

Leukocyte antigen-related inhibition attenuates palmitate-induced insulin resistance in muscle cells

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Abstract

Palmitate has been shown to induce insulin resistance in skeletal muscle cells. The aim of this study was to investigate the role of the leukocyte common antigen-related (*LAR*) gene in palmitate-induced insulin resistance in C2C12 cells. A stable C2C12 cell line was generated using *LAR* short hairpin RNA. The levels of *LAR* protein and phosphorylation of insulin receptor substrate-1 (*IRS1*) and Akt were detected by western blot analysis. 2-Deoxyglucose uptake was measured in *LAR* knockdown and control cells using D-[2-³H]glucose. *LAR* protein level was decreased by 65% in the stable cell line compared with the control cells. Palmitate (0.5 mM) significantly induced *LAR* mRNA (65%) and protein

levels (40%) in myotubes compared with untreated cells. Palmitate significantly reduced insulin-stimulated glucose uptake in both the control and *LAR* knockdown cells by 33 and 51% respectively. However, *LAR* depletion improved insulin-stimulated glucose uptake in myotubes treated with palmitate. Furthermore, the inhibition of *LAR* prevented palmitate-induced decreases in phosphorylation of *IRS1*^{Tyr632} and Akt^{Ser473} in C2C12 cells. In conclusion, these results reveal that palmitate induces *LAR* expression in C2C12 cells. We also provided evidence that the inhibition of *LAR* attenuates palmitate-induced insulin resistance in myotubes.

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Introduction

Insulin resistance plays a major role in the development of some diseases such as type 2 diabetes (T2D) and metabolic syndrome (Czech 2002). Resistance to the cellular actions of insulin causes reduced glucose uptake in muscle, increased glucose production and VLDL secretion in the liver, and enhanced lipolysis in adipose tissue (Meshkani & Adeli 2009). Muscle insulin resistance has a key role in whole body glucose homeostasis, as almost 80% of insulin-dependent glucose uptake in the body occurs in this tissue (Pessin & Saltiel 2000).

At the molecular level, the mechanisms underlying insulin resistance in muscle are not well understood. An elevated plasma-free fatty acid (FFA) level has been proposed to induce insulin resistance in muscle of obese and insulin-resistant subjects (Reynoso *et al.* 2003). The glucose fatty acid cycle is a mechanism that has been proposed, but it appears that defects in post-receptor signaling are the major cause of insulin resistance in target tissues (Bakhtiyari *et al.* 2010). Several reports from human and animal studies suggest that increased FFA levels result in insulin signaling defects in muscle cells (Dresner *et al.* 1999, Kruszynska *et al.* 2002). A decreased autophosphorylation of the insulin receptor has been reported

in muscle and adipose tissues of T2D patients. Also, reduced expression of PI3 kinase (PI3-K) and PKB has been described in skeletal muscle of lean and obese individuals (Björnholm *et al.* 1997). Also, we have recently demonstrated that palmitate and an inflammatory state can induce insulin resistance by increasing the expression of protein tyrosine phosphatase non-receptor 1 (PTPN1) in muscle cells (Bakhtiyari *et al.* 2010, Parvaneh *et al.* 2010, MohammadTaghvaei *et al.* 2011).

PTPases are a large family of enzymes that have critical roles in many cellular functions (den Hertog *et al.* 2008). Leukocyte antigen-related (*LAR*) is a receptor-like tyrosine phosphatase with a broad tissue distribution (den Hertog *et al.* 2008). *LAR* has a prominent role in nervous system development that is conserved throughout evolution (Chagnon *et al.* 2004). *LAR* is also expressed in insulin-responsive tissues such as muscle, liver, and adipose tissues. In liver cells, *LAR* has a high expression, whereas a low expression level was observed in skeletal muscle (Norris *et al.* 1997). *LAR* is expressed as a 200 kDa proprotein and spans the plasma membrane once with an extracellular amino-terminus. *LAR* catalytic activity is most likely negatively regulated by dimerization (Chagnon *et al.* 2004).

LAR has some roles in different cellular functions such as establishing and maintaining neuronal networks, apoptosis,

and glucose homeostasis (den Hertog *et al.* 2008). There are several lines of evidence that LAR acts as inhibitor of insulin action. Overexpression studies of LAR showed that this phosphatase can negatively regulate insulin receptor and Shc phosphorylation, as well as the PI3-K and MAPK pathways (Kulas *et al.* 1995, 1996). Insulin receptor substrate-1 (IRS1) is also a substrate for LAR *in vitro* (Calera *et al.* 2000, Goldstein *et al.* 2000). In addition, antisense inhibition of LAR gene expression in cultured cells enhanced insulin signaling (Kulas *et al.* 1995, Force *et al.* 2004). Lar-deficient mice exhibited significantly lower levels of plasma glucose as well as a reduced rate of hepatic glucose production in the fasting state (Ren *et al.* 1998). A significant resistance to insulin-stimulated glucose disposal and suppression of hepatic glucose output were also observed in this animal model (Ren *et al.* 1998). Although LAR is not required for embryonic development, it seems to be necessary for mammary gland development (Schaapveld *et al.* 1997). Furthermore, obese, insulin-resistant, and T2D patients showed a high expression of LAR in their muscle and liver tissues (Ahmad *et al.* 1995, 1997). Taken together, these studies reveal that LAR negatively regulates insulin signaling and that elevated expression of LAR might contribute to the pathogenesis of insulin resistance. However, the main responsible factor for the induction of LAR in insulin target tissues especially in muscle is not completely understood. In this study, we aimed to investigate the importance of palmitate in the expression of LAR in C2C12 muscle cells. We also studied the role of LAR in palmitate-induced insulin resistance by inhibiting its expression in muscle cells.

Materials and Methods

Materials

C2C12 myoblasts were purchased from the Pasteur Institute of Iran. DMEM, fetal bovine serum (FBS), and horse serum were obtained from Gibco-BRL. Other cell culture reagents were from Sigma-Aldrich. The RNA isolation kit, primer assays for LAR, and β -actin were purchased from Qiagen. SYBR Green PCR kit was purchased from Takara (Shiga, Japan). Anti-mouse LAR antibody and anti-goat IgG-HRP were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). HRP-linked mouse IgG, chemiluminescence western blotting detection kit, and [3 H]2-deoxyglucose ([3 H]2-DOG) were purchased from GE Healthcare (Heidelberg, Germany). All other chemicals were from Merck Chemicals. Tissue culture vessels and disposable plastic wares were purchased from Greiner Bio-One (Frickenhhausen, Germany).

Methods

Cell culture C2C12 myoblast was purchased from the Pasteur Institute of Iran. Myoblasts were maintained at 37 °C (in an atmosphere of 5% CO₂) in DMEM (Gibco) containing 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin.

Differentiation of myoblasts into myotubes was induced when the cells had achieved 70–90% confluence by replacing the media with DMEM containing 2% horse serum. All treatments were performed at the end of the fourth day of differentiation.

FFA treatment Lipid-containing media were prepared by a modification of the method of Svedberg *et al.* (1990). Palmitate was dissolved in ethanol and then diluted with pre-warmed DMEM containing fatty acid-free BSA (1% w/v). C2C12 myotubes were treated with vehicle and 0.25 and 0.5 mM palmitate for 16 h.

Gene expression analysis After treatments, the cells were harvested and total RNA was extracted using RNeasy mini kit. Total RNA was reverse transcribed using MMulv reverse transcriptase and random hexamer primer. LAR expression level was quantified using QuantiTect primer assay for LAR and QuantiFast SYBR Green PCR Master Mix. The levels of LAR transcript were normalized relative to β -actin. The amplification protocol for 40 cycles was as follows: 10 s at 95 °C for initial activation, 5 s at 95 °C for denaturation, and 20 s at 60 °C for annealing/extension.

Western blot analysis Tissue lysate was prepared by homogenization in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 1 mM Na-EDTA, and 1 mM phenylmethylsulphonyl fluoride) supplemented with protease inhibitor cocktail (Roche). For detection of phosphoprotein, a buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM Na₄P₂O₇, 2 mM NaVO₄, and protease inhibitor cocktail was used. After determining protein concentrations in the supernatant, equal amounts of protein were subjected to SDS-PAGE, followed by transfer onto PVDF membrane. Blocking was carried out through overnight incubation at 4 °C with 5% nonfat dry milk in TBS with 0.5% Tween 20. Blots were incubated with antibodies against LAR, p-IRS1 (Tyr632), and IRS1 (Santa Cruz Biotechnology); Akt and phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, MA, USA); and β -actin (Abcam, Cambridge, MA, USA). Antibody binding was visualized using a chemiluminescent substrate. Each experiment was performed at least three times.

Plasmid preparation Double-stranded oligonucleotides containing an inverted repeat of the LAR siRNA sequence (5'-AGATTCGGTGGCTACCAGGTCACCTAT-3') were designed using the pSilencer insert design tool and cloned into pRS vector. The scrambled sequence short hairpin RNA (shRNA) vector was used as a control. The presence of the insert was confirmed by digestion of pRS vector with BamHI and HindIII.

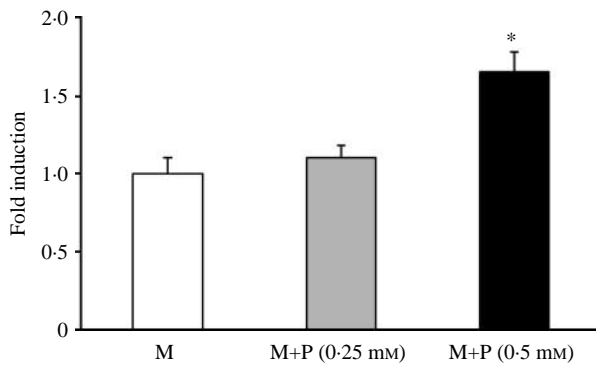


Figure 1 The effect of palmitate on *LAR* mRNA expression in the differentiated C2C12 cells. After differentiation, the myotubes were exposed to 0.25 and 0.5 mM BSA-complexed palmitate (M+P) or BSA alone (M), as control, for 16 h. *LAR* mRNA levels were quantified by real-time PCR and were normalized relative to β -actin. * $P < 0.01$ vs M.

Myoblast transient transfection C2C12 myoblasts were transiently transfected with *LAR* shRNA and the scrambled shRNA plasmids using the calcium phosphate precipitation technique. In brief, a day before transfection, 5×10^5 cells/well were seeded in 0.1% gelatin-coated six-well cluster plates. The plates were transfected with 15 μ g shRNA and pEGFP-C1 plasmids (as control for transfection efficiency).

Stable transfection For stable transfection, 48 h after transfection, myoblasts were trypsinized and divided into fresh medium containing 4 μ g/ml puromycin (Sigma–Aldrich). The cells were divided such that they are no more than 25% confluent and then were nourished with selective medium every 3–4 days. In the next step, the puromycin-resistant colonies were pooled and nourished with the selection medium for 2 weeks. Stable myoblasts were harvested and the *LAR* protein levels were assessed using western blot.

Glucose uptake assay To assess the effect of *LAR* knockdown on insulin resistance induced by palmitate, glucose uptake assays were performed. Glucose uptake measurements were performed in triplicate and in three independent experiments. In brief, after 4 days of differentiation, the myotubes were treated with 0.5 mM palmitate for 16 h followed by a serum starvation of 2–3 h in DMEM plus 0.1% BSA. Myotubes were then treated with or without 100 nM insulin for 30 min and washed two times with wash buffer (20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, and 1 mM $CaCl_2$). Myotubes were then incubated in the transport buffer (wash buffer containing 0.5 mCi [3H]2-DOG/ml and 10 μ M 2-DOG) for 10 min. The cells were lysed in 0.05 M NaOH, followed by liquid scintillation counting. Aliquots of each well were used to determine protein concentration.

Statistical analysis All statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA). Comparisons

among all groups were performed with the one-way ANOVA test. If statistical significance was found, the Tukey's *post hoc* test was performed. Values of $P < 0.05$ were considered statistically significant. Results are expressed as mean \pm S.E.M. of at least three independent experiments.

Results

The effects of palmitate on LAR mRNA and protein levels in the differentiated C2C12 cells

To investigate the effect of palmitate on *LAR* mRNA expression, the differentiated C2C12 cells were treated with BSA and 0.25 and 0.5 mM palmitate for 16 h. Palmitate (0.25 mM) did not significantly alter *LAR* mRNA expression, but 0.5 mM palmitate significantly increased *LAR* mRNA level (65%, $P < 0.01$) in the myotubes compared with the cells treated with BSA (Fig. 1). Similar to mRNA levels, only 0.5 mM palmitate significantly enhanced *LAR* protein level (40%, $P < 0.01$) compared with the untreated cells (Fig. 2).

Inhibiting the expression of LAR in C2C12 cells

To address the role of *LAR* in palmitate-induced insulin resistance, *LAR* knockdown C2C12 cells were generated. pRS and EGFP-C1 plasmids were successfully delivered to myoblasts with high efficiency using the calcium phosphate precipitation technique. Pooled *LAR* shRNA colonies with 65% reduction in *LAR* protein level and the scrambled shRNA colonies were selected for further investigations (Fig. 3).

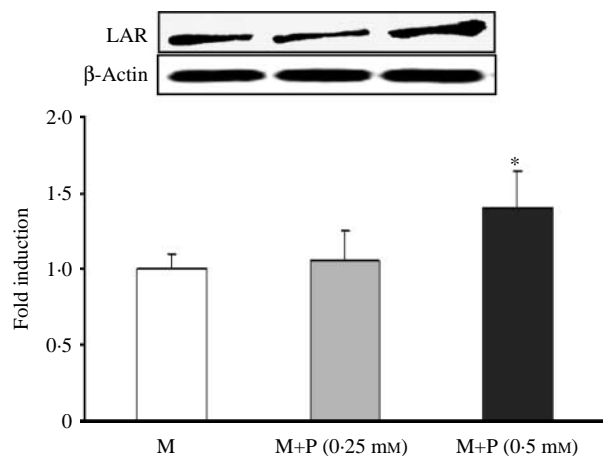


Figure 2 The effect of palmitate on *LAR* protein level in C2C12 myotubes. After differentiation, the myotubes were exposed to 0.25 and 0.5 mM BSA-complexed palmitate (M+P) or BSA alone (M), as control, for 16 h. Protein levels were quantified by western blot and were normalized relative to β -actin. * $P < 0.01$ vs M.

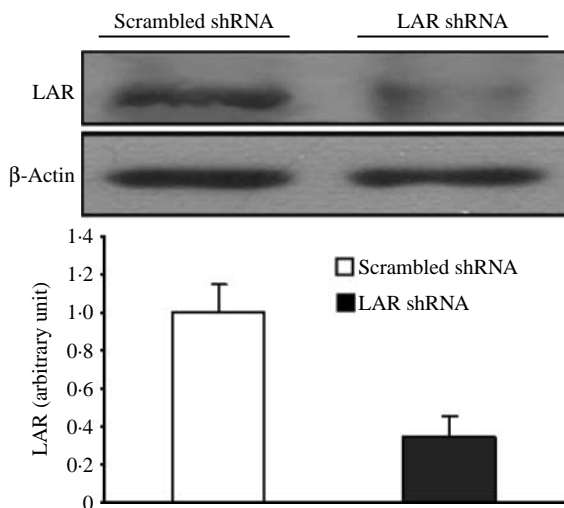


Figure 3 Knockdown of LAR in C2C12 cells following stable transfection with shRNA expression pRS plasmid. After selection of stable clone, western blot was performed with antibodies against LAR and β -actin. Protein levels of LAR were normalized relative to protein level of β -actin.

Enhancement of glucose uptake in LAR knockdown cells treated with palmitate

To determine the significance of LAR in muscle insulin resistance, [3 H]2-DOG uptake was measured. In the basal condition, LAR knockdown stable and control cells did not show a significant difference in the uptake of glucose (Fig. 4). Insulin stimulation led to an increase in glucose uptake in both cell lines, whereas LAR knockdown cells showed much higher sensitivity to insulin in comparison with the control cells (93 vs 35%, $P < 0.001$). Insulin-stimulated glucose uptake was decreased by palmitate in both the control and the knockdown cells by 33 ($P < 0.01$) and 51% ($P < 0.01$) respectively. Importantly, in the simultaneous presence of insulin and palmitate, LAR knockdown cells remained 40% ($P < 0.01$) more sensitive to insulin than the control cells. These results indicated that the inhibition of LAR induces a modest increase in glucose uptake in C2C12 cells even in the presence of palmitate.

Enhancement of insulin signaling by inhibiting LAR

In order to provide more evidence for the importance of LAR inhibition in palmitate-induced muscle insulin resistance, we assessed the insulin signaling pathway in LAR knockdown cells. The results showed that LAR knockdown cells had higher insulin-induced phosphorylation of IRS1 (Tyr632) compared with the control cells (40%, $P < 0.01$). Palmitate (0.5 mM) reduced IRS1 phosphorylation in control cells (35%, $P < 0.01$), whereas the inhibition of LAR-prevented palmitate reduced IRS1 phosphorylation. Interestingly, the LAR knockdown cells in the presence of

palmitate had 70% ($P < 0.01$) higher IRS1 phosphorylation levels compared with the controls (Fig. 5). We also assessed the effects of palmitate on Akt phosphorylation in the cells (Fig. 6). Akt phosphorylation was reduced by palmitate in both the control and the knockdown cells by 45% ($P < 0.001$); however, the LAR knockdown cells showed a higher Akt phosphorylation (65%, $P < 0.01$) compared with the control cells. Taken together, these results provide evidence that LAR is a mediator of palmitate-induced insulin resistance in muscle cells.

Discussion

An increased FFA level is one of the mechanisms leading to insulin resistance in muscle. It is now commonly accepted that FFAs, especially palmitate, reduce the tyrosine kinase activity of the insulin receptor in muscle cells (Reaven 1988, Czech 2002). However, the target molecules for FFAs in insulin signaling are not completely understood. We have recently demonstrated that palmitate can induce insulin resistance in muscle cells by enhancing the expression of PTP1B (MohammadTaghvaei *et al.* 2011, 2012). In this study, we provided more evidence that LAR is also a new mediator of palmitate-induced muscle insulin resistance. Here, we showed that 0.5 mM palmitate can induce LAR mRNA and protein levels in myotubes, whereas the lower concentration of this fatty acid seems to have no effect on LAR expression. It should be noted that this concentration of palmitate (0.5 mM) has been previously shown to induce insulin resistance in skeletal muscle cells (Lee *et al.* 2006, Coll *et al.* 2008). The results of this experiment imply that a high concentration of palmitate can be suggested as one of the factors that increase LAR expression in muscle of obese and diabetic patients (Ahmad *et al.* 1995).

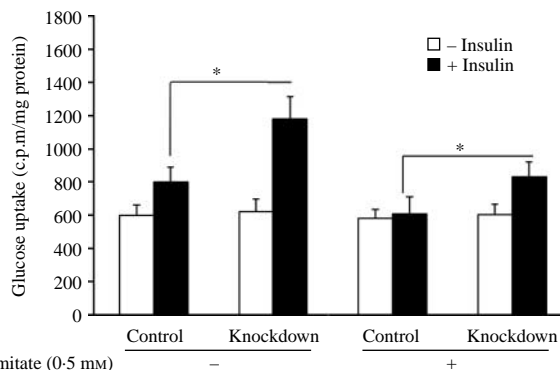


Figure 4 The effect of palmitate on glucose uptake in LAR knockdown stable and control C2C12 cells. Myotubes were grown in the absence and presence of 0.5 mM palmitate for 16 h followed by serum starvation for 2 h in 1% FBS-DMEM. Then, glucose uptake was measured in the presence or absence of insulin and cytochalasin B. The figure shows representative data gained from mean \pm s.e.m. of three independent experiments, * $P < 0.01$.

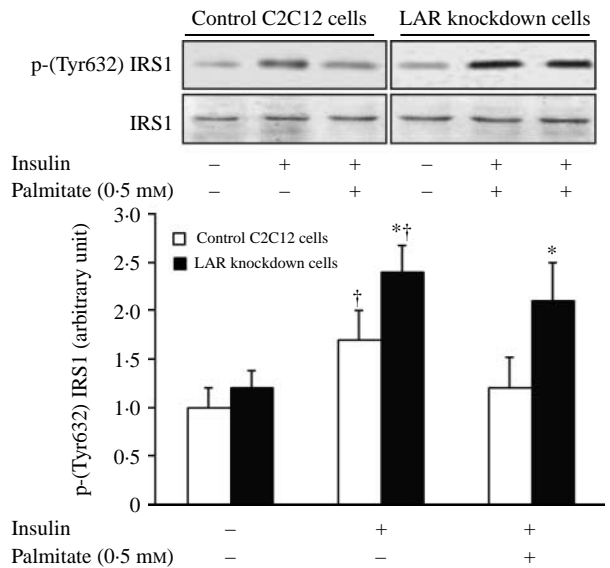


Figure 5 The effect of palmitate on tyrosine 632 phosphorylation status of IRS1 in LAR knockdown and the control cells expressing the scrambled shRNA. Myotubes were treated with 0.5 mM palmitate for 16 h. Before harvesting, the cells were incubated in the presence or absence of 100 nM insulin for 15 min. Cell lysates were prepared and subjected to western blot using specific antibodies. The figure shows representative data gained from the mean \pm S.E.M. of three independent experiments, * P <0.05 vs control cells expressing the scrambled shRNA, † P <0.05 vs the cells treated with palmitate.

Several *in vivo* and *in vitro* studies have been conducted to show the effect of LAR on insulin signaling (Kulas *et al.* 1995, 1996, Goldstein *et al.* 2000); however, little is known about the direct role of LAR in insulin resistance when a fatty acid is present. To investigate more precisely the importance of LAR in palmitate-induced insulin resistance, a stable cell line expressing LAR shRNA was generated. This stable cell line showed a 35% protein level of the control C2C12 cell. To study the influence of LAR inhibition on the insulin signaling pathway, two key molecules (IRS1 and Akt) of this pathway were selected. IRS1 phosphorylation indirectly leads to activation of Akt by activating PI3-K (Esposito *et al.* 2001). Phosphorylation of Akt is an important factor in insulin-induced glucose metabolism in the muscle and liver (Hirata *et al.* 2003). In this study, as expected, insulin stimulated the phosphorylation of Akt and IRS1 in the knockdown and control cells. Palmitate led to a decrease in the level of phosphorylation of IRS1 and Akt, and these findings are in concordance with the previous studies showing that palmitate induces insulin resistance in skeletal muscle by inhibiting the activity of molecules of the insulin signaling pathway (Storz *et al.* 1999, Chavez & Summers 2003, Bakhtiyari *et al.* 2010). However, it appears that the inhibition of LAR ameliorates the effect of palmitate on insulin signaling molecules in myotubes. Even in the presence of palmitate, LAR knockdown cells had higher IRS1 and Akt phosphorylation

compared with the cells receiving the scrambled shRNA. It is worthy to note that the effect of LAR on the insulin signaling pathway has been shown previously in different cell culture and *in vivo* studies. It has been reported that LAR overexpression leads to decreased IRS1 phosphorylation *in vitro* (Calera *et al.* 2000, Goldstein *et al.* 2000). Antisense suppression of LAR enhanced insulin-dependent insulin receptor phosphorylation and PI3-K activity in hepatoma cells (Kulas *et al.* 1995, Mooney *et al.* 1997). Furthermore, insulin-stimulated phosphorylation of IRS1 and IRS2 was decreased in muscle of transgenic mice that overexpress LAR in skeletal muscle (Zabolotny *et al.* 2001). However, in contrast to the results of this study, one study showed that knockdown of LAR induces post-receptor defects with reduced insulin-induced activation of Akt and phosphorylation of insulin receptor and IRS1 in HEK293 cells (Mander *et al.* 2005). The difference between these results and those herein may be due to use of different cell types. We also assessed glucose uptake in knockdown and control cells. We observed that the inhibition of LAR increased glucose uptake even when the myotubes were treated with palmitate. This finding is consistent with the other study in mice with muscle-specific overexpression of LAR. Transgenic mice showed reduced insulin signaling, decreased glucose uptake in muscle, and impaired whole body glucose disposal (Zabolotny *et al.* 2001). All together, these findings suggest that LAR, as previously shown, is a negative regulator of

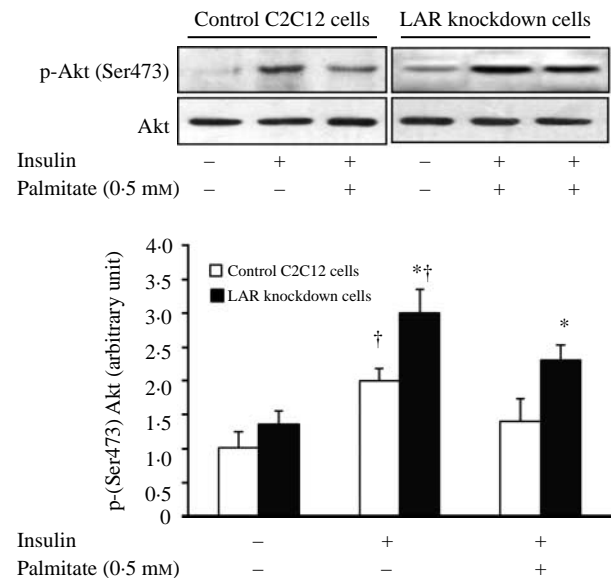


Figure 6 The effect of palmitate on serine 473 phosphorylation status of Akt in LAR knockdown and the control cells expressing the scrambled shRNA. Myotubes were treated with 0.5 mM palmitate for 16 h. Before harvesting, the cells were incubated in the presence or absence of 100 nM insulin for 15 min. Cell lysates were subjected to western blot using specific antibodies. The figure shows representative data gained from the mean \pm S.E.M. of three independent experiments, * P <0.05 vs control cells expressing the scrambled shRNA, † P <0.05 vs the cells treated with palmitate.

insulin signaling and its inhibition ameliorates palmitate-induced insulin resistance in muscle cells by increasing the activity of key molecules of the insulin signaling pathway.

In summary, the data presented here indicate that palmitate might be the responsible factor for the overexpression of LAR in insulin resistance state. Our results also show that the LAR knockdown attenuates insulin resistance induced by palmitate in C2C12 cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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