PLIN2 inhibits insulin-induced glucose uptake in myoblasts through the activation of the NLRP3 inflammasome

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Received January 8, 2015; Accepted June 29, 2015

DOI: 10.3892/ijmm.2015.2276

Abstract. Impaired lipid metabolism and inflammatory pathways have individually been implicated in the development of insulin resistance in skeletal muscle; however, little evidence is available to date linking the two in this context. In this study, we explored a potential molecular mechanism underlying insulin resistance in myoblasts mediated by the crosstalk between lipid accumulation and inflammatory pathways. We examined the influence of perilipin 2 (PLIN2), one of the most highly expressed lipid droplet-associated proteins in skeletal muscle, on glucose uptake and on the nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome *in vitro*. PLIN2 overexpression in C2C12 cells led to an increased expression of NLRP3, caspase-1 and interleukin (IL)-1β, along with an impaired insulin-induced glucose uptake. This defect was remedied by the RNAi-mediated knockdown of NLRP3 expression. We also found that insulin receptor substrate-1 (IRS-1), a component of insulin signaling, was negatively regulated by NLRP3 and IL-1β, and that IL-1β inhibited insulin-induced glucose uptake in myoblasts. These results suggest that PLIN2 inhibits insulin-induced glucose uptake by activating NLRP3, caspase-1 and IL-1β, leading to a decreased IRS-1 expression. This study provides *in vitro* evidence supporting an association between lipid metabolism and inflammatory pathways in the pathogenesis of insulin resistance in skeletal muscle, and suggests potential therapeutic targets that warrant further investigation.

Introduction

Skeletal muscle accounts for the majority of insulin-stimulated glucose uptake and is thus a key organ for the development of insulin resistance. Disordered lipid metabolism is considered a major contributor to skeletal muscle insulin resistance (1). Impaired insulin signaling, as observed in subjects with obesity and type 2 diabetes, is strongly associated with an excess accumulation of triacylglycerols (TAGs) within muscle fibers in skeletal muscle (2-5). TAGs are mainly stored in neutral lipid droplets (LDs), coated by lipid droplet-associated proteins which are referred to as perilipins (6,7). One of the 5 perilipins (PLINs), PLIN2, is a marker for LDs in human skeletal muscle, and the levels of intramuscular PLIN2 and triglycerides are closely correlated. Studies have reported increased TAG accumulation and lipid droplet formation when PLIN2 is overexpressed *in vitro* (8-10). Conversely, the knockdown of PLIN2 in macrophages has been shown to decrease the size and number of cellular lipids and LDs (8). A previous study using a rat model of diabetes demonstrated that increased levels of Plin2 in skeletal muscle correlated with insulin resistance when the rats were fed a high-fat diet (11). Plin2 antisense oligonucleotides have been shown to protect mice from insulin resistance when fed high-fat diets (12). These studies indicate a connection between PLIN2 dysregulation and the development of insulin resistance; however, the molecular mechanisms underlying this response are not yet fully understood.

Inflammation also contributes to the development of insulin resistance (13-15). The nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome is a group of protein complexes that sense a diverse set of host-derived stimuli, such as damage-associated molecular patterns (DAMPs) and control the production of important pro-inflammatory cytokines, such as interleukin (IL)-1β (16). The NRLP3 inflammasome enhances insulin resistance by triggering inflammation in adipose tissue in subjects with obesity (16,17).

In this study, we found that PLIN2 overexpression led to the activation of the NLRP3 inflammasome in C2C12 cells, providing evidence that the PLIN2 inhibition of insulin-induced glucose uptake is linked to both defects in lipid metabolism and to inflammation.

Materials and methods

Cell culture. C2C12 mouse myoblasts (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (both from

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Key words: PLIN2, insulin resistance, skeletal muscle, NLRP3 inflammasome

Sigma-Aldrich). All cells were maintained in a 5% CO_2 incubator at 37˚C. After the cryopreserved cells were thawed, they were subcultured for at least 2 passages, and experiments were performed at 24 h post-seeding.

Preparation of PLIN2 expression constructs. Human PLIN2 cDNA, including the open reading frame and 3' untranslated region (UTR), was amplified using *PfuTurbo* DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) with primers that included the 5' *Xho*I and *Xba*I restriction sites. The polymerase chain reaction (PCR) primers were as follows: forward, 5'-ctcgagaagaaaaatggcatccgttgcagttgatcca-3' and reverse, 5'-tctagaaactggtctatcctgcagtgaattttattgaattc-3'. The thermal cycling conditions were as follows: an initial denaturation step at 95˚C for 2 min, followed by 35 cycles of amplification (95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 2 min), and a final extension at 72˚C for 10 min. Afterwards, an A nucleotide base overhang was generated by the addition of Ex Taq DNA polymerase (Clontech, Mountain View, CA, USA). The PCR product was inserted into the pGEM-T Easy Vector (Promega, Madison,WI, USA). For the construction of the mammalian expression vectors, TA clones were subcloned into the *Xho*I and *Xba*I sites in the pCS2$^+$ vector, which was kindly provided by Dr Louis Kunkel (Division of Genetics and Genomics, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA).

Cell transfection. To induce the overexpression of PLIN2 in the C2C12 cells, the cells (at 70% confluence) were transfected with the pCS2$^+$ vector expressing full-length human *PLIN2* using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. An empty pCS2$^+$ vector was transfected into the C2C12 cells as a control. To reduce endogenous NLRP3 expression, the C2C12 cells were transfected with 80 pmol of siRNA oligonucleotides (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) using Lipofectamine 2000 reagent. Non-targeted siRNA oligonucleotides (Santa Cruz Biotechnology, Inc.) were used as controls. The C2C12 cells were also co-transfected with the pCS2$^+$ vector expressing PLIN2 and siRNA oligonucleotides targeting NLRP3. At 48 h post-transfection, the cells were harvested to extract the protein, and 25 μg of protein were used for the detection of PLIN2 or NLRP3 by western blot analysis.

Western blot analysis. The cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.5) with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) (both from Santa Cruz Biotechnology, Inc.), and 25 μg of protein were separated by electrophoresis. The resolved protein was transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h to reduce non-specific binding and then incubated with antibodies to NLRP3 (sc-66846), caspase-1 (sc-56036), IL-1β (sc-7884), β-actin (sc-47778; all from Santa Cruz Biotechnologies, Inc.) and PLIN2 (ab52355; Abcam, Cambridge, UK) at 4˚C overnight. After washing 3 times with TBS containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (ab6728, ab97051; Abcam), and the signals were visualized using an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific) on a ChemiDoc XRS$^+$ imaging system (Bio-Rad, Hercules, CA, USA).

Measurement of intracellular triglyceride levels. At 48 h post-transfection with either *PLIN2*-expressing vector or the empty vector, the C2C12 cells were harvested in radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnologies, Inc.). The intracellular triglyceride levels were measured in the cell lysates using a colorimetric triglyceride quantification kit (ab65336; Abcam) according to the manufacturer's instructions at a 570 nm wavelength on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). C2C12 cells incubated with 400 μM palmitic acid (Sigma-Aldrich) for 24 h were also subjected to this triglyceride assay as a positive control.

Glucose uptake assay. At 48 h post-transfection with either *PLIN2*-expressing vector or the empty vector, C2C12 cells were washed twice with Krebs-Ringer-Phosphate-HEPES (KRPH) buffer (20 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl and 4.7 mM KCl, pH 7.4) and starved of glucose by incubation with KRPH buffer containing 0.2% bovine serum albumin (BSA) at 37˚C for 40 min. The cells were then stimulated with 100 nM insulin (Sigma-Aldrich) for 30 min in KRPH buffer supplemented with 0.2% BSA. Glucose transport was determined by subsequent stimulation with 2-deoxy-D-glucose-6-phosphate (2DG6P) at a final concentration of 0.1 mM for 20 min, as previously described (18). The reaction was terminated by washing the cells 4 times with ice-cold phosphate-buffered saline (PBS). The cells were lysed in lysis buffer, and glucose uptake was assessed using the Glucose Uptake Colorimetric assay kit (MAK083; Sigma-Aldrich) in accordance with the manufacturer's instructions. The absorbance was measured at a 412 nm wavelength on a microplate reader (BioTek Instruments, Inc.). To confirm the effects of IL-1β treatment on glucose uptake, the C2C12 cells were incubated with murine IL-1β (PeproTech, Rocky Hill, NJ, USA) at 10, 25 and 50 ng/ml for 24 h. The cells were then subjected to the glucose uptake assay described above.

Reverse transcription PCR (RT-PCR). Total RNA was extracted from the C2C12 cells in each experimental group using TRIzol reagent (Life Technologies). At 48 h post-transfection with *PLIN2*-overexpressing vector and a combination of *PLIN2*-expressing vector with either *NLRP3* siRNA or non-targeted control siRNA, the C2C12 cells were stimulated with 100 nM insulin for 30 min. This was followed by RNA extraction to measure the expression levels of insulin receptor substrate-1 (IRS-1). RNA was also extracted from the C2C12 cells treated with murine IL-1β at concentrations of 10, 25 and 50 ng/ml. First-strand cDNA was constructed with an oligo(dT) primer using the Superscript III first-strand synthesis system (Life Technologies) for RT-PCR. PCR was performed using Ex Taq DNA polymerase as follows: an initial denaturation step at 95˚C for 2 min, followed by 35 cycles of amplification (95˚C for 30 sec, 56˚C for 30 sec, 72˚C for 1 min) using the DNA Engine System (Bio-Rad). *IRS-1* (209 bp) was amplified using the following primers: forward, 5'-CGATGGCTTCTCAGA CGTG-3' and reverse, 5'-CAGCCCGCTTGTTGATGTTG-3'. The internal control gene, *GAPDH* (395 bp), was amplified

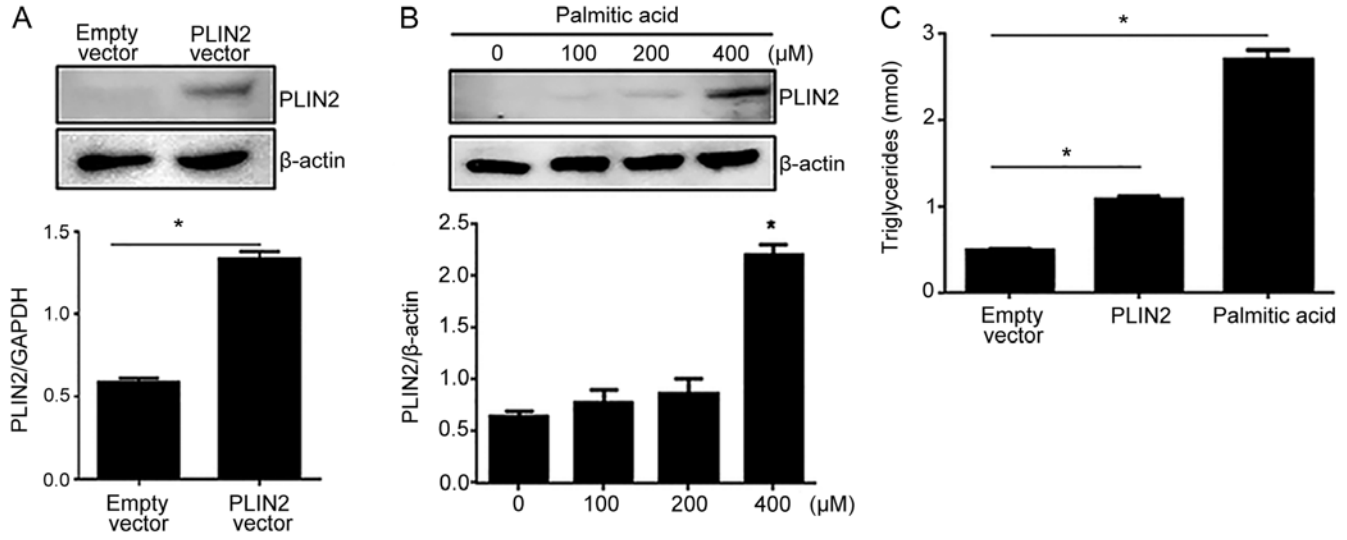

Figure 1. Perilipin 2 (PLIN2) induces triglyceride accumulation in C2C12 cells. (A) C2C12 cells transfected with empty vector or *PLIN2*-expressing vector were lysed in lysis buffer, and the cell lysates were assessed by western blot analysis. Histogram shows the protein expression of PLIN2, normalized to β-actin, as determined by densitometric analysis. (B) PLIN2 protein levels in C2C12 cells treated with 0, 100, 200 and 400 μM palmitic acid for 24 h. Cells treated with 400 μM palmitic acid demonstrated a significant induction of PLIN2 in comparison with the cells treated with 0, 100 or 200 μM palmitic acid. The β-actin protein signal was used for normalization. (C) Triglyceride levels in C2C12 cells transfected with empty vector or PLIN2-expressing vector were examined. C2C12 cells incubated with 400 μM palmitic acid were used as positive controls. The data are expressed as the means ± SEM. *P<0.05.

using the following primers: forward, 5'-GTC TTC TCC ACC ATG GAG AAG GCT-3' and reverse, 5'-CAT GCC AGT GAG CTT CCC GTT CA-3'.

Statistical analysis. Values are expressed as the means ± standard error of the mean (SEM). Statistical significance was analyzed using the Student's t-test with GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). For all analyses, a value of P<0.05 was considered to indicate a statistically significant difference.

Results

PLIN2 overexpression leads to the cellular accumulation of triglycerides. To determine the effects of PLIN2 on the accumulation of intracellular triglycerides, we overexpressed full-length human *PLIN2* in the C2C12 cells using 2 methods: transient transfection and treatment with palmitic acid. Palmitic acid is a saturated fatty acid that activates peroxisome proliferator-activated receptor γ (PPAR-γ), which in turn acts as a transcriptional activator of PLIN2 (19-21). Western blot analysis demonstrated a significant induction of PLIN2 expression following transfection with the *PLIN2*-overexpressing vector (Fig. 1A). Various concentrations (0, 100, 200 and 400 μM) of palmitic acid were used to mimic PLIN2 induction. Western blot analysis indicated that treatment of the C2C12 cells with 400 μM of palmitic acid resulted in significantly higher PLIN2 levels compared to treatment with 0, 100 or 200 μM of palmitic acid (Fig. 1B). We then measured the intracellular triglyceride levels in the C2C12 cells in which the overexpression of *PLIN2* was induced using either method: transient transfection with the vector expressing *PLIN2* or treatment with 400 μM palmitic acid, the latter serving as a positive control. Both treatments yielded intracellular triglyceride levels that were significantly higher than those in the cells transfected with the empty vector; however, the cells treated with palmitic acid displayed a more dramatic elevation in intracellular triglyceride levels than the cells transfected with the *PLIN2*-overexpressing vector (Fig. 1C).

PLIN2 overexpression impairs insulin-stimulated glucose uptake. Since increased levels of intracellular triglycerides are associated with an impaired glucose uptake (22), we hypothesized that glucose uptake would be reduced in C2C12 cells that overexpress PLIN2. Following transfection with the *PLIN2*-expressing vector, the C2C12 cells were subjected to an insulin-stimulated glucose uptake assay. C2C12 cells transfected with the empty vector responded with a significant escalation of glucose uptake in response to insulin stimulation, whereas the C2C12 cells that overexpressed PLIN2 exhibited an impaired glucose uptake in response to insulin stimulation (Fig. 2A).

PLIN2 impairs insulin-stimulated glucose uptake activity through the activation of the NLRP3 inflammasome. We then investigated the potential involvement of the NLRP3 inflammasome in PLIN2-induced insulin resistance. This pathway has been implicated in recognizing certain non-microbial 'danger signals' that lead to caspase-1 activation and the subsequent production of IL-1β. As excessive fat is also known to activate the NLRP3 inflammasome (17), we hypothesized that this inflammasome would be activated by accumulating levels of triglycerides in C2C12 cells that overexpress PLIN2. PLIN2 overexpression led to an increased protein expression of NLRP3, caspase-1 and IL-1β, suggesting the activation of the NLRP3 inflammasome (Fig. 2B and C). To confirm that PLIN2 affects cellular glucose uptake through the activation of the NLRP3 inflammasome, we performed *NLRP3* knockdown experiments by transfection with siRNA. siRNA targeting NLRP3 effectively reduced the endogenous expression of NLRP3 protein (Fig. 3A). Subsequently, the C2C12 cells were co-transfected with

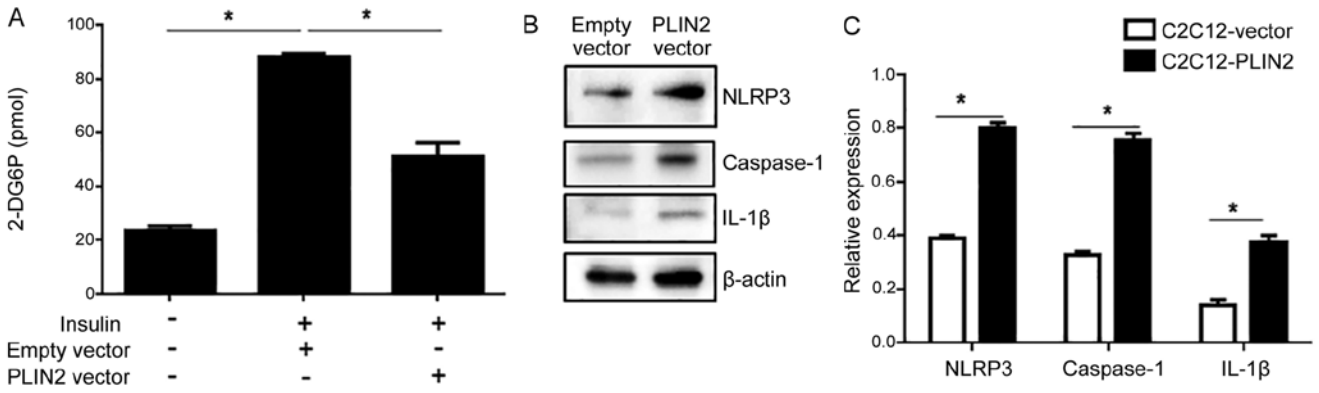

Figure 2. Perilipin 2 (PLIN2) overexpression impairs insulin-induced glucose uptake but activates the nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome. (A) C2C12 cells were transfected with either the empty vector or *PLIN2*-expressing vector and incubated with 100 nM insulin for 30 min. After a 20-min pulse with 2-deoxy-D-glucose-6-phosphate (2-DG6P), glucose uptake was assessed. C2C12 cells that overexpressed PLIN2 showed a significant decrease in glucose uptake. (B) Expression of NLRP3, caspase-1 and interleukin-1β (IL-1β) in C2C12 cells overexpressing PLIN2 was assessed by western blot analysis. (C) Densitometric analysis of (B). The data are expressed as the means ± SEM. *P<0.05.

PLIN2-expressing vector and *NLRP3* siRNA, followed by a glucose uptake assay. *NLRP3* knockdown resulted in decreased levels of NLRP3, caspase-1 and IL-1β in the cells overexpressing PLIN2 (Fig. 3B), and increased insulin-stimulated glucose uptake (Fig. 3C). The non-targeted control siRNA did not increase glucose uptake in the cells that overexpressed PLIN2.

Suppression of IRS-1 by IL-1β mediates the effects of the NLRP3 inflammasome on insulin-induced glucose uptake. IRS-1, one of the major substrates of the insulin receptor kinase, is essential for the activatation of PI3K in response to insulin, which leads to the phosphorylation of protein kinase B (also known as Akt) and subsequent glucose uptake (23). The downregulation of IRS-1 seems to be the major mechanism involved in the alteration of insulin signaling and glucose transport (24). Thus, we examined the effects of *PLIN2* and *NLRP3* manipulations on the mRNA expression levels of *IRS-1* in the C2C12 cells. PLIN2 overexpression (with transfection with control siRNA) led to a decrease in IRS-1 expression. However, *PLIN2* overexpression accompanied by *NLRP3* knockdown via siRNA transfection was associated with an increased *IRS-1* expression in response to insulin stimulation (Fig. 3D). IL-1β, the final step in the NLRP3 inflammasome, has been demonstrated to reduce insulin-induced glucose uptake in adipocytes by affecting IRS-1 expression (18,25). To determine whether IL-1β treatment affects insulin-induced glucose uptake in myoblasts, we treated the C2C12 cells with IL-1β at concentrations of 0, 10, 25 and 50 ng/ml for 24 h prior to measuring insulin-induced glucose uptake. IL-1β was found to inhibit glucose transport upon insulin stimulation in a dose-dependent manner (Fig. 4A). We also found that IL-1β decreased endogenous IRS-1 expression in the C2C12 cells (Fig. 4B).

Discussion

Skeletal muscle is a major organ which is involved in insulin-stimulated glucose uptake and thus in the development of insulin resistance. Previous studies have demonstrated that excess lipid accumulation contributes to the pathogenesis of insulin resistance, thus suggesting potential therapeutic interventions (2,26,27). Aside from the lipid overload hypothesis, inflammation has been considered to be a pivotal contributor to insulin resistance. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6 and monocyte chemoattractant protein-1 (MCP-1) have been identified as promoters of insulin resistance; however, these findings were mainly related to adipose tissue (13,28).

In this study, we attempted to identify an association between lipid accumulation and the inflammatory pathway that leads to insulin resistance in C2C12 cells. We found that the knockdown of *NLRP3* attenuated PLIN2-induced insulin resistance in C2C12 cells. PLIN2 is a lipid droplet-associated protein that is abundantly expressed in skeletal muscle. It is well known that the expression of PLIN2 correlates with the accumulation of intramuscular lipids (8-10). We observed in this study that C2C12 cells which overexpressed PLIN2 contained significantly greater amounts of triglycerides. Furthermore, we also noted that *PLIN2* overexpression led to the upregulation of IL-1β through NLRP3-caspase-1 upregulation.

The NLRP3 inflammasome consists of a group of protein complexes that are composed of the Nod-like receptor protein NLRP3 (also termed cryopyrin or NALP3), adapter proteins, a caspase recruitment domain (Cardinal) and caspase-1. The NLRP3 inflammasome has been implicated in the production of mature IL-1β and IL-18 in response to a variety of signals (16,17). One of the signals, DAMPs, is host-derived and is released as a result of perturbations of tissue homeostasis caused by microbial or non-microbial insults, conveying a general sense of tissue under stress (16,17). Although the normal activation of the NLRP3 inflammasome contributes to host defense, excessive activation results in inflammatory diseases mediated by the pro-inflammatory cytokine, IL-1β (16,17). It has been previously reported that the NLRP3 inflammasome instigates obesity-induced autoinflammation and insulin resistance (29). Considering that muscle is a critical organ involved in insulin sensitivity, we can speculate that the NLRP3 inflammasome links excess fat and insulin resistance in myoblasts. In the present study, we demonstrated that C2C12 cells overexpressing PLIN2 exhibited increased levels of NLRP3, caspase-1 and IL-1β. As PLIN2 impaired glucose uptake activity, this indicates a potential correlation between NLRP3 and glucose uptake in C2C12 cells in response to insulin. The knockdown of *NLRP3*

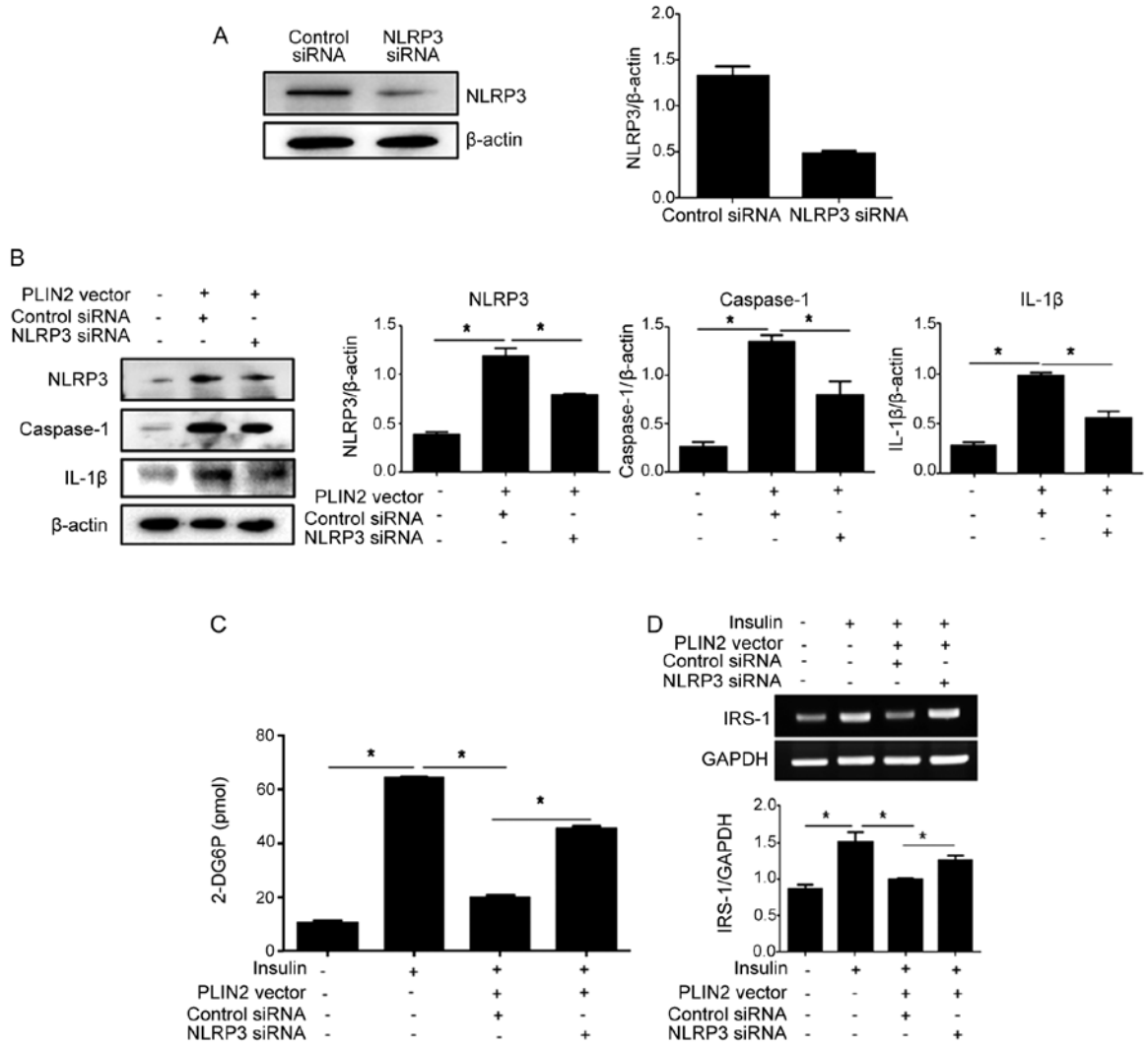

Figure 3. Nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) silencing inhibits perilipin 2 (PLIN2)-induced insulin resistance. (A) Endogenous NLRP3 expression was reduced by targeted siRNA knockdown and quantified by western blot analysis. (B) Expression of NLRP3, caspase-1 and interleukin-1β (IL-1β) after NLRP3 silencing in PLIN2 overexpressing C2C12 cells. Histograms represent densitometric analysis. (C) Glucose uptake was measured in C2C12 cells from each experimental group. C2C12 cells subjected to NLRP3 knockdown exhibited a recovery of glucose uptake along with PLIN2 overexpression. (D) mRNA expression of insulin receptor substrate-1 (*IRS-1*) in C2C12 cells from each experimental group was confirmed by endpoint PCR. *IRS-1* expression was decreased in *PLIN2*-overexpressing C2C12 cells exposed to insulin, whereas *NLRP3* knockdown increased the levels of *IRS-1*. The data are expressed as the means ± SEM. *P<0.05. 2-DG6P, 2-deoxy-D-glucose-6-phosphate.

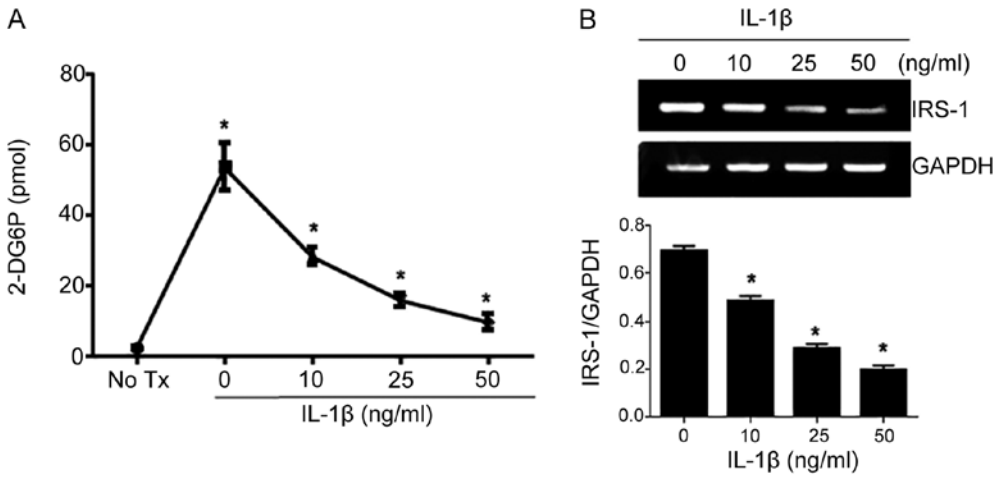

Figure 4. Interleukin-1β (IL-1β) impairs the glucose uptake in response to insulin in C2C12 cells and the expression of insulin receptor substrate-1 (IRS-1). (A) C2C12 cells were treated with IL-1β at concentrations of 10, 25 and 50 ng/ml for 24 h, and insulin-induced glucose transport was then measured. (B) mRNA expression of *IRS-1* was confirmed in C2C12 cells incubated with IL-1β at concentrations of 10, 25 and 50 ng/ml for 24 h. The histogram indicates densitometric analysis. The data are expressed as the means ± SEM. *P<0.05. 2-DG6P, 2-deoxy-D-glucose-6-phosphate. No Tx, No treatment.

by RNA interference significantly inhibited insulin resistance which is typically induced in PLIN2-overexpressing C2C12 cells, suggesting that the NLRP3 inflammasome mediates PLIN2-induced insulin resistance. Decreased levels of IRS-1 have been reported to alter insulin signaling in adipocytes and myocytes (24,25). In this study, we observed that IRS-1 expression was decreased in C2C12 cells that overexpressed PLIN2 in the context of insulin exposure, whereas *NLRP3* knockdown increased the expression of IRS-1. IL-1β, the final product of the NLRP3 inflammasome, has been reported to reduce insulin-induced glucose uptake in adipocytes, mainly by inhibiting IRS-1 expression (18). In the present study, we observed the inhibitory effect of IL-1β on the expression of IRS-1 in C2C12 cells, and on this basis we suggest that inflammatory cytokines directly target muscle cells, as well as adipocytes in the context of insulin signaling disturbances.

In conclusion, the present study provides a fresh view of the role of LDs and associated proteins in cellular metabolism by demonstrating the following novel insights. First, insulin-mediated cellular glucose uptake inversely correlated with PLIN2 expression in myoblasts. Second, components of the NLRP3 inflammasome were significantly upregulated in *PLIN2*-overexpressing myoblast cells, while the downregulation of *NLRP3* inhibited PLIN2-induced insulin resistance. Taken together, these data suggest that PLIN2 influences cellular glucose uptake and transport by interacting with the NLRP3 inflammasome. Since insulin resistance can be characterized by inefficient glucose uptake in muscle and fat cells, our findings provide a molecular mechanism that links cellular lipid contents and the inflammatory response underlying the pathogenesis of insulin resistance. Future studies using animals fed a high-fat diet may help determine whether the suppression of the activation of the NLRP3 inflammasome would protect the animals against type 2 diabetes mellitus.

Acknowledgements

The authors thank the Department of Pediatrics at the University of Florida College of Medicine for supporting this study.

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