stimulation of C9 cells with 10 nM insulin for 10 min induced an evident Akt phosphorylation at Thr^{308} and Ser^{473} residues, an effect that was inhibited by both CLA isomers (Fig. 1A-1B and 1C-1D, respectively). As can be observed in Fig. 1, this effect cannot be explained by the degradation of Akt protein since total Akt showed no significant reduction throughout stimulation with CLA isomers. Interestingly, while the phosphorylation state of Akt at Thr^{308} was affected by both isomers in a time-dependent manner, the insulin-induced Akt phosphorylation at Ser^{473} was affected only by t10,c12-CLA but not by c9,t11-CLA, except only at the highest time of stimulation (60 h). Neither of the two isomers alone promoted any Akt phosphorylation (Suppl. Figs. S1A-S1B). Thus, our data confirm previous findings on the role of t10,c12-CLA on insulin resistance, but it also shows a significant effect, not previously reported, of c9,t11-CLA on desensitization of insulin signaling, affecting Akt phosphorylation.

In order to determine whether the effect of CLA isomers on insulin-induced Akt phosphorylation affects the regulation of a downstream substrate of Akt, we examined the state of phosphorylation of GSK-3α/β. As expected, we found that phosphorylation of GSK-3α/β induced by insulin in C9 cells was reduced by pretreatment with both CLA isomers, although only the effect of t10,c12-CLA was time-dependent (Fig. 2A), while c9,t11-CLA diminished GSK-3 phosphorylation after 48 h of pretreatment (Fig. 2B).

We also found that insulin-induced glycogen synthase (GS) activity, observed by its dephosphorylation at Ser^{641}, was affected by both CLA isomers, promoting its phosphorylation, as observed in Fig. 2C. These results indicate the ability of CLA isomers to desensitize insulin signaling and its main downstream effectors in hepatic cells, such as Akt, GSK3, and GS.

3.2. CLA isomers decrease insulin-induced activation of IR and IRS

To determine whether CLA isomers impair insulin signaling at the level of IR and IRS-1 activation, we evaluated the insulin-induced phosphorylation of both proteins at Tyr^{1158} (Fig. 3A-3B) and Tyr^{632}, (Fig. 3C-D) respectively. As shown in Fig. 3A-3B, pretreatment of C9 cells with 50 μM c9,t11- or 50 μM t10,c12-CLA, for the indicated times, decreased IR activation. In contrast, insulin-induced phosphorylation of IRS-1 at Tyr^{632} was impaired only by the t10,c12-CLA isomer in a time-dependent manner (Fig. 3D), while the c9,t11-CLA isomer just affected the phosphorylation of IRS-1 partially, after 48 h of pretreatment (Fig. 3C). Interestingly, none of these effects can be associated with changes in the IR or IRS-1 protein expression levels (Fig. 3A-3D), suggesting a different mechanism involved in CLA-induced insulin resistance in this hepatic cell model.

Although our results suggest a specific effect of CLA isomers on insulin signaling, we assessed the impact of the non-conjugated linoleic acid (LA) on insulin-induced Akt phosphorylation. As shown in Suppl. Fig. S2, LA did not affect insulin-induced Akt (phosphorylation at Thr^{308}) or IRS (phosphorylation at Tyr^{632}) activation after 24 h of treatment. However, as previously shown [24,25], at 48 h of stimulation LA induced an evident sensitization effect on insulin-induced Akt and IRS phosphorylation (Suppl. Fig. S2). In contrast, palmitate, which has been associated to insulin resistance in different tissues [14,15,26], induced impairment of insulin-induced Akt phosphorylation in C9 cells (Suppl. Fig. S3).

3.3. CLA isomers induce phosphorylation of IRS-1 at Ser^{612} and Ser^{307}

Early studies have demonstrated the importance of IRS-1 phosphorylation at serine/threonine residues as a critical mechanism in the impairment of insulin actions [20,27]. To determine whether CLA isomers induce phosphorylation of IRS-1 at serine residues, C9 cells were exposed to c9,t11- or t10,c12-CLA from 24 to 60 h and phosphorylation of Ser^{612} and Ser^{307}, two potential phosphorylation sites involved in the inhibition of insulin signaling, was determined by immunoblotting. As shown in Fig. 4, both isomers were found to increase IRS-1 serine phosphorylation on these residues. Interestingly, we observed a different pattern of phosphorylation response depending on the type of isomer used. While the c9,t11-CLA isomer reached a maximum effect on Ser^{612} and Ser^{307} phosphorylation at 24 h (~3.0-fold increase over control, $p < 0.05$) (Fig. 4A and C, respectively), and subsequently decreased closely to baseline, the t10,c12-CLA isomer promoted an increase in phosphorylation of Ser^{612} and Ser^{307} after 24 h stimulation, effect that was maintained up to 60 h (~9.0-fold increase over control, $p < 0.05$) (Fig. 4B and D, respectively). These data suggest that both CLA isomers could impair insulin signaling due to serine phosphorylation of IRS-1.

3.4. PKCε activation is involved in the CLA-mediated inhibition of insulin signaling

To examine whether CLA isomers mediate PKCε activation in C9 cells, we determined phosphorylation of PKCε at Ser^{729}, which has been associated with FFA-mediated activation [28,29]. Cells exposed to c9,t11-CLA showed time-dependent PKCε phosphorylation that reached a maximum after 48 h stimulation (~6.0-fold increase over control, $p < 0.05$), an effect that was continued up to 60 h (Fig. 5A). In contrast, cells treated with t10,c12-CLA reached a peak at 24 h stimulation (~5.5-fold increase over control, $p < 0.05$) and then declined in a time-dependent manner (Fig. 5B). These data confirm that in C9 cells both CLA isomers can activate PKCε, suggesting its participation in the inhibitory effects of CLA on insulin signaling.

To determine the role of PKCε in the CLA isomers-mediated regulation of insulin signaling, the dominant-negative mutant of PKCε (PKCε-DN) was transfected, and C9 cells were pretreated with c9,t11- or t10,c12-CLA isomers. We found that the adverse effects of both CLA isomers on insulin-induced Akt Thr^{308} phosphorylation were improved (Fig. 6A and B). Similarly, the previous observed inhibitory effect of t10,c12-CLA isomer on insulin-induced Akt Ser^{473} phosphorylation at 24 h (Fig. 1D), was also inhibited by transfection of the PKCε-DN (Fig. 6C). Thus, altogether these findings suggest that PKCε modulates the effect of CLA isomers on insulin-stimulated phosphorylation of Akt.

We next evaluated whether PKCε is participating as part of the mechanism by which CLA isomers are mediating insulin resistance in C9 cells. For this purpose, we also examined the effect of the PKCε-DN on CLA-mediated phosphorylation of IRS-1 at Ser^{612}. Transfection of this mutant yielded a significant decrease in the CLA isomers-induced

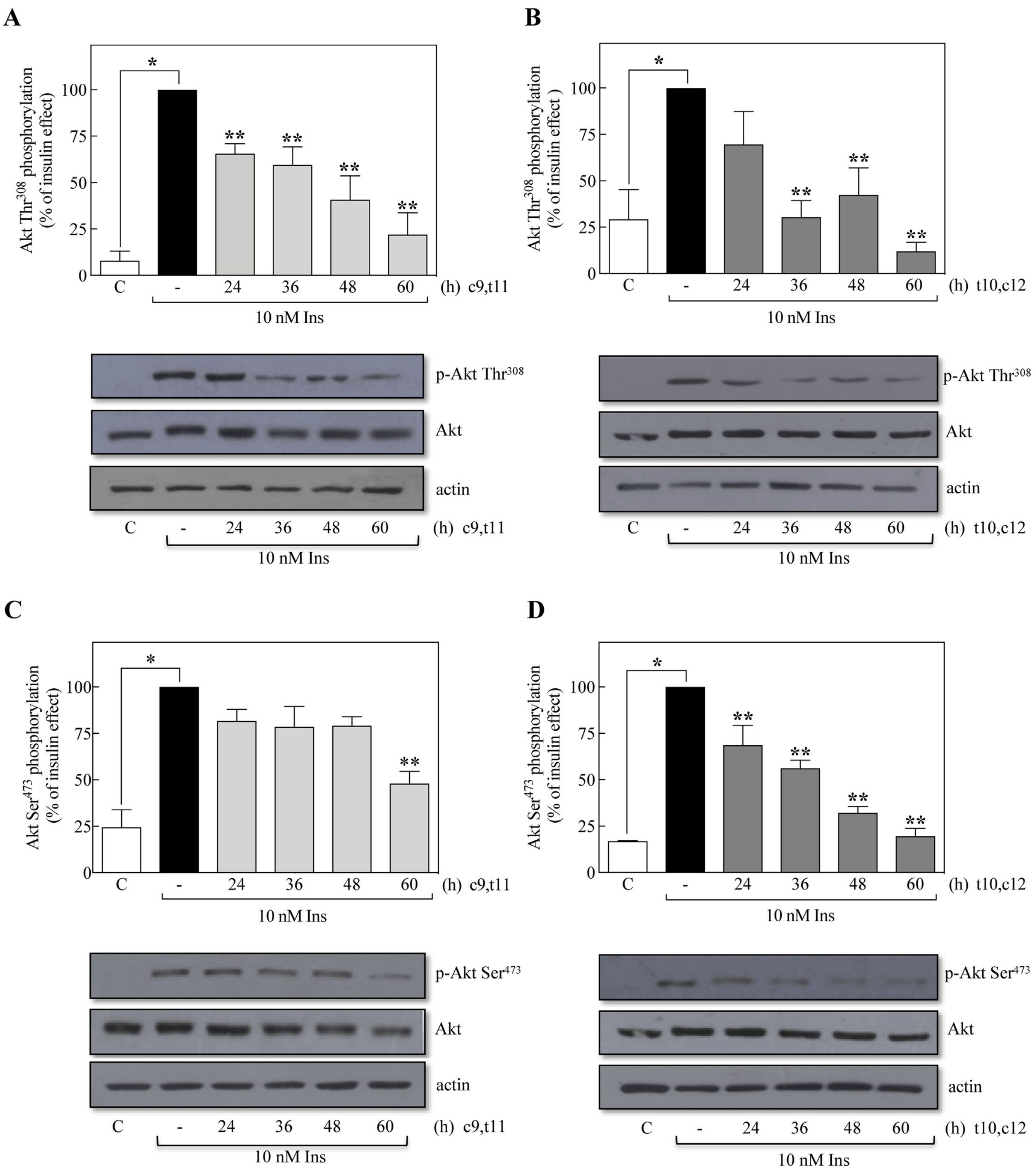

Fig. 1. Effect of c9,t11-CLA and t10,c12-CLA isomers on insulin-induced Akt phosphorylation. C9 cells were incubated with 50 μM c9,t11-CLA (A and C) or 50 μM t10,c12-CLA (B and D) at 37 °C, for the indicated times, and then stimulated with 10 nM insulin for 10 min. Cell extracts were subjected to immunoblot analysis using anti-p-Akt Thr^{308} (A and B) or anti-p-Akt Ser^{473} (C and D). Akt was used as total protein and actin as loading control. Data represent the mean ± SEM of five individual experiments. The lower panels show representative immunoblots. $^{*}p < 0.05$ vs C (control); $^{**}p < 0.05$ vs insulin (−). Ins, insulin.

IRS-1 Ser^{612} phosphorylation (Fig. 7A and B), indicating that phosphorylation of IRS-1 at Ser^{612} plays a critical role in the activation of PKC to modulate the interaction of IRS-1 with the insulin receptor.

To strengthen the role of PKCε in the actions of CLA isomers due to the restricted overexpression of the PKCε-DN versus endogenous PKCε observed in the above experiments (~2.5-fold increase over control, $p < 0.05$, Fig. 6D), which limits the endogenous PKCε interference, we decided to silence PKCε expression with pSuper-PKCε RNAi [22]. As is observed in Fig. 8, the silencing of PKCε (~7.5-fold decrease over control, $p < 0.05$, Fig. 8C) prevented the effect of c9,t11- (Fig. 8A) or

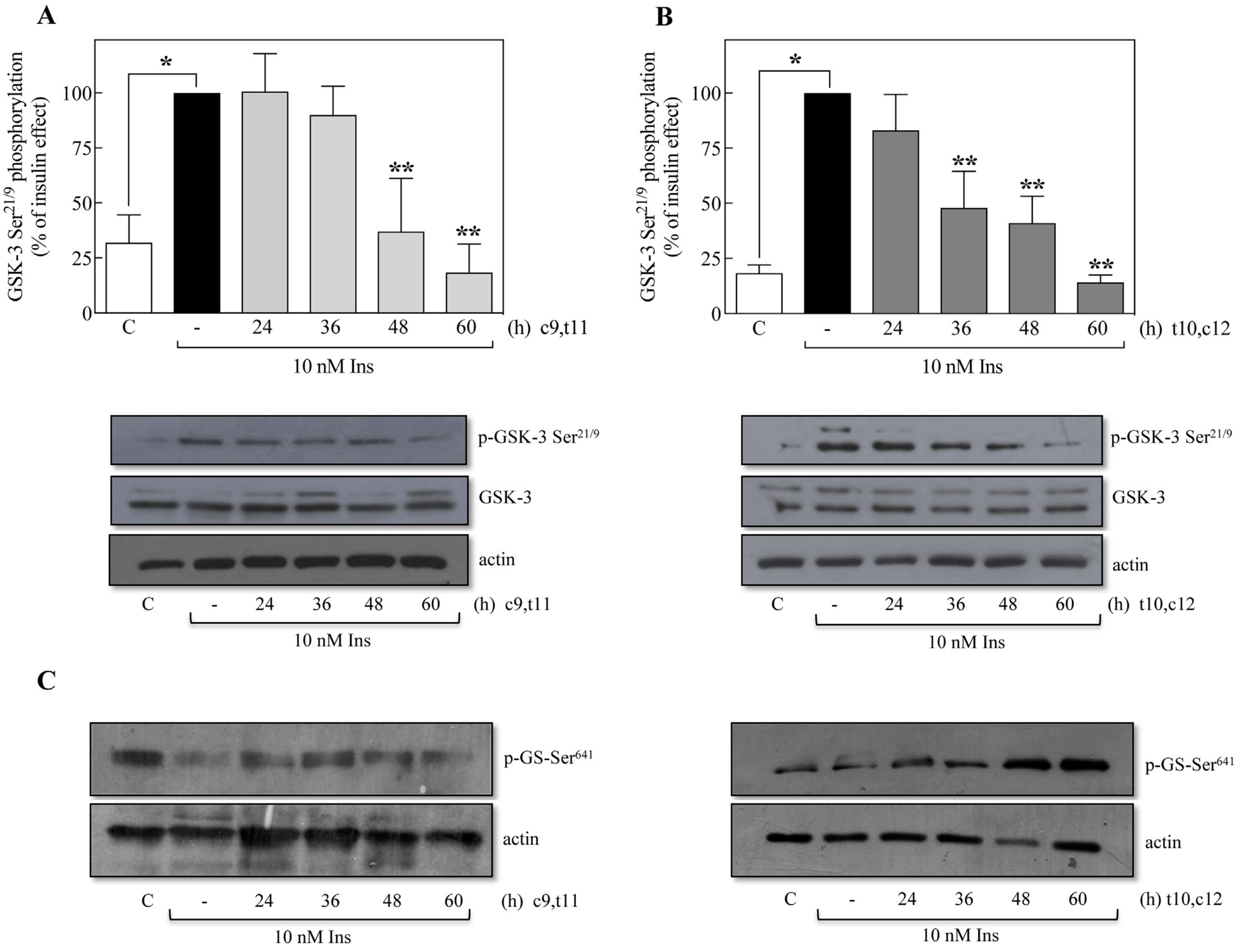

Fig. 2. Effect of c9,t11-CLA and t10,c12-CLA isomers on insulin-induced GSK-3 phosphorylation. C9 cells were incubated with 50 μM c9,t11-CLA (A) or 50 μM t10,c12-CLA (B) at 37 °C, for the indicated times, and then stimulated with 10 nM insulin for 10 min. Cell extracts were subjected to immunoblot analysis using anti-p-GSK-3 $Ser^{21/9}$. GSK-3 was used as total protein and actin as loading control. Data represent the mean ± SEM of five individual experiments. The panels on the right show representative immunoblots. $^{*}p < 0.05$ vs C (control); $^{**}p < 0.05$ vs insulin (−). Ins, insulin.

t10,c12-CLA (Fig. 8B) isomers on insulin-induced Akt phosphorylation, in a similar way as observed with the use of the PKCε-DN, thereby further emphasizing the role of this kinase in the actions of both isomers.

3.5. CLA isomers induce lipid-droplets accumulation in C9 cells

In order to determine the effect of both CLA isomers on hepatic lipid accumulation, C9 cells were treated for 48 h with c9,t11- or t10,c12-CLA, stained with Oil Red O dye and then examined by confocal microscopy (Fig. 9). The treatment of C9 cells with CLA isomers induced the formation of numerous spherical cytosolic vesicles containing the Oil Red O dye, suggesting their identity as neutral lipid droplets (Fig. 9B and C). While the control, in the absence of the CLA isomers only exhibited a low frequency of small and isolated lipid-droplets (Fig. 9A), the treatment with c9,t11-CLA isomer induced the production of such lipid droplets with different sizes and specifically distributed in the cytoplasm (Fig. 9B). Although most of the cells exhibited small lipid droplets, certain cells (arrow) had big lipid droplets located near the nucleus. In contrast, treatment with t10,c12-CLA isomer produced large lipid droplets in all cells, heavily stained by the Oil Red O dye (Fig. 9C). Even more, it was possible to find lipid-droplets aggregates distributed throughout the cytoplasm. In all cases, stained vesicles were found exclusively in the cytoplasm (multiple arrows), while the nuclei remained empty of the Oil Red O labeling (merge). In support of these results, quantification of relative fluorescence intensities of lipid staining indicates a clear increase by the action of both isomers of CLA (Fig. 9D). When C9 cells were exposed to 50 μM LA for 24 h, we observed the presence of a limited number of small vesicles distributed throughout the cytoplasm (Suppl. Fig. S4B). Interestingly, after 48 h incubation with LA, the size of the lipid droplets increased, and the presence of lipid aggregates was found in the cytoplasm and nearby the nuclei (arrow) (Suppl. Fig. S4C), although in smaller amounts compared to treatment with CLA isomers (Fig. 9). C9 cells untreated with LA did not show the presence of lipid droplets (Suppl. Fig. S4A).

4. Discussion

In the present study, we investigated the effect of c9,t11- and t10,c12-CLA isomers on insulin signaling in C9 cells, a normal rat hepatic cell model that retains an epithelial phenotype and exhibits endogenous expression of IR [20]. C9 cells provide a valuable model when studying the molecular mechanisms associated with insulin signaling in hepatocytes [20,30–33]. Our results indicate that both CLA isomers

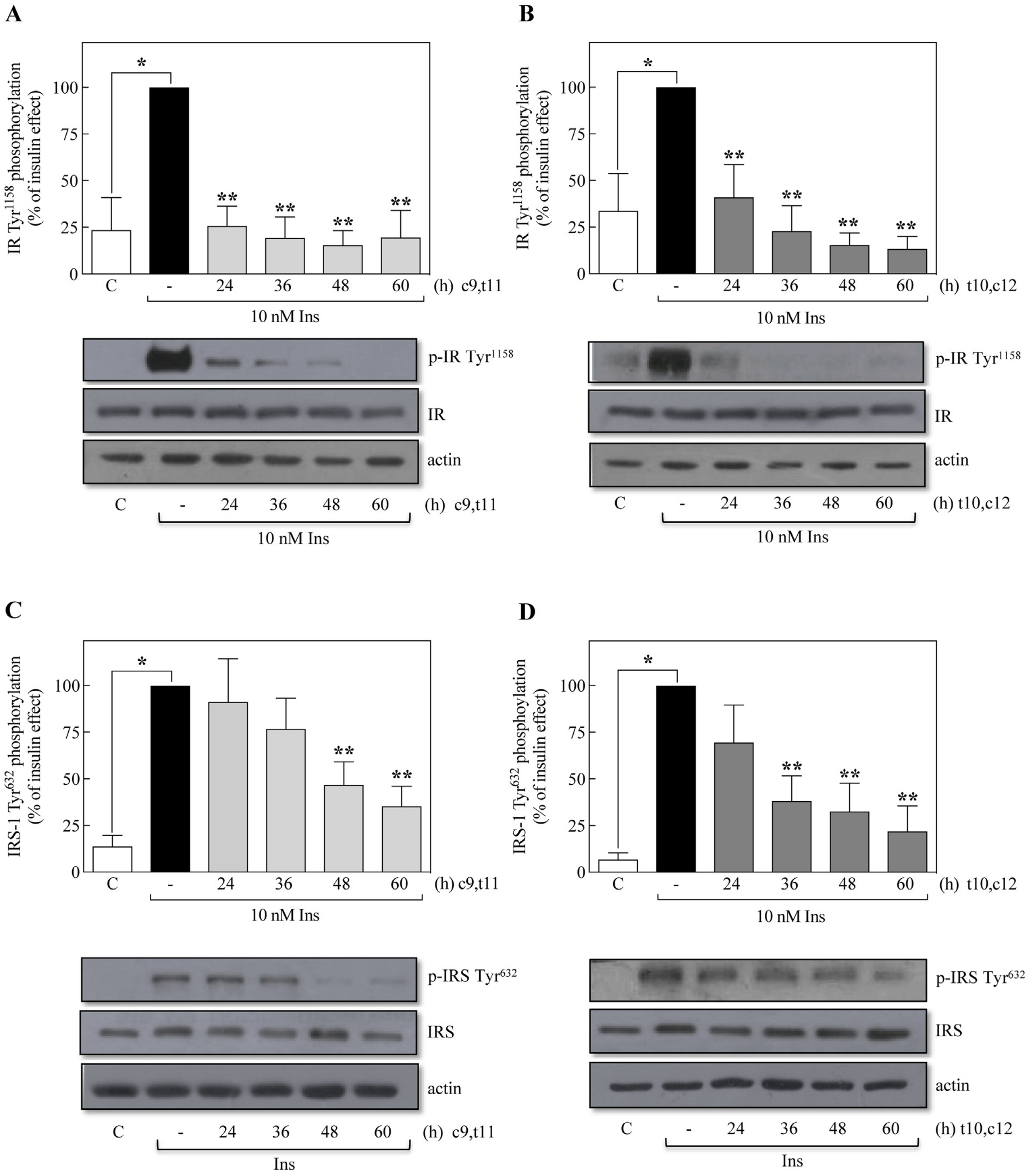

Fig. 3. Effect of c9,t11-CLA and t10,c12-CLA isomers on insulin-induced IR and IRS-1 phosphorylation. C9 cells were incubated with 50 μM c9,t11-CLA (A and C) or 50 μM t10,c12-CLA (B and D) at 37 °C, for the indicated times, and then stimulated with 10 nM insulin for 10 min. Cell extracts were subjected to immunoblot analysis using anti-p-IR Tyr^{1158} (A and B) or anti-p-IRS-1 Tyr^{632} (C and D). IR (A and B) or IRS-1 (C and D) were used as total protein and actin as loading control. Data represent the mean ± SEM of five individual experiments. The lower panels show representative immunoblots. *$p < 0.05$ vs C (control); **$p < 0.05$ vs insulin (−). IR, insulin receptor; IRS-1, insulin receptor substrate-1; Ins, insulin.

inhibit insulin-induced Akt/GSK3 signaling through phosphorylation of IRS-1 at Ser^{612}, using a mechanism that involves activation of PKCε. We additionally found that CLA isomers induce lipid droplet accumulation and modify the morphology of C9 cells. In contrast to these observations, LA, which has been associated with protection against obesity-induced insulin resistance and improved glucose tolerance [24,25], induced an evident sensitization effect on insulin-induced signaling in hepatic C9 cells.

Akt is an essential mediator in the metabolic actions of insulin, particularly in the regulation of glucose metabolism in the liver, where it promotes glycogen synthesis and suppresses gluconeogenesis [34]. In the present study, we found that when C9 cells were treated with c9,t11- or t10,c12-CLA, insulin-induced phosphorylation of Akt at Thr^{308} and Ser^{473}, two of the main activation sites for this enzyme, was impaired. Intriguingly, we observed a more consistent time-dependent inhibitory effect of both isomers in Akt phosphorylation at Thr^{308} rather than Ser^{473}, although the role of both phosphorylation sites in the activation of specific cellular responses mediated by Akt is not yet fully

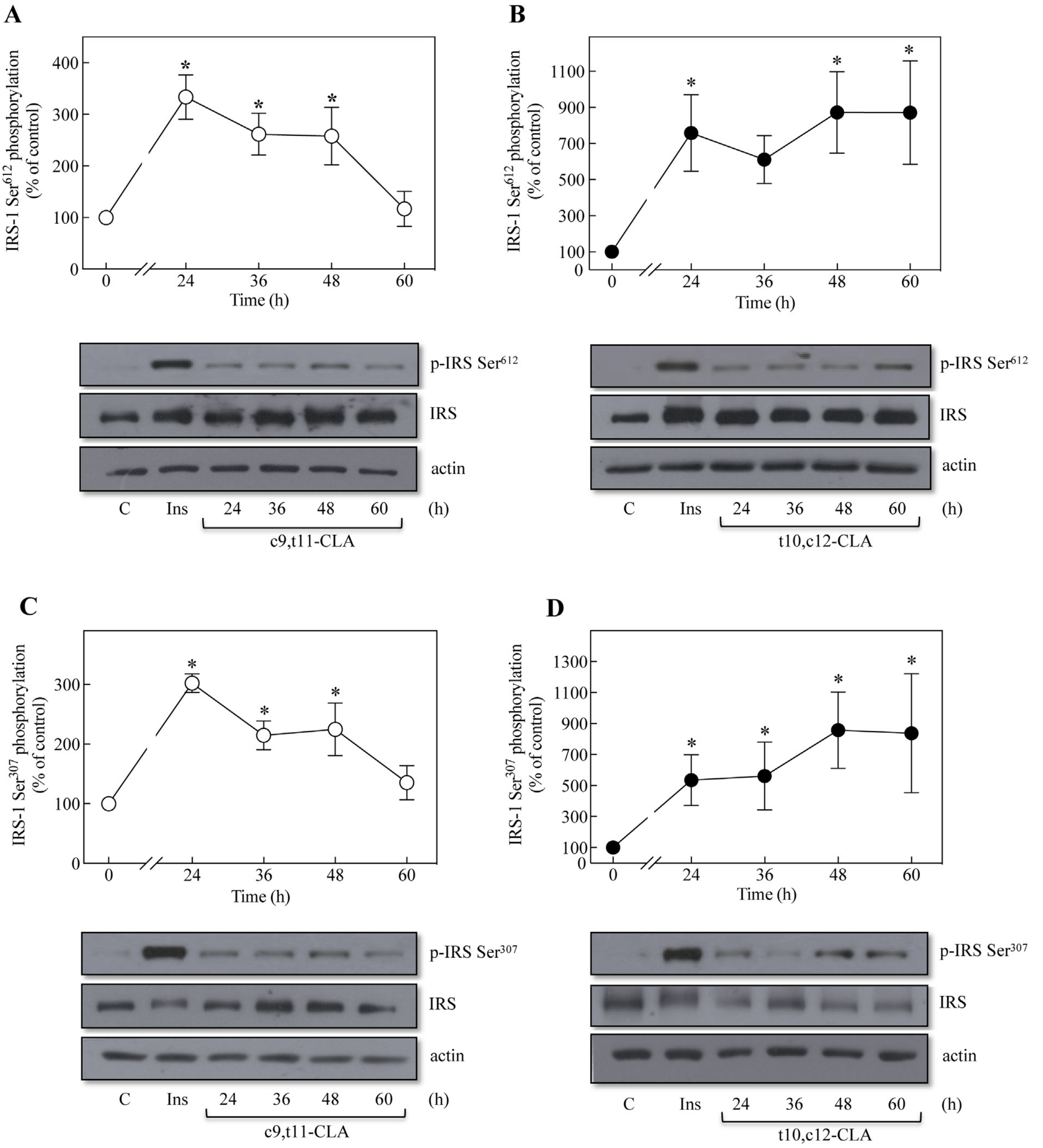

Fig. 4. CLA isomers induce phosphorylation of IRS-1 at Ser^{612} or Ser^{307}. C9 cells were stimulated with 50 µM c9,t11-CLA (open circles, A and C) or 50 µM t10,c12-CLA (solid circles, B and D) at 37 °C, for the indicated times. Cell extracts were subjected to immunoblot analysis using anti-p-IRS-1 Ser^{612} (A and B) or anti-p-IRS-1 Ser^{307} (C and D). IRS-1 was used as total protein and actin as loading control. Data represent the mean ± SEM of five individual experiments. The lower panels show representative immunoblots in which the effect of insulin (10 nM for 10 min) was used as a control stimulus. $^*p < 0.05$ vs 0 (control). IRS-1, insulin receptor substrate-1; Ins, insulin.

understood. Some reports have suggested that Thr^{308} phosphorylation is associated with the metabolic actions of Akt without the requirement of Ser^{473} phosphorylation [35,36]. In a previous report from our group, it was shown that insulin induces greater phosphorylation of Thr^{308} residue than Ser^{473} in C9 cells [20]. These results, in agreement with our present findings, allow us to suggest that insulin-mediated Akt Thr^{308} phosphorylation could play an important role in the metabolic actions of Akt. In this sense, its alteration mediated by both CLA isomers would have a more significant impact on the regulation of primary downstream targets of Akt in hepatic cells, including GSK3 and GS, two essential regulators of glycogen synthesis.

An essential factor contributing to the development of insulin resistance is the phosphorylation of serine/threonine-residues of IR and IRS-1 proteins [37–39]. IR/IRS-1 hyperphosphorylation decreases tyrosine phosphorylation and reduces IR kinase activity and interaction with downstream substrates such as PI3K, altering the phosphorylation and activation of Akt [40,41]. Furthermore, it has been reported that IR/IRS-1 serine/threonine phosphorylation accelerates their degradation. Thus, we examined how CLA isomers could decrease insulin-induced IR and IRS-1 activity, and found that activation of the IR and its substrate were affected due to a significant decrease in tyrosine phosphorylation of both proteins with no changes in their expression.

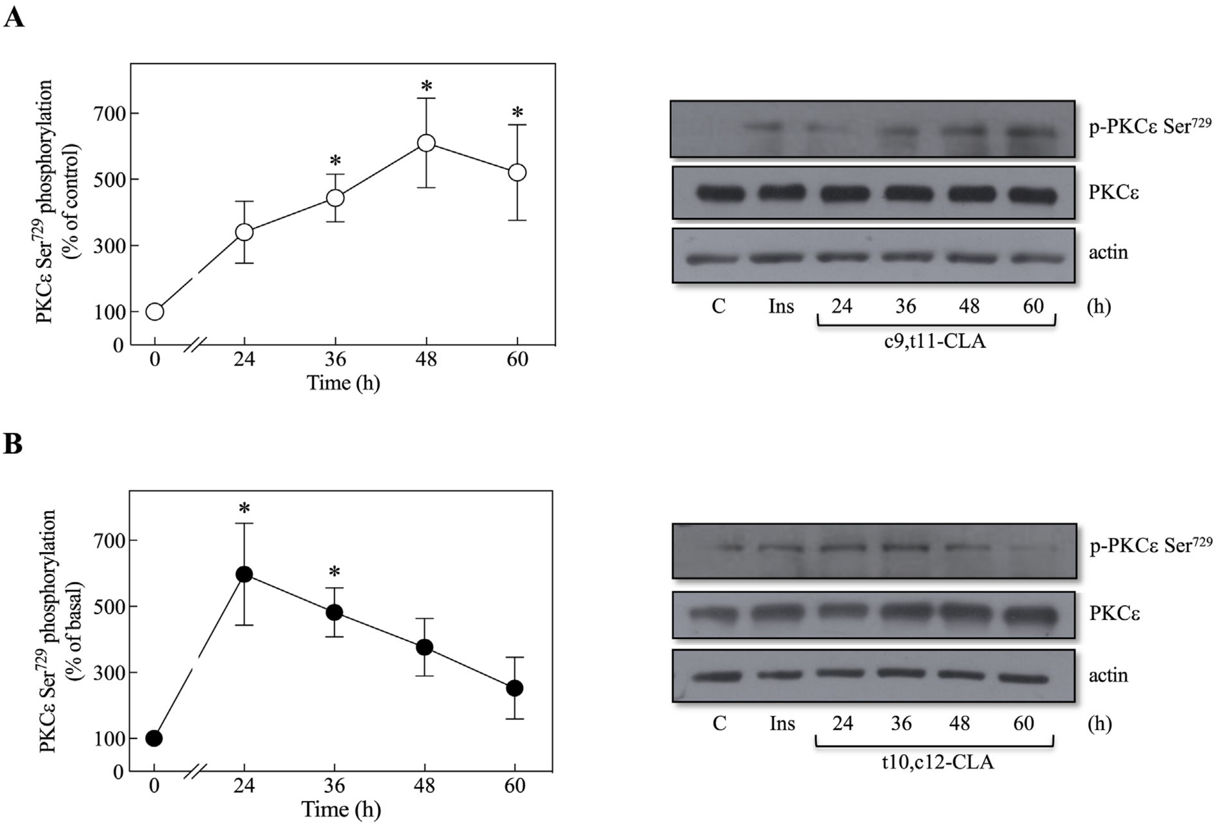

Fig. 5. CLA isomers induce phosphorylation of PKCε. C9 cells were incubated with 50 μM c9,t11-CLA (open circles, A) or 50 μM t10,c12-CLA (solid circles, B) at 37 °C, for the indicated times. Cell extracts were subjected to immunoblot analysis using anti-p-PKCε Ser^{729}. PKCε was used as total protein and actin as loading control. Data represent the mean ± SEM of five individual experiments. The panels on the right show representative immunoblots in which the effect of insulin (10 nM for 10 min) was used as a control stimulus. *$p < 0.05$ vs 0 (control). Ins, insulin.

Although in the present study we did not evaluate the mechanism involved in the impairment of IR tyrosine phosphorylation by both CLA isomers, serine/threonine phosphorylation undoubtedly represents a probable mechanism of IR regulation in C9 cells [37,42]. The activation of PKC has been shown to cause an increase in the serine/threonine phosphorylation of the IR at some residues, although the critical regulatory site(s) have not yet been identified [43]. A recent study by Petersen et al. [39] has shown that phosphorylation of the IR at Thr^{1160} through PKCε mediates hepatic insulin resistance in mice with a high-fat diet (HFD). Interestingly, IR Thr^{1160} phosphorylation would impair IR kinase activity by destabilizing its active conformation. In this regard, a more detailed study is required to determine the mechanism involved in regulation at the level of IR.

Experimental evidence has shown that the decrease in tyrosine phosphorylation of IRS-1/2 could be considered as an indicator of insulin-resistant states such as obesity and DM2 [44], although the molecular mechanisms associated with this process are not clearly understood. Nevertheless, the role of serine/threonine phosphorylation of IRS-1 is thought to be related to the impairment of its tyrosine phosphorylation, interaction with the IR, and downstream propagation of insulin signaling [45–48]. There are some reports in adipose and hepatic tissues of the CLA isomers´ effects on serine phosphorylation of IRS-1; however, the results are still controversial, and very different between them [49,50]. In the present study, we observed a simultaneous increase in the phosphorylation of IRS-1 at Ser^{612} and Ser^{307}, findings that suggest that both isomers of CLA impaired insulin-signaling pathway in C9 cells.

Under insulin resistance conditions, different protein kinases have been identified as regulators of IRS function through phosphorylation of several of its serine/threonine residues, including mTOR/S6K1, MAPK, JNK, IKKβ and different isoforms of PKC, among others [51]. In this context, various reports have suggested that PKC is an important regulator of insulin signaling, mediated by different kinds of stimuli, including HFD [14,19,52]. Interestingly, hepatic insulin resistance could be attributed to activation of PKCε, since this protein is the predominant PKC isoform activated in the liver following an HFD [13]. Moreover, lipid-induced hepatic insulin resistance could be prevented by knocking-down PKCε [16]. In the present report, we found a substantial contribution of PKCε activation in the treatment effect of t10,c12- and c9,t11-CLA isomers; both CLA isomers significantly increased the phosphorylation levels of PKCε at Ser^{729}. In agreement with this, dietary CLA increased hepatic DAG, membrane-associated PKCε, and promoted the development of hepatic insulin resistance [19].

It has been shown that activation of PKC stimulates the serine/threonine phosphorylation of IRS-1 [53]. In a high-fat-fed rat model, Samuel et al. [13] demonstrated that PKCε activation decreased IRS-1/IRS-2 tyrosine phosphorylation, a mechanism involved in hepatic fat accumulation. We observed in C9 hepatic cells that when we transfected the dominant negative of PKCε and/or induced the silencing of the expression of this protein, the inhibitory effect of both CLA isomers in Akt-Thr^{308} phosphorylation was prevented, and the phosphorylation of IRS-1 at Ser^{612} decreased with the transfection of the dominant negative of PKCε. Thus, our results suggest for the first time that PKCε could be responsible for CLA isomers´ effects in impairing the insulin signaling pathway through a mechanism that involves IRS-1 phosphorylation at Ser^{612}. There is evidence that phosphorylation of residue Ser^{612} in the IRS-1 protein is required for the ability of PKC to regulate the insulin-stimulated tyrosine phosphorylation of this protein negatively. This serine residue is in proximity to Tyr^{608}, a residue known to be phosphorylated by the IR and to play a significant role in PI3K/IRS-1 interaction [54].

Our data show that both CLA isomers induced the phosphorylation

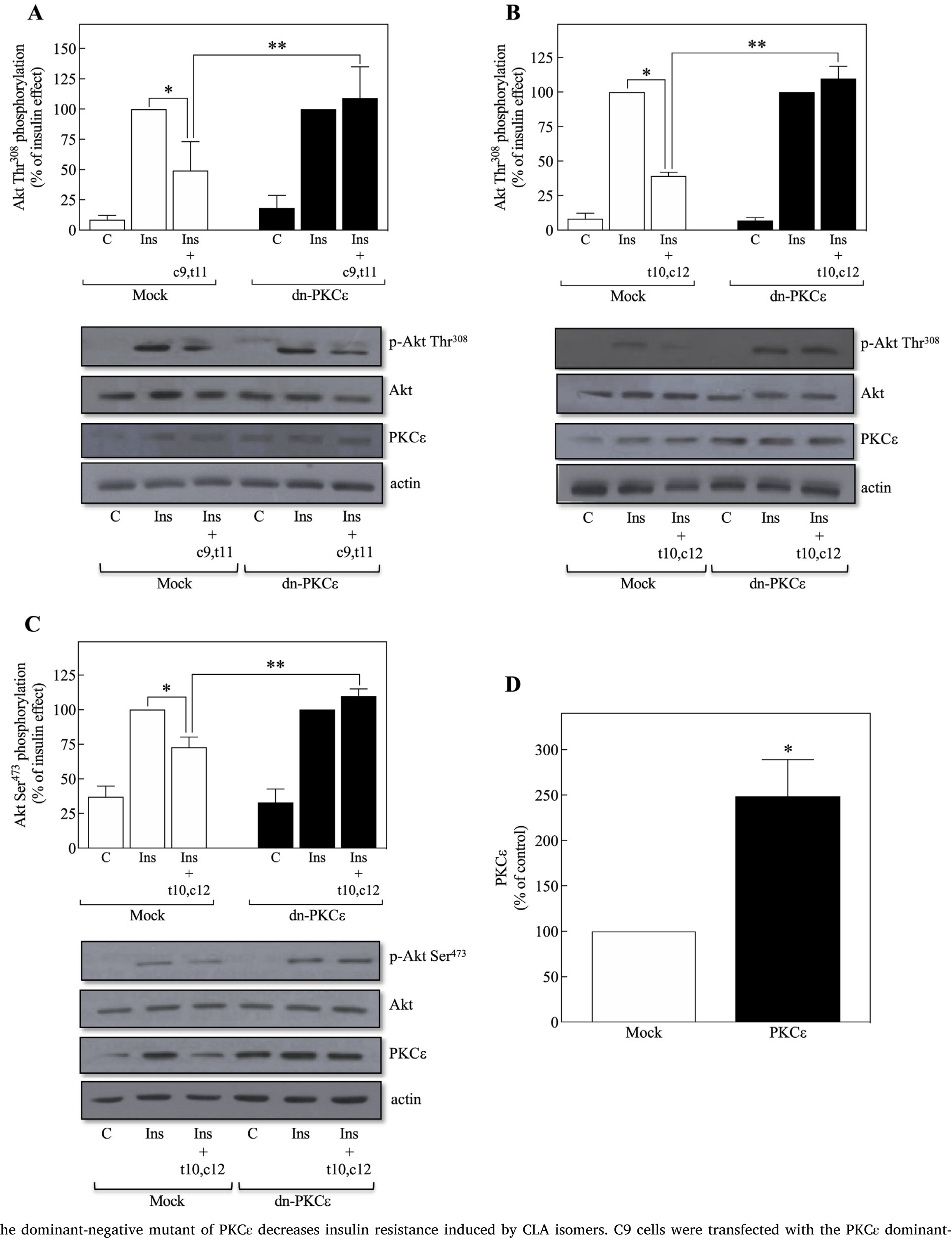

Fig. 6. The dominant-negative mutant of PKCε decreases insulin resistance induced by CLA isomers. C9 cells were transfected with the PKCε dominant-negative plasmid (PKCε-DN) or pcDNA3 plasmid as a control (mock). Then, cells were incubated with 50 μM c9,t11-CLA (A) or 50 μM t10,c12-CLA (B and C) for 24 h and stimulated with 10 nM insulin for 10 min. Cell extracts were subjected to immunoblot analysis using anti-p-Akt Thr^{308} (A and B), anti-p-Akt Ser^{473} (C), or anti-PKCε (A-C). Akt was used as total protein and actin as loading control. (D) The graph shows the increase of PKCε protein due to the plasmid in C9 cells. Data represent the mean ± SEM of five to six individual experiments. The panels on the right show representative immunoblots. *$p < 0.05$ vs Ins (A-C) or Mock (D); **$p < 0.05$ vs Ins + c9,t11-Mock (A) or Ins + t10,c12-Mock (B and C). Ins, insulin.

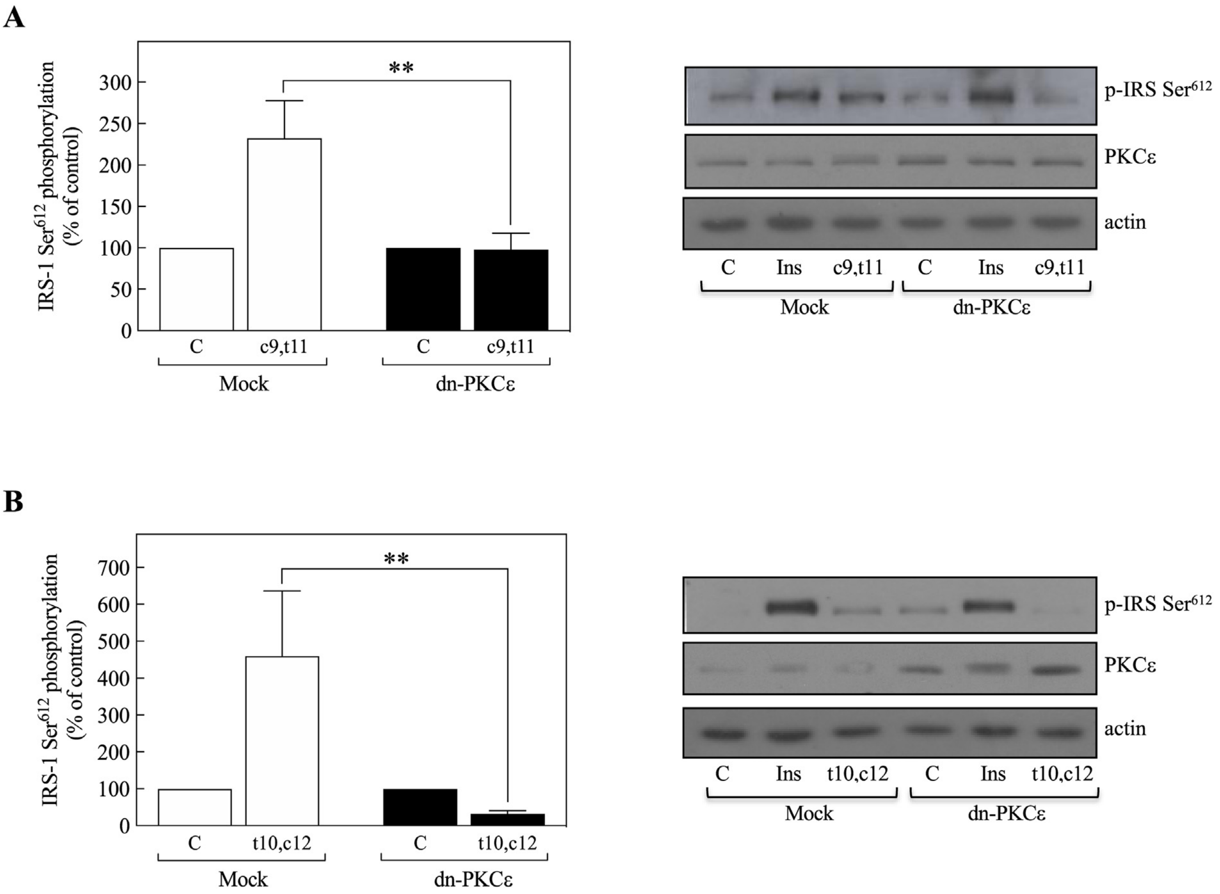

Fig. 7. CLA isomers-stimulated IRS-1 Ser^{612} phosphorylation was reduced in HEK 293 cells overexpressing a PKCε dominant-negative mutant. C9 cells were transfected with the PKCε dominant-negative plasmid (PKCε-DN) or pcDNA3 plasmid as a control (mock). Then, cells were incubated with 50 µM c9,t11-CLA (A) or 50 µM t10,c12-CLA (B) for 24 h. Cell extracts were subjected to immunoblot analysis using anti-p-IRS-1 Ser^{612} or anti-PKCε (A-B). Total actin was used as loading control. Data represent the mean ± SEM of five individual experiments. The panels on the right show representative immunoblots in which the effect of insulin (10 nM for 10 min) was used as a control stimulus. $**p < 0.05$ vs c9,t11-Mock (A) or t10,c12-Mock (B). Ins, insulin.

of IRS-1 on Ser^{307}. Although classically this site has been identified as a major JNK and IKKβ phosphorylation site, several reports have indicated that PKCε is an upstream regulator of both kinases, which allow us to suggest that both CLA isomers in C9 could promote phosphorylation of IRS-1 at this particular residue [55–57].

It is noteworthy that palmitate, a saturated fatty acid classically associated with insulin resistance in different cell types [26,55,58,59], also promoted the alteration of insulin-induced Akt phosphorylation in our cell model (Suppl. Fig. S3). Although in the present study we did not determine the molecular mechanisms by which palmitate induces insulin resistance, and saturated fatty acids could have specific mechanisms to impair insulin signaling, previous studies have consistently evidenced the involvement of different PKC isoforms, particularly PKCε, PKCθ, and PKCδ, in the actions of palmitate [14,15,59]. Thus, it is possible that palmitate, as well as CLA and other fatty acids, can engage the activation of PKC as a key regulator of their actions, very likely due to the generation of lipid intermediates as a product of their metabolism, (mainly ceramides and diacylglycerol that lead to the activation of various PKC isoforms) in different cell types. In most cases, PKC activation has been associated with serine/threonine phosphorylation of IR and IRS-1, leading to a decrease in tyrosine phosphorylation and activity [16]. Associated with PKC activation, fatty acids have been frequently related to endoplasmic reticulum stress and JNK/IKK activation also as the main mechanism for mediating insulin resistance.

Hepatic insulin resistance is also associated with different alterations that may contribute to the impairment of glucose metabolism. Adkins et al. [17] reported that mice fed with a t10,c12-CLA diet develop hepatic insulin resistance, associated with a significant decrease in the Akt Thr^{308} phosphorylation levels and with the development of severe hepatic macrosteatosis. Other reports showed that mice fed with a diet containing CLA developed severe steatosis, with increased levels of triacylglycerol and DAG in the liver [60]. In this context, there are reports about the deleterious effect of the t10,c12-CLA isomer in the expression of genes associated with hepatic lipid accumulation. It has been shown that mice fed with t10,c12-CLA present a downregulation of genes related to β-oxidation, such as CPT-1 and ACOX1 [5,17]. In contrast, it was reported that under similar feed conditions, CLA promoted the up-regulation of genes associated with fatty acids synthesis, including SREBP1, ACC, FASN and SCD1 [5,17].

We observed an increased fat accumulation in C9 cells treated with both CLA isomers; our findings differ from other reports in which only the t10,c12-CLA isomer is described as responsible for the development of insulin resistance and hepatic steatosis [5,60]. In our study, we observed by confocal microscopy the presence of significant lipid droplets stained with Oil Red O dye in cells treated with c9,t11- and t10,c12-CLA isomers, suggesting that both CLA isomers induce lipid accumulation in C9 cells. Contrary to those findings, we observed smaller lipid droplet accumulation in C9 cells stimulated with LA, suggesting that this fatty acid does not promote lipid accumulation in our model, as previously shown [61–63]. Interestingly, the activation of pro-inflammatory signaling pathways has been reported as a consequence of the deposition of lipids in the liver. Those pathways involve the activation of kinases associated with the negative regulation of the insulin pathway like IKK

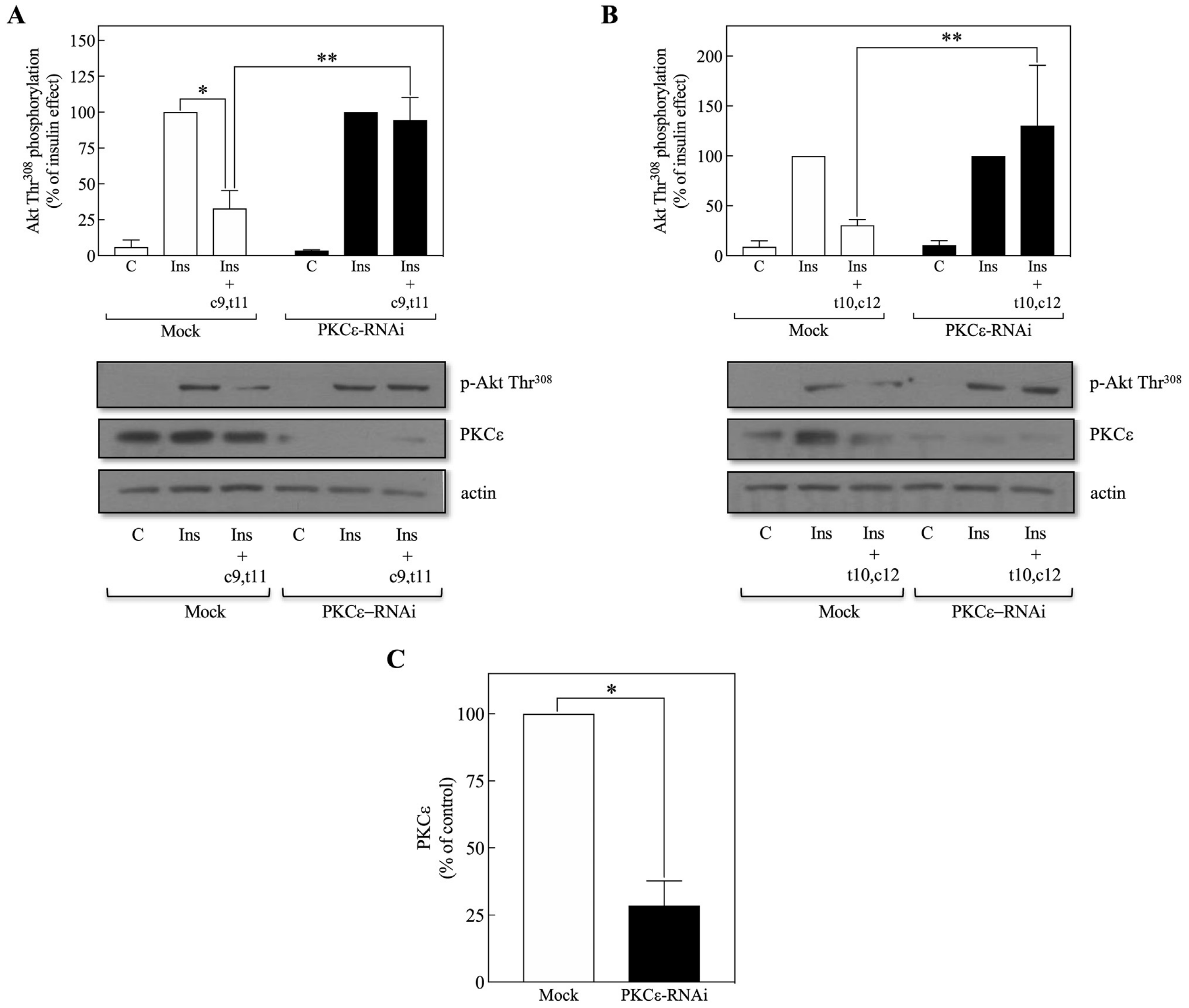

Fig. 8. The silencing of PKCε decreases insulin resistance induced by CLA isomers. To silence the expression of PKCε, cells were transiently transfected with pSUPER-PKCepsilon RNAi (PKCε-RNAi) or pcDNA3 plasmid as control (Mock). Then, cells were incubated with 50 μM c9,t11-CLA (A) or 50 μM t10,c12-CLA (B) for 24 h and stimulated with 10 nM insulin for 10 min. Cell extracts were subjected to immunoblot analysis using anti-p-Akt Thr308 (A and B), anti-PKCε (A and B). Actin was used as loading control. (C) The graph shows the knockdown of PKCε in C9 cells. Data represent the mean of three individual experiments. The panels on the right show representative immunoblots. *$p < 0.05$ vs Ins (A) or Mock (C); **$p < 0.05$ vs Ins + c9,t11-Mock (A) or Ins + t10,c12-Mock (B). Ins, insulin.

and JNK, which, like PKC, can also promote serine phosphorylation of IRS [64].

Moreover, using transmission electron microscopy (TEM) (Supplementary Methodology), we observed the presence of spherical cytosolic vesicles, suggesting their identity as neutral lipid droplets in cells treated with both CLA isomers (Suppl. Fig. S5). These observations are similar to our results described previously by confocal microscopy, and suggest that c9,t11- and t10,c12-CLA isomers could be involved in the induction of hepatic steatosis process in our model accompanied by insulin resistance, in agreement with previous studies that have reported severe hepatic macrosteatosis, and morphological changes in mice fed a diet containing CLA [17,65].

In conclusion, our findings indicate that the c9,t11- and t10,c12-CLA isomers impair insulin-induced IRS/PI3K/Akt/GSK3 signaling by a mechanism that depends on PKCε. Activation of this kinase, possibly due to elevated intracellular levels of DAG, leads to phosphorylation of IRS-1 at Ser^{612} and Ser^{307}, the latter by a possible indirect mechanism involving PKC/JNK activation. These effects are associated with decreased insulin signaling, promoting the inability of insulin to trigger glycogen synthesis in hepatic cells and the development of liver damage, including hepatic steatosis.

Authors´ contributions

JAO-R and AR-G conceived the project. JAO-R, AR-G and RM-F designed experiments. AR-G, CJR-F and JH-A carried out experiments. JAO-R, AR-G, RM-F, NG-N, GQ-G, J-WS and EL-S analyzed and discussed data. JAO-R and AR-G wrote the manuscript. All the authors read and approved the final manuscript.

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Conflict of interest

None of the authors has any potential conflict of interest to declare.

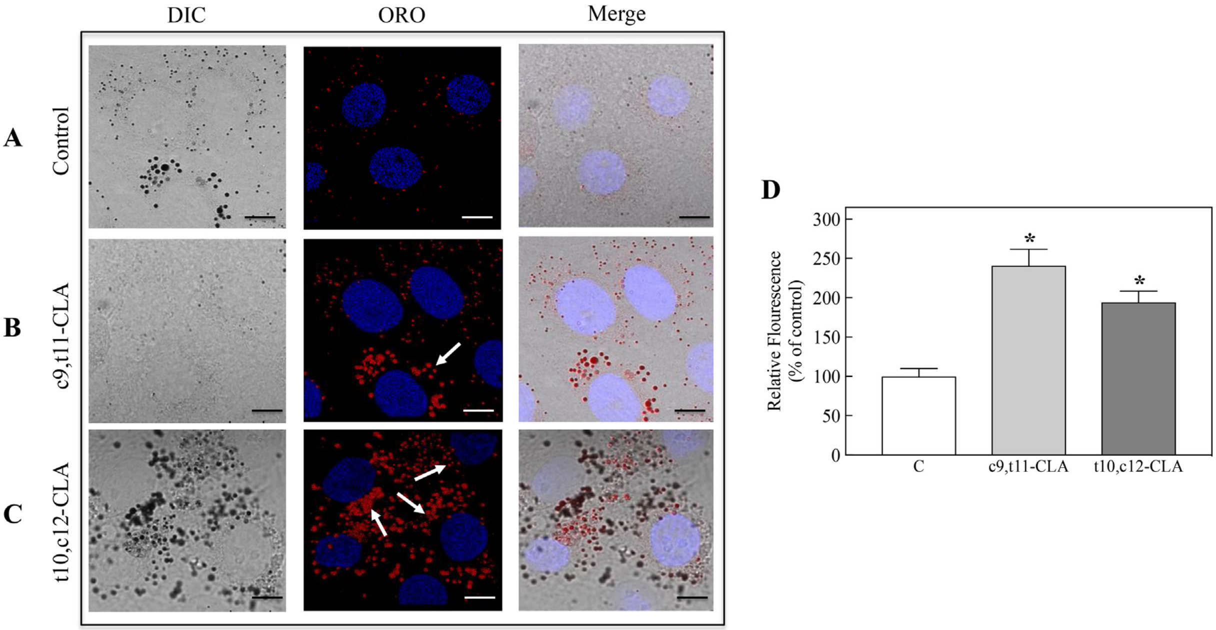

Fig. 9. Effect of CLA isomers on lipid accumulation in C9 cells. Cells were incubated without (A) or with 50 μM c9,t11- (B) or 50 μM t10,c12-CLA (C) at 37 °C, for 48 h. After stimulation, cells were fixed, and lipid droplets and nuclei were stained with Oil Red O dye and DAPI, respectively, and visualized with a Zeiss LMS-700 confocal microscope, using a 3D-surface configuration. Differential interference contrast (DIC) microscopy. Oil Red O (ORO). Merge. Scale bars: 10 μm. (D) Lipid accumulation in cells was determined using the free-ImageJ Software. Data represent the mean ± SEM of three individual experiments. *$p < 0.05$ vs C (control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.10.013.

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