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Palmitate induces SHIP2 expression via the ceramide-mediated activation of NF-κB, and JNK in skeletal muscle cells

Sattar Gorgani-Firuzjaee [a,b], Saeideh Ahmadi [a], Reza Meshkani [a,*]

[a] Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
[b] Student Scientific Research Center (SSRC), Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran

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ABSTRACT

Aims: Elevated plasma free fatty acids impair the insulin signaling by induction of the expression of protein phosphatases. However, the effect of palmitate on SH2-containing inositol 5′-phosphatase 2 (SHIP2) expression has not been investigated. Here we investigated the effects of palmitate on SHIP2 expression and elucidated the underlying mechanisms in skeletal muscle cells.
Main methods: SHIP2 mRNA and protein levels were measured in C2C12 myotubes exposed to palmitate. Specific inhibitors were used to identify the signaling pathways involved in SHIP2 expression.
Key findings: The results showed that 0.5 mM palmitate significantly upregulates the mRNA and protein levels of SHIP2 in C2C12 cells. To address the role of palmitate intracellular metabolites in SHIP2 expression, the myotubes were treated with palmitate in the presence of ceramide and diacylglycerol synthesis inhibitors. The results demonstrated that only ceramide synthesis inhibition could prevent palmitate-induced SHIP2 expression in these cells. In addition, the incubation of muscle cells with different concentrations of C2-ceramide dose-dependently enhanced SHIP2 expression. Furthermore, the inhibition of both JNK and NF-κB pathways could prevent ceramide-induced SHIP2 expression in myotubes.
Significance: These findings suggest that palmitate contributes to SHIP2 overexpression in skeletal muscle via the mechanisms involving the activation of ceramide-JNK and NF-κB pathways.

1. Introduction

Type 2 diabetes, one of the most common endocrine diseases, is caused by decreased insulin sensitivity and beta cell dysfunction [1]. The skeletal muscle has a major role in insulin-stimulated glucose uptake and is, therefore, one of the important sites of insulin resistance in the body. Obesity and sedentary life style are suggested as the risk factors for insulin resistance and other metabolic diseases such as atherosclerosis and hypertension [2]. An elevated plasma free fatty acids (FFAs) along with the impairment in glucose uptake and decreased insulin sensitivity has been reported during the development of insulin resistance in skeletal muscle [1]. In vivo studies also reveals that elevated FFAs can induce both inflammation [3] and insulin resistance [1,4], whereas decreasing the plasma FFA levels resulted in lowering of insulin resistance [5]. Although the underlying mechanisms of FFA-induced insulin resistance are not well understood, several mechanisms including increased FFA metabolites such as ceramide and diacylglycerol [6], the activation of protein tyrosine phosphatases (PTPs) such as protein tyrosine phosphatase 1B (PTP1B) and leukocyte common antigen-related (LAR) [1,3,7–9], and the induction of inflammatory response [3,9] have been proposed.

The insulin signaling pathway has important role in cellular metabolism, growth, and differentiation. Insulin binding to its receptor leads to receptor autophosphorylation and the tyrosine phosphorylation of insulin receptor substrates (IRSs) [10–12]. The activated IRSs associates with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3 kinase), resulting in the activation of the p110 subunit [12]. PI3 kinase converts PIP_2 (4,5) to PIP_3 (3,4,5) a crucial lipid second messenger in various insulin metabolic actions [13]. PIP_3 activates Akt and this results in induction of insulin-dependent glucose uptake in insulin target tissues such as skeletal muscle and fat cells [12]. SH2-containing inisitol 5′-phosphatase 2 (SHIP2) is a lipid phosphatase which dephosphorylates PIP_3 and convert it to PIP_2 [10].

In vitro and in vivo studies have provided the evidence that SHIP2 is one of the negative regulators of the insulin signaling pathway [14]. Mice lacking the SHIP2 gene have increased sensitivity to insulin [15]. In addition, human studies have shown that the polymorphisms of the SHIP2 gene are associated with the metabolic syndrome in British and French populations [16]. The

* Corresponding author.
E-mail address: rmeshkani@tums.ac.ir (R. Meshkani).

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expression of SHIP2 is elevated in muscle tissue of type 2 diabetic and obese subjects [17]. However, the exact molecular mechanisms underlying this overexpression are not fully understood in skeletal muscle cells.

The purpose of this study was to investigate the effect of palmitate on the expression of SHIP2 and elucidate the underlying mechanisms in skeletal muscle cells. Exposure of the cells to palmitate led to increased SHIP2 expression in C2C12 cells. The mechanism by which palmitate increased the expression of SHIP2 seems to be through the activation of ceramide-JNK and NF-κB pathways.

2. Material and method

2.1. Materials

C2C12 myoblasts were purchased from the Pasteur Institute of Iran. Tissue culture media, penicillin, and streptomycin were purchased from Invitrogen. The RNA isolation kit, primers for SHIP2, and β-actin were purchased from Qiagen. SYBR Green PCR kit was purchased from Takara (Shiga, Japan). Antibodies were obtained from Cell Signaling. Chemiluminescence western blotting detection kit was purchased from GE Healthcare (Heidelberg, Germany). All specific inhibitors were from CalBiochem.

2.2. Cell culture

C2C12 myoblasts were maintained at 37 °C (in an atmosphere of 5% CO_2) in Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Berlin, Germany) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin–streptomycin. Myoblasts differentiated into myotubes by changing the media with 2% horse serum. Lipid-containing media were prepared by Svedberg et al. method [18]. Briefly, sodium palmitate was dissolved in 50% (v/v) ethanol, diluted in DMEM containing 1% (w/v) fatty acid free BSA to the final concentration and was incubated in 37 °C for 2 h while being shaken. Then, the cells were treated with palmitate for 16 h. Inhibitors were used 1 h before the treatment with palmitate.

2.3. Real-time PCR

Total RNA was extracted using RNeasy mini kit. Total RNA (1 μg) was reverse transcribed using Fermentas reverse transcriptase. Real-time PCR was conducted using a RotorGene 3000 instrument (Corbett Research, Australia). SHIP2 expression level was assessed by QuantiTect primer (Qiagen). The data were normalized against β-Actin transcript level and analyzed by delta delta Ct.

2.4. Western blot analysis

C2C12 cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 1 mM Na-EDTA, 1 mM PMSF supplemented with complete EDTA-free protease inhibitor cocktail). 30 μg of total protein was run on a SDS–PAGE gel and transferred onto PVDF membranes. The blots were probed with anti-SHIP2 specific antibody, followed by the incubation with anti-mouse HRP-conjugated secondary antibody. The membranes were developed using enhanced chemiluminescence and protein bands were quantified using Image J software.

2.5. Statistical analysis

The data are presented as mean ± SD of at least three independent experiments. The statistical analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL). Comparisons among all groups were performed with one-way analysis of variance (ANOVA) test. If significant differences were found, a post hoc Bonferroni test was carried out. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. The effects of palmitate on SHIP2 expression in C2C12 cells

We first assessed the SHIP2 mRNA expression in C2C12 cells treated with BSA, 0.25 and 0.5 mM palmitate for 16 h. When the cells were treated with 0.25 mM palmitate, no significant change was observed, whereas 0.5 mM palmitate significantly upregulates SHIP2 mRNA level (81%, $P < 0.01$) compared to control cells (Fig. 1A). In addition, 0.5 mM palmitate could significantly upregulate SHIP2 protein level by 70% compared to untreated cells (Fig. 1B).

3.2. The role of ceramide and diacylglycerol pathways in palmitate-induced SHIP2 over expression

It has been reported that palmitate activates several signaling pathways by conversion to its metabolites such as diacylglycerol and [19] ceramide [20]. To identify the mechanism of palmitate-induced SHIP2 expression in muscle cells, we first evaluated the role of de novo ceramide synthesis inhibition in the SHIP2 gene and protein expression. Simultaneous treatment of the cells with palmitate and myriocin (de novo ceramide synthesis inhibitor) significantly prevented the upregulation of the SHIP2 mRNA and protein levels in myotubes (Fig. 2A and B). We also observed that the exposure of myotubes to different concentrations of the cell-permeable C2-ceramide significantly increased the SHIP2 protein level (Fig. 2C), while 100 μM ceramide caused about 4-fold increase in the SHIP2 protein level compared to the control cells. These data suggest that the de novo ceramide synthesis pathway is involved in palmitate-mediated upregulation of the SHIP2 in skeletal muscle cells.

It has been shown that the elevation of plasma FFA may lead to increased diacylglycerol and subsequent activation of PKC in skeletal muscle cells [21]. To test the role of this pathway in SHIP2 expression, calphostin C, a strong and specific inhibitor of PKC was used. The results demonstrated that 100 μM calphostin C could not prevent palmitate-induced SHIP2 expression in C2C12 cells (Fig. 2A and B). These data imply that the DAG-PKC activation may not be involved in the effects of palmitate on SHIP2 expression.

3.3. Role of NF-κB in palmitate-induced SHIP2 expression

Since it has been reported that the C2C12 exposure to palmitate activates NF-κB [22] and this activation can induce the expression of some protein phosphatases such as PTP1B [3,7,23], we next examined whether the activation of this transcription factor is involved in palmitate-mediated SHIP2 overexpression in muscle cells. To test this hypothesis, we used the NF-κB inhibitor, parthenolide, which specifically inhibits NF-κB activation by preventing the IkB degradation. The data showed that parthenolide significantly reduced the SHIP2 mRNA and protein levels in the presence of ceramide (Fig. 3A and B). Taken together, these findings suggest that SHIP2 overexpression by palmitate is at least partly mediated through the activation of NF-κB in C2C12 cells.

3.4. Importance of MAPK signaling in ceramide-induced SHIP2 expression

To investigate the role of MAPK signaling in ceramide-induced SHIP2 expression, we used specific inhibitors of c-Jun N-terminal kinase (JNK), p38, and ERK1/2 pathways. To determine which of

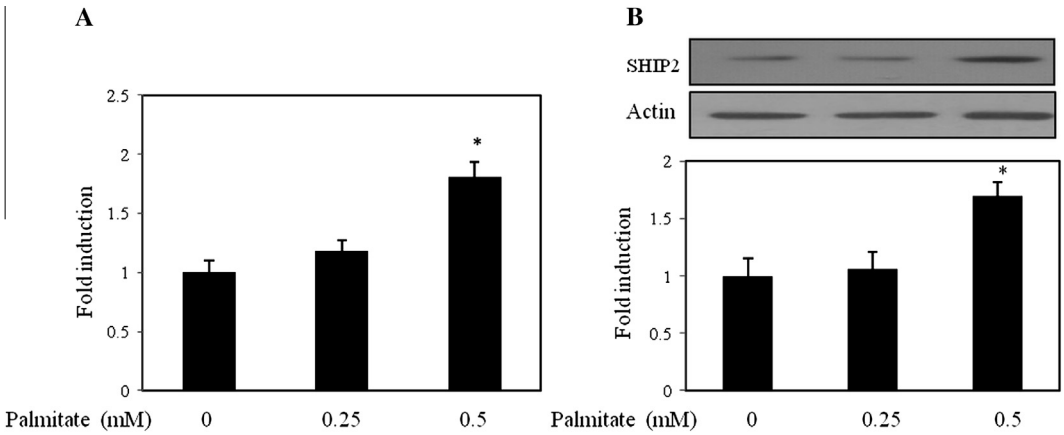

Fig. 1. The effects of palmitate on SHIP2 mRNA and protein levels. (A) After differentiation, the myotubes were exposed to 0.25 and 0.5 mM palmitate for 16 h. SHIP2 mRNA expression was quantified by real-time PCR and normalized relative to β-actin mRNA expression. $^{*}P < 0.01$ vs untreated cells. (B) Protein levels were quantified by western blot and normalized relative to β-actin protein level. $^{*}P < 0.01$ vs untreated cells.

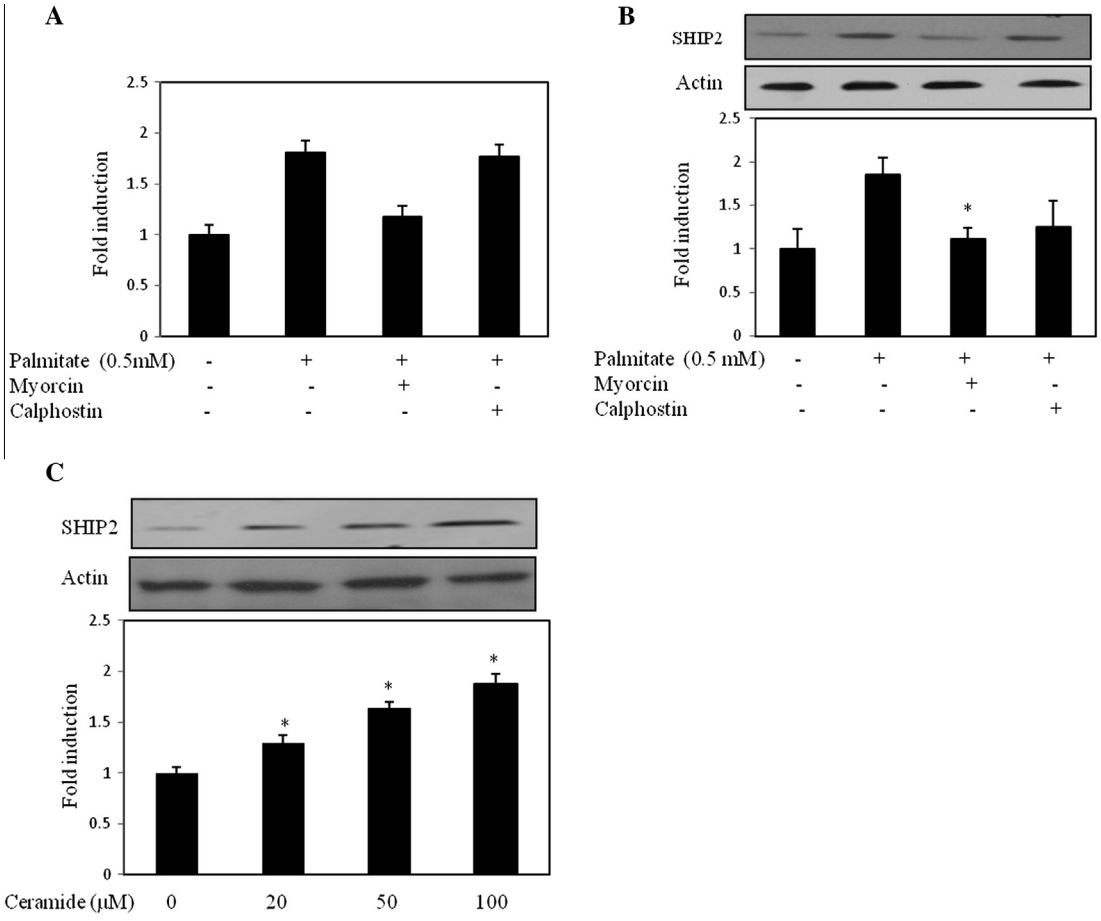

Fig. 2. The role of ceramide and diacylglycerol pathways in palmitate-induced SHIP2 expression. (A) After differentiation, the myotubes were incubated in the presence of palmitate, ceramide, calphostin and myriocin for 16 h. SHIP2 mRNA levels were quantified by real-time PCR and normalized relative to β-actin mRNA expression. $^{*}P < 0.01$ vs palmitate treated cells. (B) Protein levels were quantified by western blot and normalized relative to β-actin protein level. $^{*}P < 0.01$ vs palmitate treated cells. (C) The myotubes were incubated in the absence or the presence of different concentrations of C2-ceramide for 16 h. $^{*}P < 0.01$ vs untreated cells.

these pathways mediates increased SHIP2 expression, we pretreated the myotubes with SP600125 (20 μM), PD98059 (20 μM) and SB202190 (10 μM) to prevent JNK, ERK and p38 pathways, respectively, prior to treatment with ceramide. The JNK inhibitor significantly reduced ceramide-induced SHIP2 expression by 60%, while the ERK and p38 inhibitors had no effect on the SHIP2 mRNA level in the presence of ceramide (Fig. 4A). We also obtained the same results when the myotubes were treated simultaneously with the inhibitors and 0.5 mM palmitate (data not shown). It is worthy to note that the JNK inhibitor also inhibited ceramide-induced SHIP2 protein level in C2C12 cells (Fig. 4B). These results suggest that the ceramide-JNK pathway may be involved in increased the expression of SHIP2 in the cells treated with palmitate.

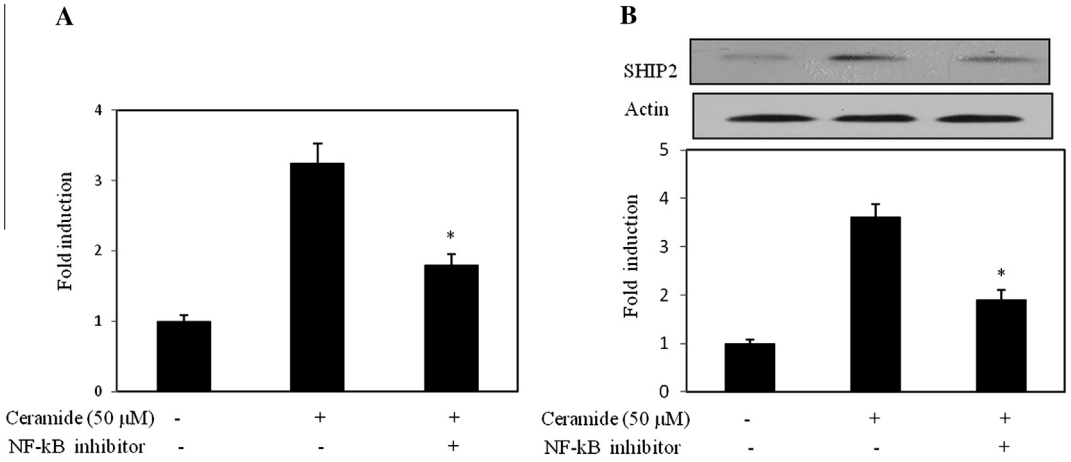

Fig. 3. Role of NF-κB in palmitate-induced SHIP2 expression. A: After differentiation, the myotubes were incubated in the presence of ceramide (50 μM) and NF-κB inhibitor for 16 h. SHIP2 mRNA levels were quantified by real-time PCR and normalized relative to β-actin mRNA expression. $^{*}P < 0.01$ vs myotubes treated with ceramide (50 μM). (B) SHIP2 protein level were analyzed with western blotting and normalized with β-actin protein level. $^{*}P < 0.01$ vs myotubes treated with ceramide (50 μM).

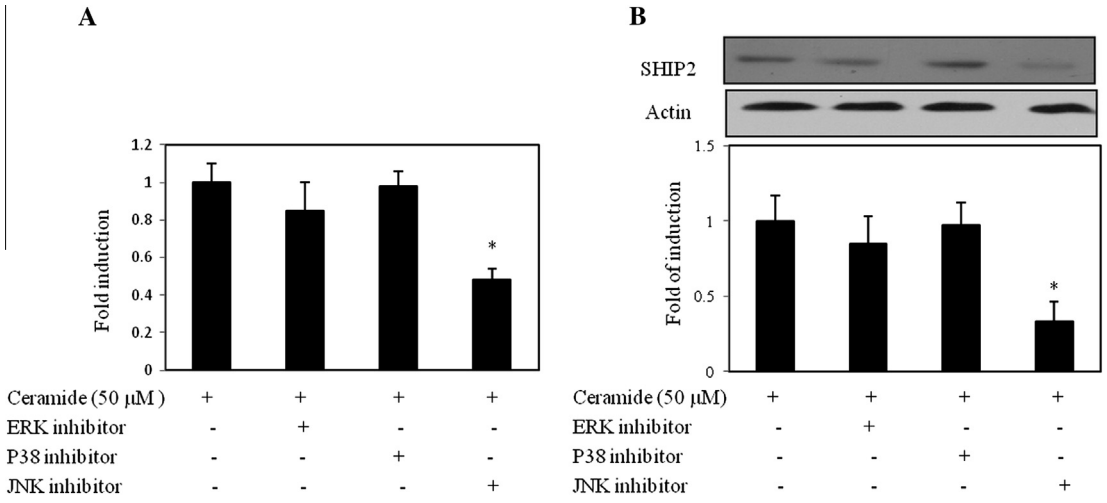

Fig. 4. Importance of MAPK signaling in ceramide-induced SHIP2 expression. After differentiation, the myotubes were incubated in the presence of ceramide (50 μM) and specific inhibitors of ERK, JNK, and P38 pathways for 16 h. SHIP2 mRNA levels were quantified by real-time PCR and normalized relative to β-actin mRNA expression. $^{*}P < 0.01$ vs myotubes treated with ceramide (50 μM). (B) SHIP2 protein level was analyzed using western blotting and normalized with β-actin protein level. $^{*}P < 0.01$ vs myotubes treated with ceramide (50 μM).

4. Discussion

Many important cellular functions such as proliferation, cell metabolism, differentiation and survival are controlled by phosphorylation and dephosphorylation of proteins [24]. Lipid phosphates such as Phosphatase and tensin homolog (PTEN) and SHIP2 regulate PI3K lipid products by dephosphorylating PIP3 to PIP_2 [2]. In vivo and in vitro evidence suggests that SHIP2 acts as negative regulator of the insulin signaling [10] and its overexpression impairs the insulin signaling by dephosphorylating the PIP3 [14,16,25]. Studies in diabetic and obese subjects revealed an elevated SHIP2 expression and activity in skeletal muscle cells [14]. Although the detailed mechanisms for SHIP2 overexpression is not well defined, however, different factors including glucose, fatty acids and inflammation can be proposed as the cause of SHIP2 overexpression in insulin resistance state. Among these factors, it appears that high FFA level especially saturated FFAs play the prominent role in the development of insulin resistance in muscle cells [6,26,27]. We previously reported that palmitate and an inflammatory state induce the PTP1B and LAR expression at the transcriptional level in skeletal muscle cells [1,3,4,7,9]. Here we have focused on SHIP2 and investigated the molecular mechanism underlying SHIP2 overexpression in C2C12 cells.

In this study we demonstrated that the effect of palmitate on SHIP2 expression is mediated by ceramide pathway. We first showed that palmitate induces SHIP2 expression at the mRNA and protein level by a high concentration of palmitate. We then demonstrated that the de novo ceramide synthesis is mostly responsible for SHIP2 overexpression in muscle cells, as myriocin (de novo ceramide synthesis inhibitor), could prevent palmitate-induced SHIP2 expression in C2C12 cells. The level of ceramide synthesis depends on the availability of long-chain saturated FFAs such as palmitate. Serine palmitoyltransferase catalyzes the condensation of palmitoyl-CoA and serine to produce 3-oxosphinganine and sequential synthesis of sphinganine, dihydroceramide, and ceramide [20].

In recent years, several in vivo and in vitro studies have investigated the role of ceramide in skeletal muscle insulin resistance. It has been reported that saturated FFAs (palmitate and stearate) promote ceramide formation in muscle cells [28,29]. An elevated muscle ceramide content has been reported from the diabetic mice induced by streptozotocin [29]. It has been found the ceramide and

sphingosine content were elevated in skeletal muscle and plasma of obese and diabetic subjects [30,31]. At the molecular level, ceramide has been shown to inhibit several distinct intermediates in the insulin signaling pathway [29] such as IRS-1 and PI3-Kinase. [28]. In addition, ceramide can inactivate the IRS1 by activation of the ERK2, P38, JNK, and IκB kinases (IκKs) pathways [32]. Furthermore, ceramide promotes the dephosphorylation of Akt/PKB by protein phosphatase 2A (PP2A) [33,34]. In addition, ceramide has been reported to induce the expression of PTP1B resulting in the dephosphorylation of IRS-1 in skeletal muscle cells [3,35]. In this study we demonstrated the involvement of the ceramide in the induction of SHIP2 expression, an intermediate of the insulin signaling pathway, and this induction as demonstrated by a previous study leads to insulin resistance in muscle cells [36]. Our results do not show that the DAG-PKC pathway is involved in the induction of SHIP2 in skeletal muscle cells. Activation of PKC could induce insulin resistance by several mechanisms including the Ser/Thr phosphorylation of both the IR [37] and IRS-1 [8] and increasing the oxidative stress and the activation of the NF-κB pathway [38]. The results presented herein show that Calphostin C, a strong and specific inhibitor of PKC, cannot prevent palmitate-induced SHIP2 expression in muscle cells. These data indicate that the DAG-PKC pathway may not be involved in palmitate-induced SHIP2 expression in skeletal muscle cells. In support of our findings, it seems that DAG derived from polyunsaturated but not saturated fatty acids is the activator of PKC [8]. Therefore, it appears that DAG participates in the regulation of insulin signaling by polyunsaturated FFAs, whereas ceramide is the principle metabolite linking saturated FFAs to insulin signaling [39].

It has been shown that ceramide can activate MAPKs pathways such as JNK, ERK and p38 in different cell cultures [30]. In the present study we provide the evidence that the effect of ceramide on SHIP2 expression might be mediated at least partly by the JNK pathway. It is of interesting to note that the same molecular mechanism appears to be operative for palmitate-induced PTP1B expression in C2C12 cells [3].

In summary, our results show that the treatment of myotubes with palmitate induce SHIP2 expression via the mechanisms involving the activation of the ceramide-JNK and NF-κB pathways. This finding supports the hypothesis that elevated palmitate and ceramide levels impair muscle insulin signaling by interfering with the intermediates of the insulin signaling pathway (Fig. S1).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.006.

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