Free Fatty Acid-Induced Muscle and Fat Cell Insulin Resistance is Attenuated by Carnosic Acid

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Abstract

Elevated blood free fatty acids (FFAs), as commonly seen in obesity, results in impaired insulin action leading to insulin resistance and Type 2 diabetes mellitus. Activation of the energy sensor AMP-activated protein kinase (AMPK) increases glucose uptake in muscle and fat, and in recent years, AMPK has been viewed as an important target to counteract insulin resistance. We reported previously that carnosic acid (CA), a polyphenol found in rosemary, increased muscle cell glucose uptake and activated AMPK. In the present study, we examined the effects of CA on palmitate-induced insulin resistant L6 myotubes and 3T3-L1 adipocytes. Exposure of cells to palmitate reduced the insulin-stimulated glucose uptake in muscle and fat cells, increased serine phosphorylation of IRS-1, and decreased the insulin-stimulated phosphorylation of Akt and GLUT4 plasma membrane levels in muscle cells. Importantly, CA attenuated the deleterious effect of palmitate and restored the insulin-stimulated glucose uptake, GLUT4 levels and the activation of Akt. CA markedly attenuated the palmitate-induced serine phosphorylation of IRS-1 and phosphorylation/activation of JNK, mTOR and p70S6K, while AMPK phosphorylation was increased even in the presence of palmitate. Our data indicate that CA has the potential to counteract the palmitate-induced muscle and fat cell insulin resistance. More studies are required to further explore the anti-diabetic potential of CA.

Background

Skeletal muscle and fat tissue are two of the primary targets of insulin and play a critical role in the maintenance of glucose homeostasis. After binding to its receptor, insulin triggers an increase in the receptor tyrosine kinase activity which leads to GLUT4 glucose transporter translocation from an intracellular storage site to the plasma membrane via downstream activation of the lipid kinase phosphatidylinositol-3 kinase (PI3K) and the serine threonine kinase Akt/PKB[1]. Impairments in the PI3K-Akt cascade leads to insulin resistance and type 2 diabetes mellitus (T2DM)[1,2]. Elevated free fatty acid (FFA) levels have been linked to insulin resistance[3,4]. FFAs increase serine phosphorylation of IRS-1 leading to reduced insulin-stimulated glucose uptake and impaired PI3K/Akt signaling in muscle and fat[3,4]. Studies have shown that several protein kinases including JNK, mTOR, and p70S6K have been implicated in mediating the FFA-induced insulin resistance [3,4].

Rosemary extract (RE) has been reported to have antioxidant, anti-inflammatory, anticancer and antidiabetic properties[5,6]. RE is composed of a variety of polyphenols, with carnosic acid (CA) found in high concentration (Figure 1). Recently we found that RE and CA, increased glucose uptake and robustly activated AMPK in healthy L6 muscle cells [7,8,9].

Rosemary and carnosic acid (CA) chemical structure.

HYPOTHESIS:

Carnosic acid prevents the FFA-induced insulin resistance in L6 muscle cells and 3T3-L1 adipocytes.

Results

CA restores the insulin-stimulated glucose uptake in palmitate treated muscle and fat cells

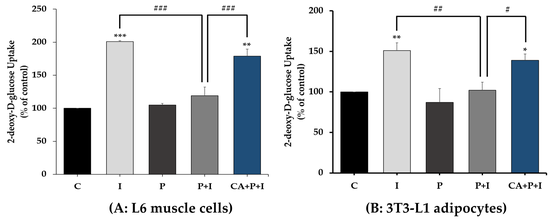


Figure 1: Fully differentiated myotubes were treated without (control, C) or with 0.2 mM palmitate (P) for 16 hours in the absence or presence of 2μM carnosic acid (CA) followed by stimulation without or with 100 nM insulin (I) for 30 min. Data are the mean of 5 independent experiments, expressed as percent of control. (* p<0.05, ** p<0.01, *** p<0.001 vs. control, # p<0.05, ## p<0.01, ### p<0.001 vs. palmitate and insulin).

CA restores the insulin-stimulated GLUT4 translocation in palmitate treated muscle cells

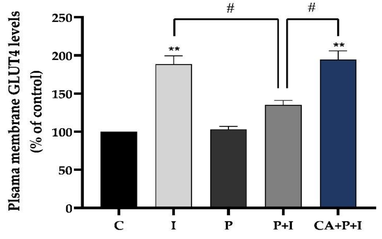


Figure 2: Myotubes were treated as in Figure 1 and a GLUT4 translocation assay was performed. Data are the mean of 3 independent experiments, expressed as percent of control. (** p<0.01 vs. control, # p<0.05 vs. palmitate and insulin.)

CA prevents the palmitate-induced phosphorylation of JNK, mTOR and p70S6K in muscle cells

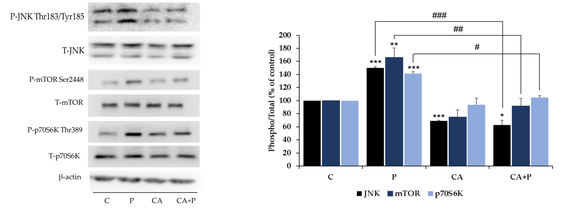


Figure 5: Myotubes were treated as in Figure 4. Data are the mean of 3 independent experiments, expressed as percent of control. (* p< 0.05, ** p<0.01, *** p<0.001 vs. control, # p<0.05 ## p<0.01, ### p<0.001 vs. palmitate alone).

CA restores the insulin-stimulated Akt phosphorylation in palmitate treated muscle cells

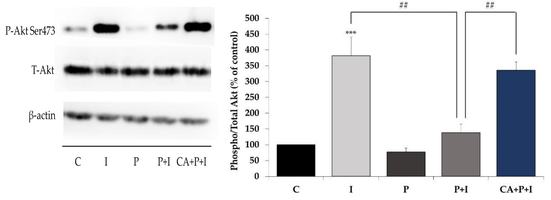


Figure 3: Myotubes were treated as in Figure 1 and western blotting was performed. Data are the mean of 3 independent experiments, expressed as percent of control. (*** p<0.001 vs. control, ## p<0.01 vs. palmitate and insulin).

CA prevents the palmitate-induced serine phosphorylation of IRS-1 in muscle cells

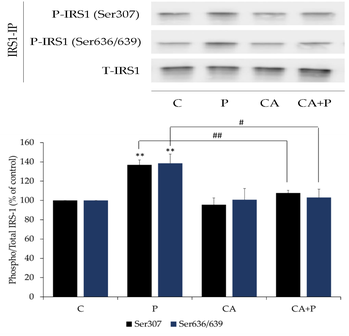


Figure 4: Myotubes were treated without (control, C) or with 0.2 mM palmitate (P) for 16 hours in the presence or absence of 2μM carnosic acid (CA). Data are the mean of 3 experiments, expressed as percent of control. (** p<0.01 vs. control, # p<0.05 ## p<0.01 vs. palmitate alone.)

CA increases the phosphorylation of AMPK and ACC in the presence of palmitate in muscle cells

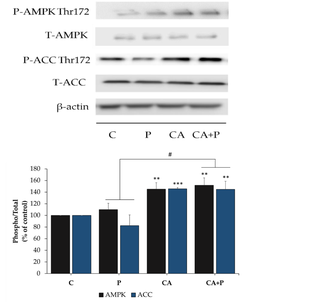
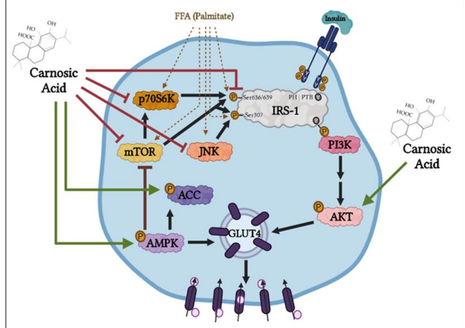


Figure 6: Myotubes were treated as in Figure 4. Data are the mean of 3 independent experiments, expressed as percent of control. (** p<0.01, *** p<0.001 vs. control, # p<0.05 vs. palmitate alone).

Summary



Our study showed that exposure of muscle cells to FFA palmitate, to mimic the elevated FFA levels seen in obesity, induced insulin resistance

Our study is the first to show that CA has the potential to counteract the palmitate-induced muscle and fat cell insulin resistance

Methods

Cell Culture and glucose uptake. L6 and GLUT4myc overexpressing L6 rat skeletal muscle cells were cultured in α-MEM and 3T3-L1 adipocytes were cultured in DMEM solution at 37^{0}C. At the end of the treatment, the cells were washed with HEPES-buffered saline (HBS) and 2-[3H]deoxy-D-glucose (2DG) uptake was measured[7,8,9].

GLUT4myc Translocation Assay. At the end of treatment, the cells were fixed with 3% paraformaldehyde, followed by incubation with primary anti-myc antibody containing blocking buffer. Next, cells were incubated with HRP-conjugated donkey anti-mouse IgG-containing secondary antibody (1:1000) and finally incubated with OPD before being stopped with 3 N HCl solution. The supernatant was collected, and the absorbance was measured using a plate reader (492 nm).

Western blotting. At the end of the treatment, the cells were lyzed with lysis buffer and solubilized in 3x SDS sample buffer, followed by separation by SDS-PAGE, transfer to PVDF and incubation with the primary antibody. HRP-conjugated secondary antibody and ChemiGLOW reagent were used to detect the protein of interest.

Acknowledgements & References

L6 parental, L6 GLUT4myc overexpressing cells and 3T3-L1 adipocytes were a kind gift from Dr A. Klip (Hospital for Sick Children, Toronto, ON, Canada). Support was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) grant to E. Tsiani.

NSERC CRSNG

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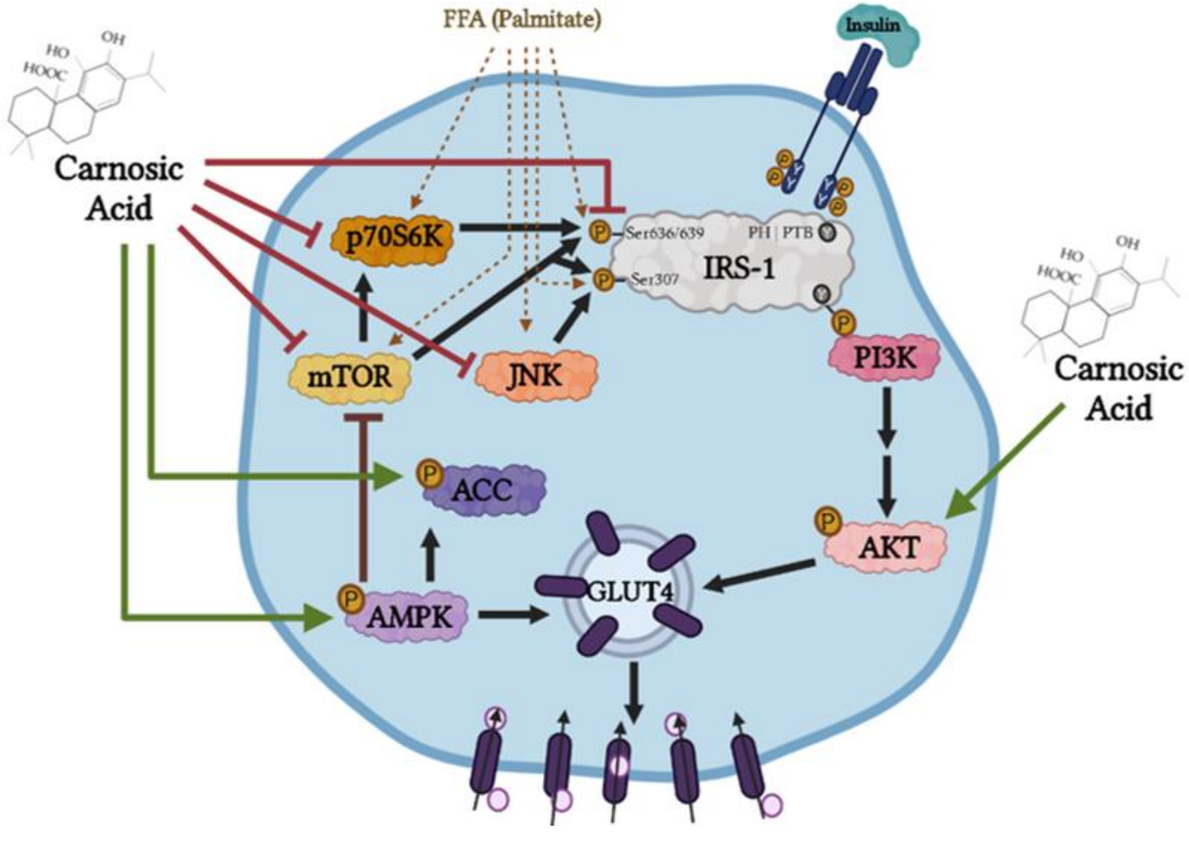
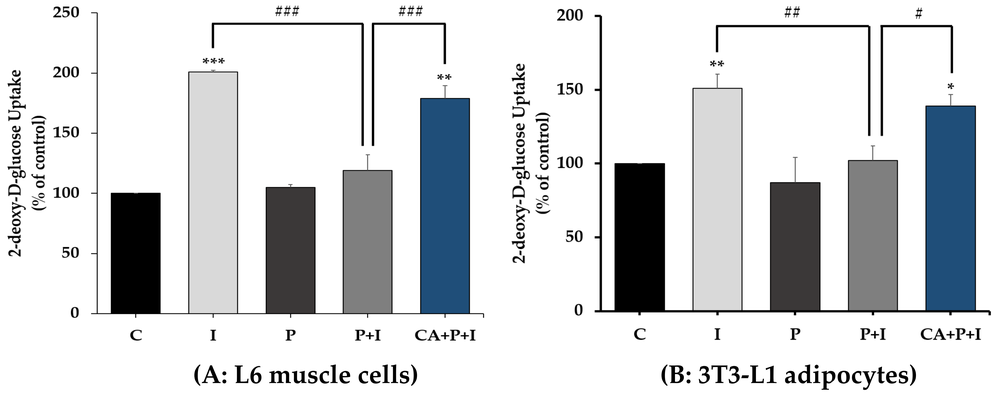

FFA (Palmitate)
Insulin
Carnosic Acid
p70S6K
Ser636/639
PH | PTB
Ser307
IRS-1
mTOR
JNK
PI3K
ACC
AKT
AMPK
GLUT4
Carnosic Acid



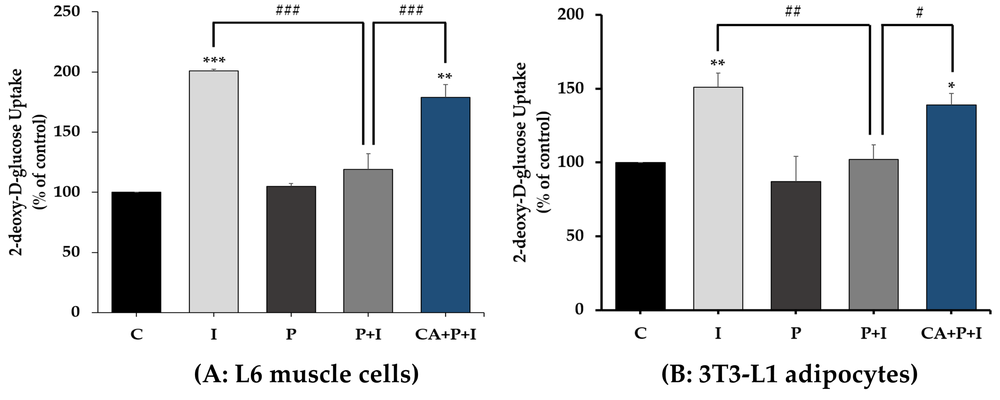


Figure 1: Carnosic acid restores the insulin-stimulated glucose uptake in palmitate treated skeletal muscle (A) and fat cells (B). Fully differentiated L6 myotubes (A) and 3T3-L1 adipocytes (B) were treated without (control, C) or with 0.2 mM palmitate (P) for 16 hours in the absence or the presence of 2 μM carnosic acid (CA) followed by stimulation without or with 100 nM insulin (I) for 30 minutes and [3H]-2-deoxy-D-glucose uptake measurements. The results are the mean ± standard error (SE) of four to six independent experiments, expressed as percent of control (** $p<0.01$, *** $p < 0.001$ vs. control, ### $p < 0.001$, ## $p < 0.01$ vs. insulin alone, # $p < 0.05$ vs. P+I).

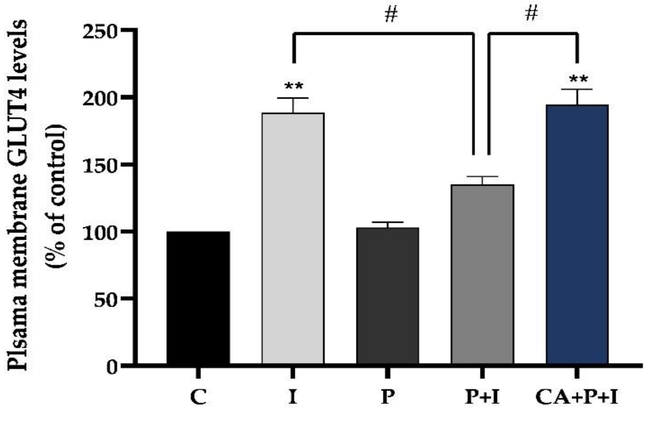


Figure 2: Effects of palmitate and carnosic acid on GLUT4 translocation. GLUT4myc overexpressing L6 myotubes were treated without (control, C) or with 0.2 mM palmitate (P) for 16 hours in the absence or the presence of 2 μM carnosic acid (CA) followed by washing, as indicated in the methods, and acute stimulation with 100 nM insulin for 30 minutes (I). After treatment, plasma membrane GLUT4 glucose transporter levels were measured. Results are the mean ± SE of three independent experiments performed in triplicate (** $p < 0.01$ vs. control, # $p < 0.05$ as indicated).

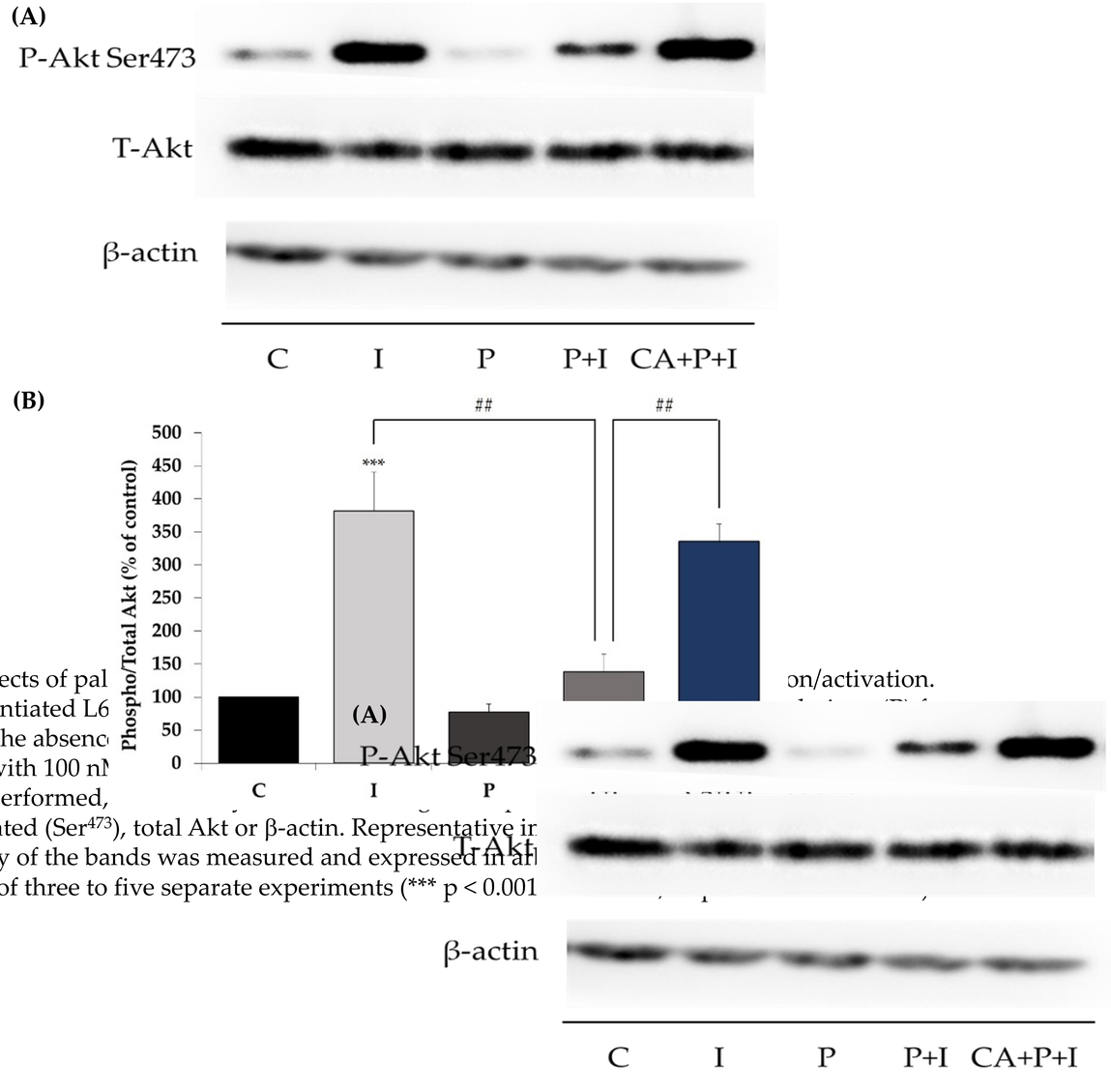
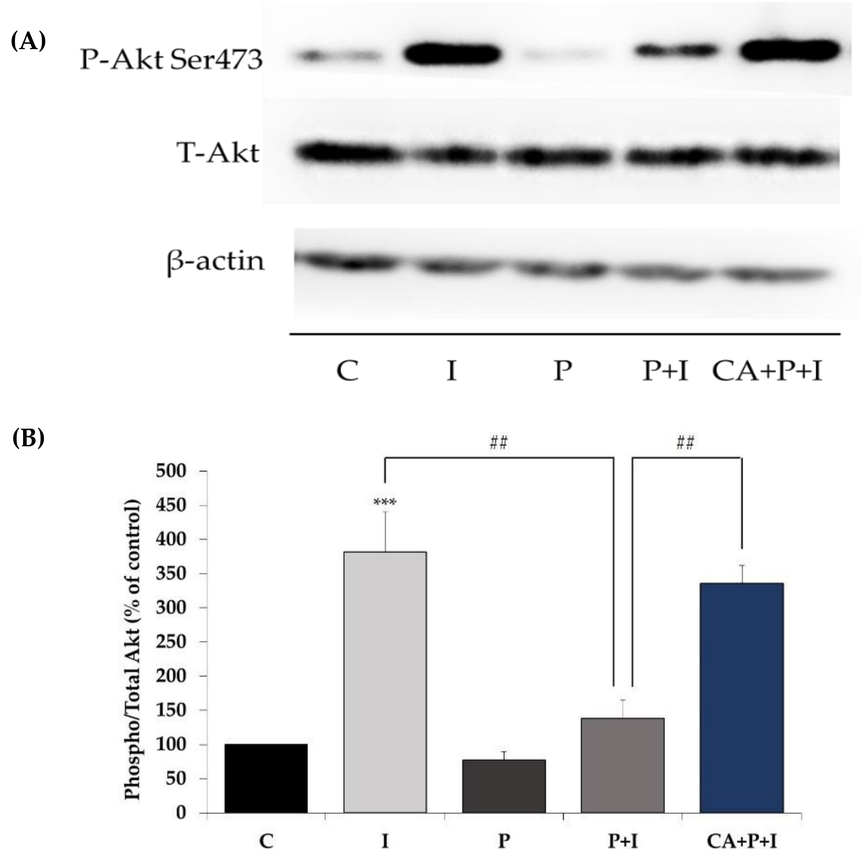
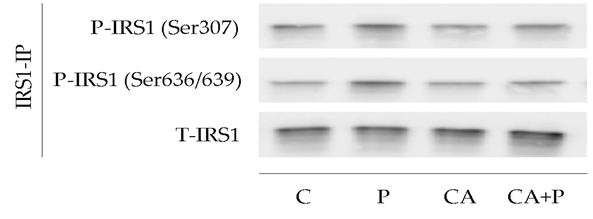
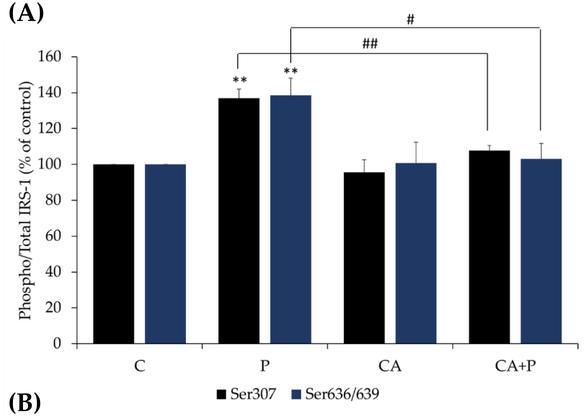


Figure 3. Effects of pal on/activation. Fully differentiated L6 16 hours in the absenc without or with 100 n PAGE was performed, phosphorylated (Ser[473]), total Akt or β-actin. Representative ir densitometry of the bands was measured and expressed in ar mean ± SE of three to five separate experiments (*** p < 0.001

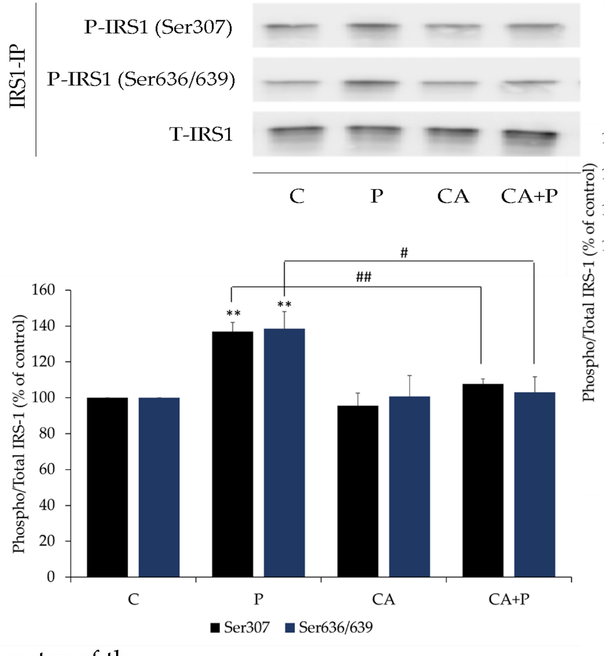




(A)



(B)



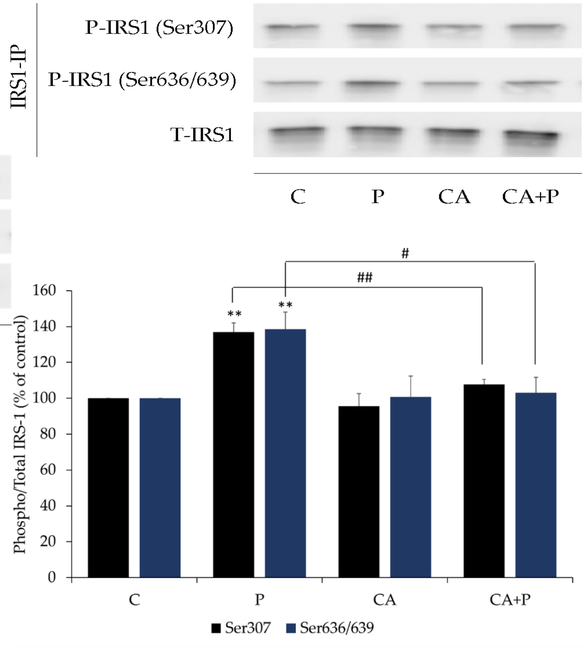
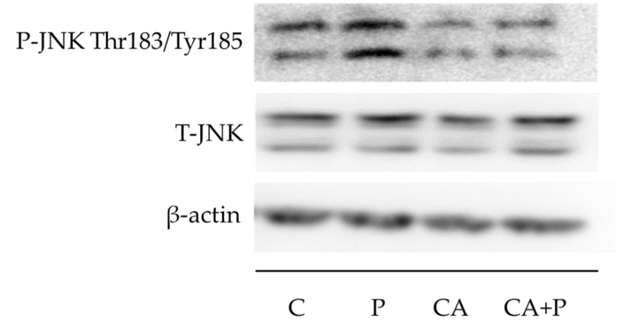
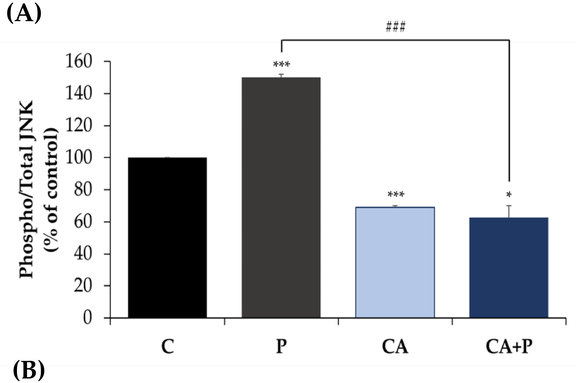
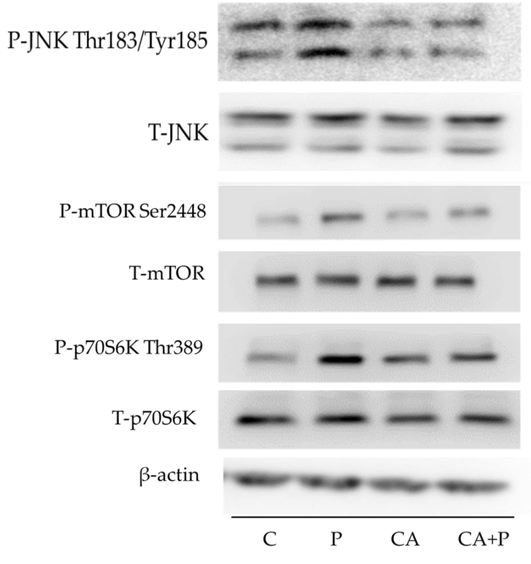
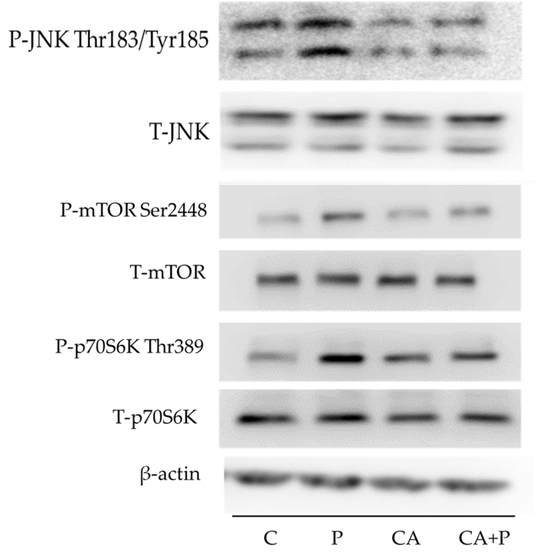


Figure 4. Effects of palmitate and carnosic acid on serine phosphorylation and e
IRS-l. Fully differentiated myotubes were treated without (control, C) or with 0
palmitate (P) for 16 hours in the absence or presence of 2 μM carnosic acid (CA
treatment, the cells were lysed, and IRS-1 immunoprecipitation was performed
SDS-PAGE and immunoblotting with specific antibodies that recognize phospl
Ser$^{636/639}$ or total IRS-1. Representative immunoblots are shown (A). The densitometry of the bands was measured and expressed in arbitrary units (B). The data is the mean ± SE of three separate experiments. (** $p < 0.01$ vs. control, ## $p < 0.01$ vs. palmitate alone).









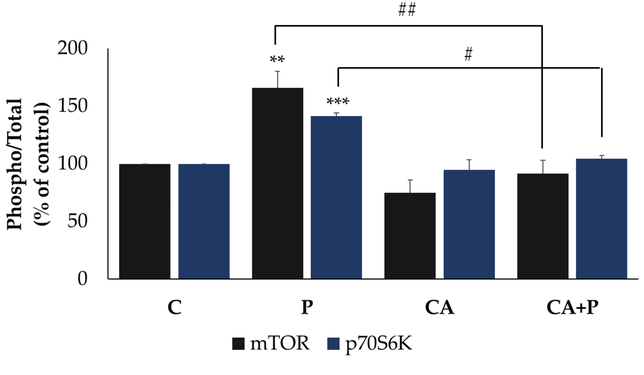


Figure 5. Effects of palmitate and carnosic acid on JNK expression and phosphorylation. Fully differentiated myotubes were treated without (control, C) or with 0.2 mM palmitate (P) for 16 hours in the absence or the presence of 2 μM carnosic acid (CA). After treatment, the cells were lysed, and SDS-PAGE was performed, followed by immunoblotting with specific antibodies that recognize phosphorylated Thr[183]/Tyr[185] or total JNK. Representative immunoblots are shown (A). The densitometry of the bands was measured and expressed in arbitrary units (B). The data are the mean ± SE of three separate experiments (*** $p < 0.001$ vs. control, ### $p < 0.001$ vs. palmitate alone).

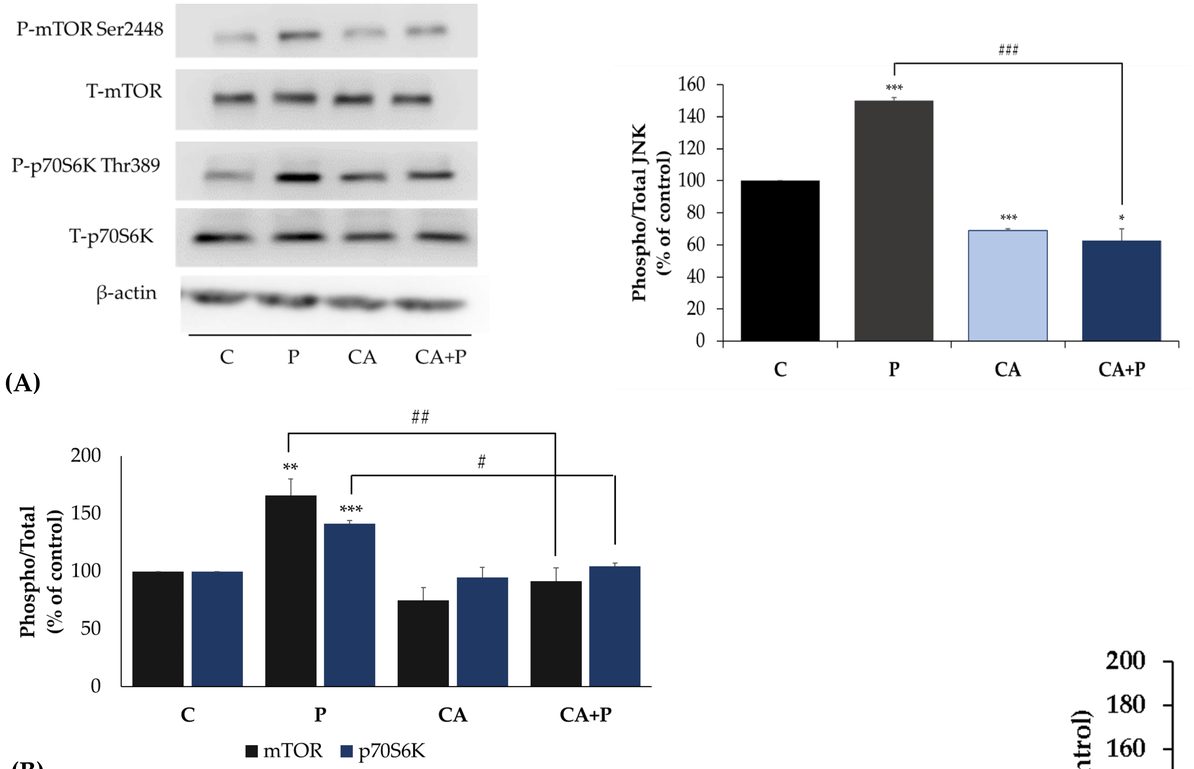
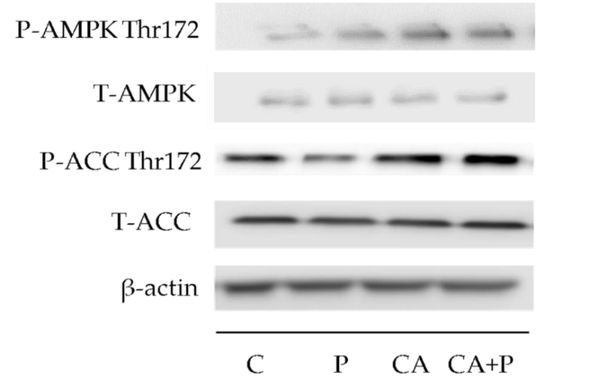
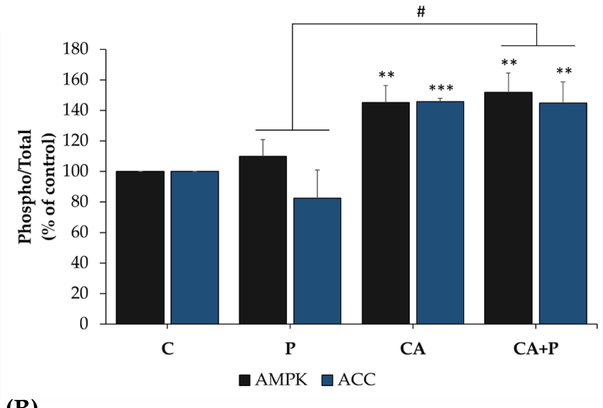
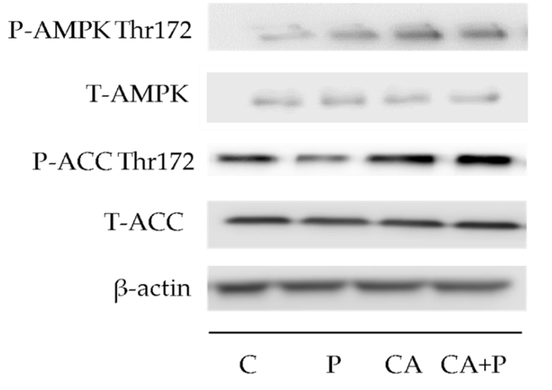
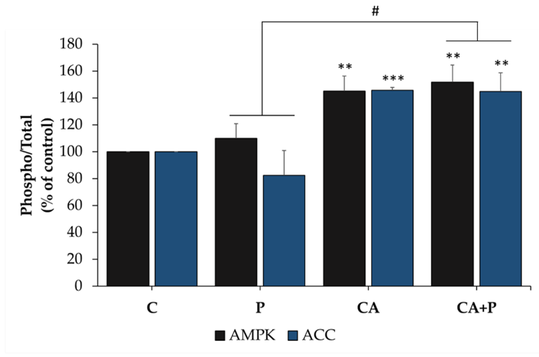


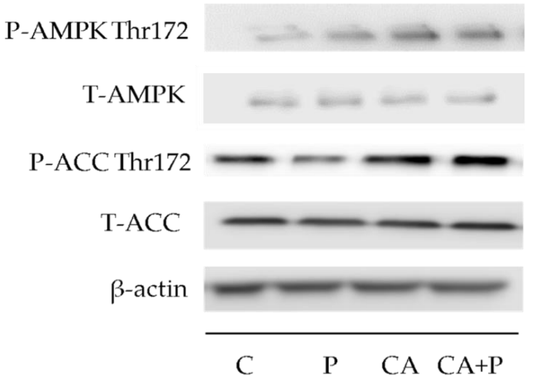
Figure 6. Effects of palmitate and carnosic acid on mTOR and p70S6K expression and phosp
Fully differentiated myotubes were treated without (control, C) or with 0.2 mM palmitate (I
hours in the absence or the presence of 2 μM carnosic acid (CA). After treatment, the cells w
and SDS-PAGE was performed, followed by immunoblotting with specific antibodies that r
phosphorylated Ser^{2448} or total mTOR and Thr^{389} or total p70S6K. Representative immunobl
shown (A). The densitometry of the bands was measured and expressed in arbitrary units (
are the mean ± SE of four separate experiments (*** $p < 0.001$ vs. control, ### $p < 0.001$ vs. p
alone).











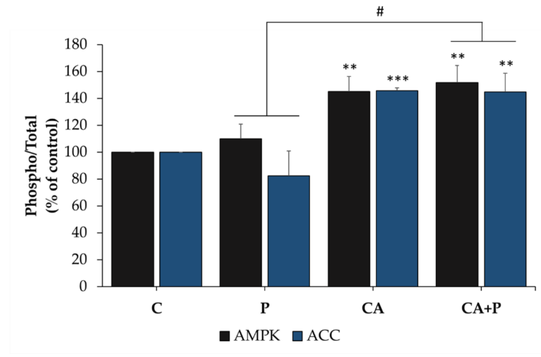


Figure 7. Effects of palmitate and carnosic acid on AMPK expression and phosphorylation. Fully differentiated myotubes were treated without (control, C) or with 0.2 mM palmitate (P) for 16 hours in the absence or the presence of 2 μM carnosic acid (CA). After treatment, the cells were lysed, and SDS-PAGE was performed, followed by immunoblotting with specific antibodies that recognize phosphorylated Thr^{172} or total AMPK and Ser^{79} or total ACC. Representative immunoblots are shown (A). The densitometry of the bands was measured and expressed in arbitrary units (B, C). The data is the mean ± SE of three separate experiments. (** $p < 0.01$, *** $p < 0.001$ vs. control, # $p < 0.05$ vs. palmitate alone).