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Reduction of endoplasmic reticulum stress using chemical chaperones or Grp78 overexpression does not protect muscle cells from palmitate-induced insulin resistance

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ABSTRACT

Endoplasmic reticulum (ER) stress is proposed as a novel link between elevated fatty acids levels, obesity and insulin resistance in liver and adipose tissue. However, it is unknown whether ER stress also contributes to lipid-induced insulin resistance in skeletal muscle, the major tissue responsible of insulin-stimulated glucose disposal. Here, we investigated the possible role of ER stress in palmitate-induced alterations of insulin action, both *in vivo*, in gastrocnemius of high-palm diet fed mice, and *in vitro*, in palmitate-treated C_2C_{12} myotubes. We demonstrated that 8 weeks of high-palm diet increased the expression of ER stress markers in muscle of mice, whereas *ex-vivo* insulin-stimulated PKB phosphorylation was not altered in this tissue. In addition, exposure of C_2C_{12} myotubes to either tuncamycine or palmitate induced ER stress and altered insulin-stimulated PKB phosphorylation. However, alleviation of ER stress by either TUDCA or 4-PBA treatments, or by overexpressing Grp78, did not restore palmitate-induced reduction of insulin-stimulated PKB phosphorylation in C_2C_{12} myotubes. This work highlights that, even ER stress is associated with palmitate-induced alterations of insulin signaling, ER stress is likely not the major culprit of this effect in myotubes, suggesting that the previously proposed link between ER stress and insulin resistance is less important in skeletal muscle than in adipose tissue and liver.

1. Introduction

Insulin resistance (IR) is a hallmark feature of type 2 diabetes and obesity. Since skeletal muscle is the major tissue responsible for whole body insulin-stimulated glucose disposal, defective action of insulin in this tissue is thought to play a major role in the aetiology of both pathologies. A key cause of muscle IR is lipotoxicity. Obese and diabetic patients have elevated plasma levels of free fatty acids (FFA) [1], and acute infusion of lipids interferes with insulin signaling [2]. In addition, muscle IR correlates with accumulation of triglycerides within skeletal muscle fibers [3], and acute incubation of myotubes with saturated lipids alters insulin signaling [4]. Potential candidates mediating the effects of saturated fatty acids on IR include (1) an increase in production of ceramides and accumulation of diacylglycerol leading to the activation of PKC [5], (2) mitochondrial dysfunction and increased oxidative stress [6], (3) inflammation and activation of the pro-inflammatory NfkB and mitogen-activated kinases [7] and (4) endoplasmic reticulum stress and activation of the unfolded protein response (UPR) [8]. Although these mechanisms could occur simultaneously and act synergistically, the molecular causes that lead to skeletal muscle IR remain elusive.

Recently, ER stress has been proposed as a central mechanism in the development of IR in liver and adipose tissue [9]. ER stress describes the accumulation of misfolded proteins that aggregate in the ER. ER stress activates the unfolded protein response (UPR) pathways in order to restore ER homeostasis. The UPR pathways was activated in liver and adipose tissue of both diet-induced and genetically ob/ob mice [8], and lipid-induced ER stress in both tissues lead to inhibition of insulin signaling [10]. Interestingly, treatment of diabetic mice with inhibitors of ER stress, such as 4-phenyl butyric acid (4-PBA) or tauroursodeoxycholic acid (TUDCA), reduced IR and restored glucose homeostasis [11]. Surprising, less is known about the role of ER stress in skeletal muscle IR. Early studies suggested that muscle ER stress is unaffected in obesity [11]. However, more recent studies demonstrated that exposure to palmitate can induce ER stress in human primary myotubes [12]. Similarly, high-fat feeding was able to activate the unfolded protein response in skeletal muscle of mice [13]. However, it is unknown whether this induction of ER stress in the presence of lipids is directly involved in muscle IR.

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Here, we investigated the role of ER stress in palmitate-induced IR both in high-palm diet fed mice and in C_2C_{12} myotubes. We used complementary strategies to control palmitate-induced ER stress and determine the impact on palmitate-induced alterations of insulin signaling. Our results demonstrate that reduction of ER stress by chemical chaperones or glucose-response protein 78 (Grp78) overexpression does not improve palmitate-induced IR in skeletal muscle cells.

2. Materials and methods

2.1. Animals

Animals were purchased from Harlan (Gannat, France) and housed under controlled temperature and humidity. All animal experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals, and regional ethic committee has approved protocols. Male C57BL/6 (6 weeks) were fed during 8 weeks either with a standard diet (SAFE, A04) or with a palm oil-enriched diet (80% A04 + 20% palm oil, palm oil was a generous gift from the French Institute of Fat and Oils and diet was prepared by SAFE). After 8 weeks, mice were sacrificed and gastrocnemius muscles were frozen. Blood glucose levels were measured using a glucometer (Roche diagnostics). For the study of insulin signaling *ex-vivo*, muscles were cut into small pieces and incubated with or without insulin (10^{-7} M) for 20 min at 37 °C [14].

2.2. C_2C_{12} cells

C_2C_{12} myoblasts were grown in DMEM supplemented with 10% fetal bovine serum under 5% CO_2 at 37 °C. Differentiation into myotubes were induced by reducing the serum to 2%. C_2C_{12} myotubes were used after 7 days of differentiation.

2.3. FFA preparation and cell treatment

Palmitate and oleate (Sigma) stock solution were prepared at 8 mM in 10% fatty acid-free BSA at 50 °C. This stock solution was diluted in serum-free culture medium to a final concentration of 200–700 μM. Tunicamycine (5 μg/ml, Sigma) was dissolved in DMSO and used as a positive control for induction of ER stress. To inhibit ER stress, C_2C_{12} myotubes were pre-treated for 9 h with TUDCA (Calbiochem, 500 μg/ml) or 4-PBA (Sigma, 20 mM). Alternatively, alleviation of ER stress was performed by adenovirus-overexpression of the chaperone Grp78 [15]. Briefly, C_2C_{12} myotubes were infected either with an adenovirus encoding GFP (as control) or Grp78 for 48 h (10^8 p.i./well), and incubated with BSA or palmitate for 18 h. For the analysis of insulin signaling, treated myotubes were incubated with or without 10^{-7} M of insulin for 20 min.

2.4. Real time RT-PCR

Total RNA from muscles or C_2C_{12} cells were purified using the TriReagent kit (applied biosystems). Target mRNA levels were measured by reverse transcription followed by real-time PCR using a Rotor-Gene™ 6000 (Corbett Research). Each essay was performed in duplicate, and values were normalized using TATA binding protein (TBP).

2.5. Western blot

Muscle biopsies were lysed in PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail, and C_2C_{12} cells in a buffer containing 200 mM NaF, 20 mM NaH_2PO_4, 150 mM NaCl, 50 mM HEPES, 4 mM $NaVO_4$, 10 mM EDTA, and 2 mM PMSF, with 1% Triton X, 10% glycerol and protease inhibitor cocktail. Proteins were separated by SDS-10% PAGE, transferred to PVDF membrane, and incubated overnight with primary antibodies. The signal was detected with a horseradish peroxidase-conjugated secondary antibody and revealed with an enhanced chemiluminescence's system (Pierce).

2.6. Statistical analyses

All data are presented as means ± SEM. Statistical significance was calculated according to unpaired Student's *t*-test. The threshold for significance was set at $p < 0.05$.

3. Results

3.1. High palm oil diet induces ER stress in skeletal muscle

We fed male C57Bl/6 mice with a standard diet (SD, 3% lipids) or a diet enriched in 20% of palm oil (HPD), in order to measure the repercussions on skeletal muscle ER stress and insulin sensitivity. After 8 weeks of feeding, HPD mice had similar body weight than SD mice (+4.6%, NS), but showed significant hyperglycemia (+54%, $p < 0.01$). Concerning ER stress, UPR markers were clearly higher in muscle of HPD mice than in SD mice. HPD significantly increased the mRNA levels of Grp78 (254%, $p = 0.01$), the spliced form of Xbp1 (sXbp1) (169%, $p = 0.02$), the activating transcription factor 3 (ATF3) (211%, $p = 0.03$) and the activating transcription factor 4 (ATF4) (131%, $p = 0.04$) (Fig. 1A). However, the mRNA level of C/EBP-homologous protein (CHOP), a late marker of ER stress, was not significantly modified by HPD compared to SD (Fig. 1A, $p = 0.28$). The protein levels of Grp78 were also induced by HPD (Fig. 1B, $p < 0.05$), confirming the induction of ER stress at protein level. To measure specifically muscle insulin sensitivity, we performed *ex-vivo* incubations of gastrocnemius muscle with or without insulin and analyzed insulin-stimulated PKB phosphorylation. As illustrated in Fig. 1C, both basal and insulin-stimulated PKB phosphorylation was not modified in HPD mice compared to SD mice, indicating that 8 weeks of 20% HPD is not sufficient to induce alterations of insulin signaling. All these data suggest that HPD-induced ER stress in skeletal muscle may precede or be unrelated to muscle IR.

3.2. Palmitate induces ER stress in C_2C_{12} myotubes

To further understand the relationship between muscle ER stress and IR, we investigated the effects of palmitate in C_2C_{12} myotubes. Firstly, we measured the effect of tunicamycine (Tn, 5 μg/ml), an inhibitor or *N*-linked glycosylation, on ER stress markers to ensure that the induction of UPR signaling could be measured in these cells. As illustrated in Supplementary Fig. 1A, Tn induced a marked increase of mRNA levels of Grp78 (20-fold), sXbp1 (60-fold), and CHOP (90-fold) after 6 h of incubation. Similarly, Grp78 protein levels, as well as the phosphorylation of eIF2α, were significantly induced by 18 h of Tn treatment (Supplementary Fig. 1B). Then, we determined whether palmitate incubation induced the same response. As shown in Fig. 2A, 200 μM palmitate significantly increased mRNA levels of all UPR markers after 6 h of incubation, while higher concentration (700 μM, 6 h) of palmitate had more pronounced effects. At both concentrations, palmitate did not increase caspase 3 activity (data not shown), indicating that palmitate did not induce apoptosis of C_2C_{12} myotubes. We then investigated time-dependent effect of palmitate on UPR marker expression. As shown in Fig. 2B, mRNA levels of all

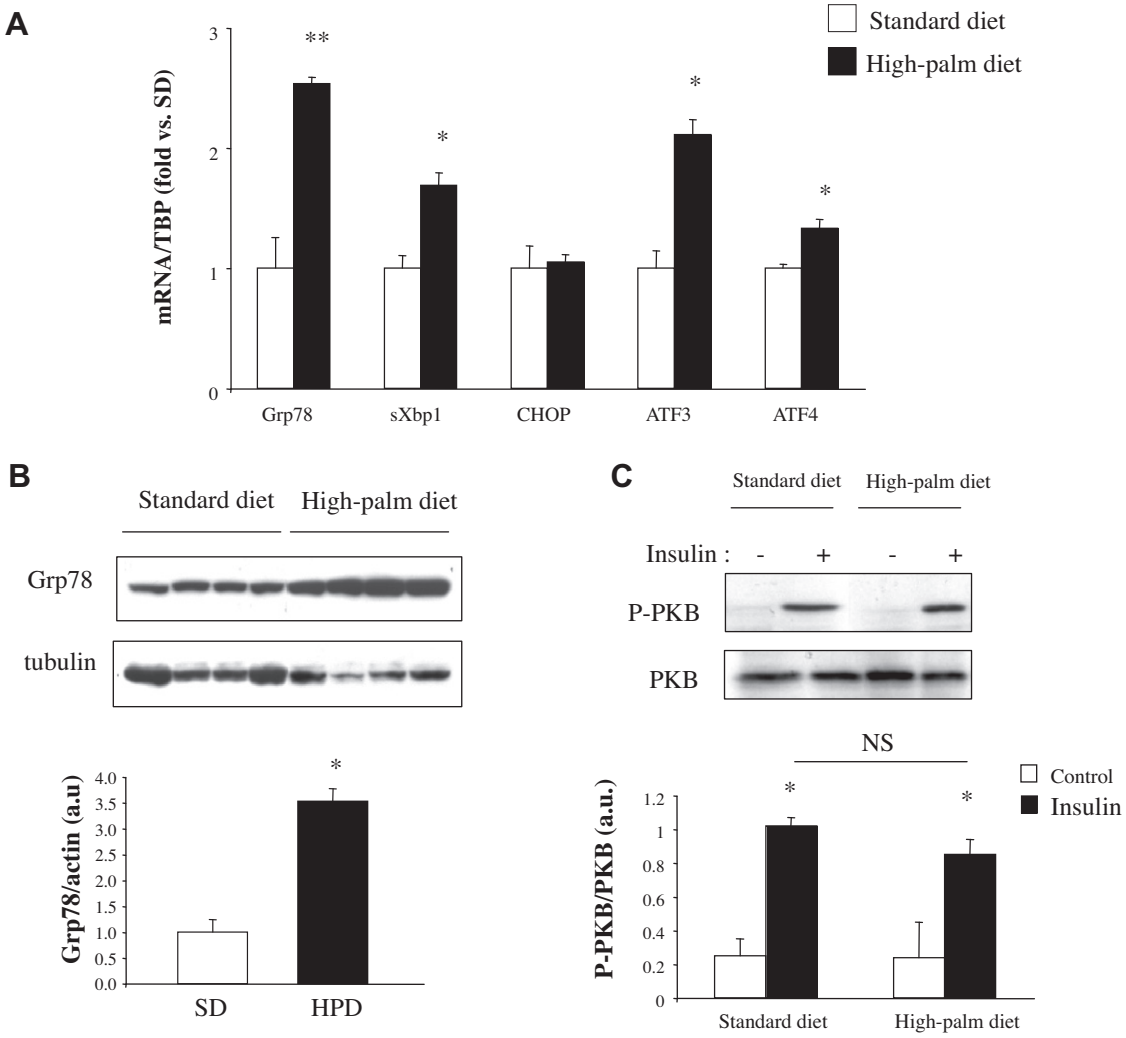

Fig. 1. Induction of ER stress markers in skeletal muscle of high-palm oil diet-fed mice. Male C57BL/6 mice were fed either with a standard diet or a high-palm diet during 8 weeks. (A) mRNA levels of ER stress markers measured by real-time RT-PCR in gastrocnemius muscle of 8 week SD and HPD fed mice. Data are expressed as mean ± SEM ($n = 5$), $^{*}p < 0.05$, $^{**}p < 0.01$ vs. SD. (B) Western-blot illustrating increased expression of Grp78 in gastrocnemius muscle of HPD mice. Downward, the phosphorylation of Grp78 is normalized to tubulin protein levels, ($n = 4$). $^{*}p < 0.05$ vs. SD. (C) Representative Western-blot showing insulin-stimulated phosphorylation of PKB (Ser473) in gastrocnemius muscle of SD and HPD mice, upon *ex-vivo* incubation. Downward, the phosphorylation of PKB is normalized to PKB protein levels, ($n = 3$). $^{*}p < 0.01$ vs. control.

UPR markers were maximally increased by palmitate (700 μM) after 18 h of incubation, expect for the splicing of Xbp1 that reached more rapidly the maximum levels (9 h). Interestingly, expression of all UPR markers returned to initial levels after 48 h of incubation (Fig. 2B), indicating that palmitate induces a transient ER stress in C_2C_{12} cells. Palmitate (200 μM, 18 h) significantly increased Grp78 protein levels (5-fold, $p < 0.0005$), and stimulated the phosphorylation of eIF2α (1.4-fold, $p < 0.01$) in C_2C_{12} cells (Fig. 2C). At opposite, oleate, a monounsaturated fatty acids, did not induce ER stress in C_2C_{12} cells and co-incubation of cells with palmitate and oleate prevented palmitate-induced ER stress (Fig. 2D). All together these results clearly demonstrate that palmitate specifically induces ER stress in C_2C_{12} cells.

3.3. Palmitate alters insulin signaling in C_2C_{12} myotubes

To estimate ER stress effects on insulin signaling, we measured the ability of both Tn and palmitate to alter insulin-stimulated PKB phosphorylation in C_2C_{12} cells. Tn treatment (5 μg/ml; 18 h) significantly inhibited insulin-stimulated PKB phosphorylation (Supplementary Fig. 1C). Interestingly, palmitate (700 μM, 18 h) also markedly reduced insulin-stimulated PKB phosphorylation in C_2C_{12} cells (Fig. 2E). Treatment with oleate did not affect insulin-stimulated PKB phosphorylation (Supplementary Fig. 1D), indicating that the effect on insulin signaling is specific to palmitate. These results suggested therefore that palmitate-induced ER stress is associated to palmitate-mediated alterations of insulin signaling.

3.4. Prevention of ER stress does not improve palmitate-induced alterations of insulin signaling

To investigate whether ER stress is involved in palmitate-induced alterations of insulin signaling, we firstly examined the effect of both TUDCA and 4-PBA, chemical chaperones known to relieve ER stress and to improve insulin sensitivity [11], C_2C_{12} cells were pre-incubated with TUDCA (500 μg/ml) for 9 h and then treated with or without Tn or palmitate during 18 h. The phosphorylation of eIF2α was measured as an indicator of ER stress level. As expected, TUDCA pre-treatment significantly reduced Tn-induced eIF2α phosphorylation (Supplementary Fig. 2A), as well as palmitate-induced eIF2α phosphorylation (Supplementary Fig. 2B). Regarding insulin signaling, TUDCA treatment did not improve the palmitate-induced reduction of insulin-stimulated PKB phosphorylation (Fig. 3A).

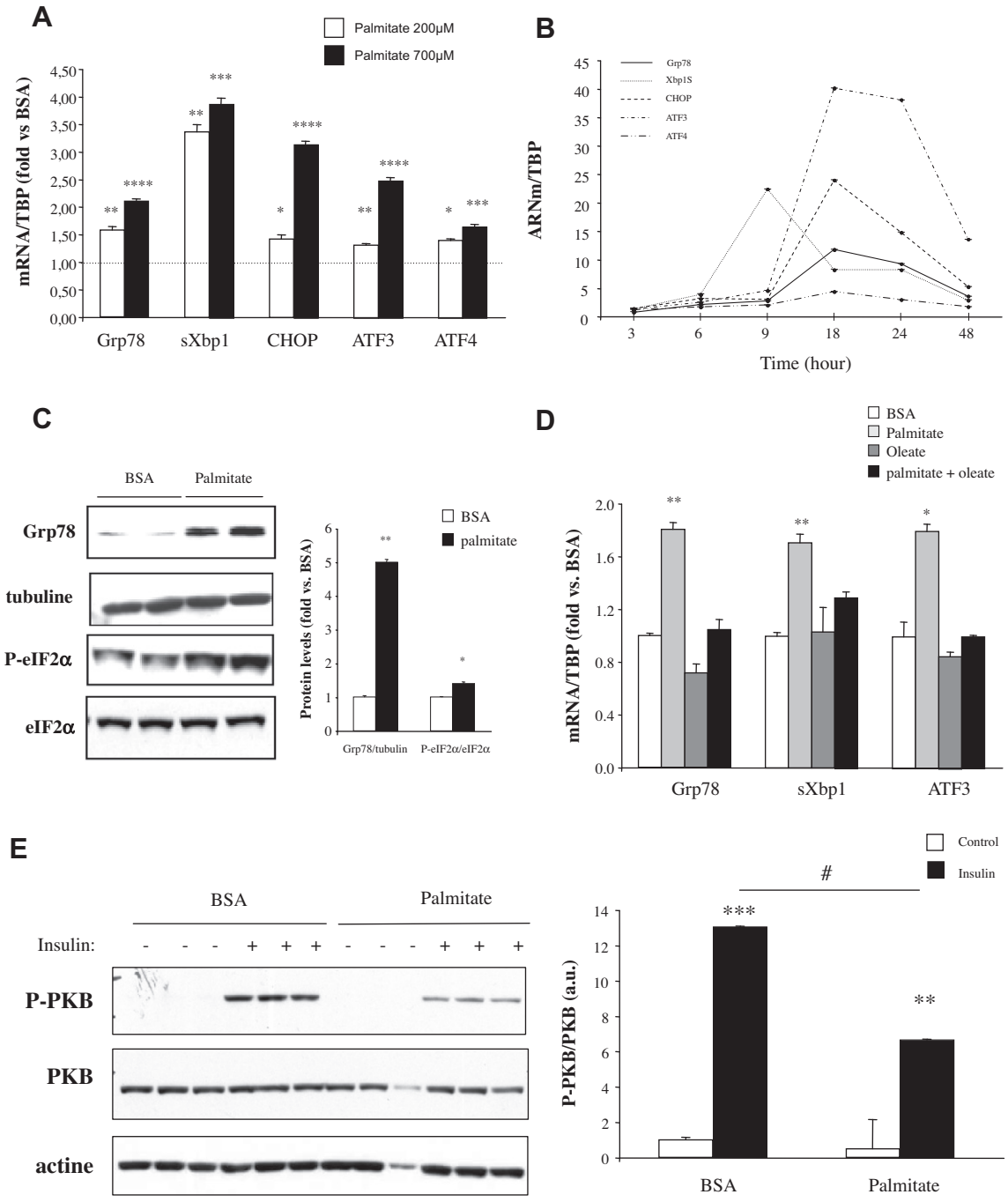

Fig. 2. Effects of palmitate on ER stress markers and insulin action in C_2C_{12} cells. (A) mRNA levels of ER stress markers measured by real-time RT-PCR in C_2C_{12} cells incubated during 6 h with BSA (as control) or palmitate (200 and 700 µM). Data are expressed as fold changes compared to BSA treatment ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.0005$ vs. BSA. (B) Time-dependant induction of mRNA levels of several ER stress markers in 700 µM palmitate-treated C_2C_{12} myotubes. Data are expressed as fold changes compared to BSA treatment ($n = 3$). (C) Western blots of ER stress markers including Grp78 and phosphorylation of eIF2α (P-eIF2α) in 700 µM palmitate-treated C_2C_{12} myotubes. Rightward of western blots, the graphs represent the Grp78 protein levels as well as the phosphorylation of eIF2α. Data are expressed as mean ± SEM ($n = 3$). $^{*}p < 0.01$, $^{**}p < 0.0005$. (D) mRNA levels of ER stress markers measured by real-time PCR in C_2C_{12} cells incubated either with palmitate (350 µM), oleate (350 µM) or a mix of both lipids (350 + 350 µM) during 18 h. Data are expressed as mean ± SEM ($n = 3$), $^{*}p < 0.01$, $^{**}p < 0.005$ vs. BSA. (E) C_2C_{12} myotubes were incubated with 700 µM palmitate during 18 h, and then stimulated with 10^{-7} M insulin during 20 min. Insulin-stimulated PKB phosphorylation was measured by Western blotting on protein lysates. Rightward of western blots, the graphs represent the quantitation and normalization of insulin-stimulated phosphorylation of PKB (Ser473) ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0005$, $^{***}p < 0.0005$ vs. respective control, $^{\#}p < 0.005$ vs. respective.

As for TUDCA, we initially pre-treated C_2C_{12} cells with 4-PBA (20 mM) for 9 h, before incubation with palmitate. However, we noted a toxic effect of the combined 4-PBA and palmitate treatment on C_2C_{12} cells (data not shown). To overcome this problem, we then pre-treated cells during 9 h with 4-PBA and remove it by changing the culture medium before palmitate (or Tn) treatment. Under these conditions, 4-PBA significantly reduced Tn-induced eIF2α phosphorylation (Supplementary Fig. 3A), and improved Tn-induced reduction of PKB phosphorylation (Supplementary Fig. 3B). Importantly, while PBA reduced palmitate-induced eIF2α phosphorylation (Supplementary Fig. 3C), it did not counteract palmitate-induced reduction of insulin-stimulated PKB phosphorylation (Fig. 3B).

Lastly, we used an alternative strategy to mitigate ER stress, by overexpressing the chaperone Grp78, as previously reported [15].

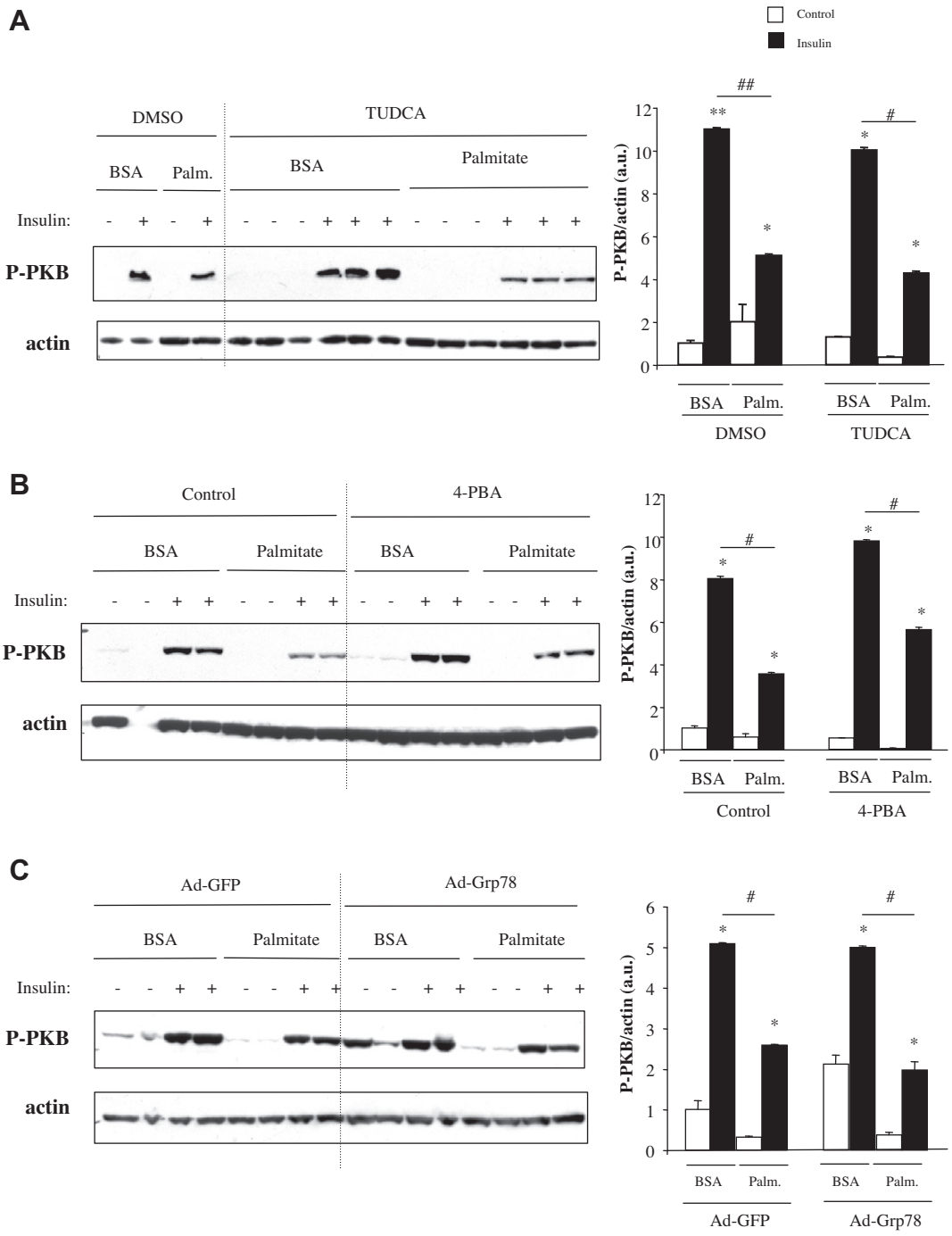

Fig. 3. Reduction of ER stress by TUDCA and 4-PBA treatments, or by adenoviral overexpression of Grp78, does not improve palmitate-induced alterations of insulin signaling. C_2C_{12} myotubes were either pretreated with 500 μg/ml TUDCA (A) or 20 mM 4-PBA (B) for 9 h, or infected for 48 h with adenovirus encoding for GFP (Ad-GFP, as control) or Grp78 (Ad-Grp78) (C), and then incubated with 700 μM palmitate for 18 h. Western blots illustrating insulin-stimulated PKB phosphorylation in palmitate-treated myotubes with or without TUDCA (A) or 4-PBA pre-treatments (B) or after the infection with Ad-GFP or Ad-Grp78 (C). Rightward of western blots, the graphs represent the quantitation and normalization of insulin-stimulated phosphorylation of PKB (Ser473) ($n = 3$). $^{*}p < 0.01$, $^{**}p < 0.005$, $^{**}p < 0.0001$ vs. respective control, $^{\#}p < 0.05$, $^{\#\#}p < 0.05$ vs. respective BSA.

As shown in Supplementary Fig. 3D, adenoviral overexpression of Grp78 significantly reduced palmitate-induced UPR markers compared to cells treated with GFP containing adenovirus, indicating that Grp78 is effective to reduce ER stress in muscle cells. However, the overexpression of Grp78 did not improve palmitate-induced reduction of insulin-stimulated PKB phosphorylation (Fig. 3C).

All together, these results demonstrated that three different strategies to reduce ER stress signaling were not able to improve palmitate-induced alterations of insulin signaling in muscle cells.

4. Discussion

Whereas altered lipid metabolism is certainly associated with skeletal muscle IR, the exact molecular mechanisms by which saturated FFA, such as palmitate, induces alterations of insulin signaling in this tissue is unclear. Surprisingly, whereas ER stress has been proposed as a central mechanism in the development of IR in liver and adipose tissue [9], there is yet no data on the implication of ER stress in muscle IR. We investigated therefore the possible role of

ER stress in lipid-induced alterations of insulin signaling in myotubes.

We demonstrated, both *in vivo* and *in vitro*, that palmitate activates the UPR pathways in skeletal muscle, suggesting that muscle cells are sensitive to palmitate-induced ER stress as recently suggested in mice [13,16]. However, another study in human indicated that 6 weeks of overfeeding with high-fat diet did not affect the expression of ER stress markers in skeletal muscle of young, lean and physically active men [17]. The discrepancy between these results is not related to a species difference, since exposure of human myotubes to palmitate also induced the expression of ER stress markers [12]. Therefore, differences in lipid-induced ER stress are most likely due to the lipid's quantity or quality used in these studies. In agreement, we observed that monounsaturated fatty acids, such as oleate, does not induced the expression of ER stress markers, and even could prevent palmitate-induced ER stress, in agreement with a recent study in L6 myotubes [13].

ER stress is well described to dampen insulin-signaling pathways in liver and adipose tissue. We demonstrated that Tn-induced ER stress is associated with reduced insulin-stimulated PKB phosphorylation and alleviation of Tn-induced ER stress by 4-PBA treatment is sufficient to improve insulin signaling in C_2C_{12} cells. Consequently, ER stress could be a molecular mechanism of IR in skeletal muscle. In a similar manner, palmitate-induced ER stress is associated with a reduction of insulin-stimulated PKB phosphorylation in C_2C_{12} myotubes. However, reduction of ER stress signaling by chemical chaperones, such as TUDCA and 4-PBA, or by the overexpression of Grp78 did not improve palmitate-induced reduction of insulin-stimulated PKB phosphorylation. Even we cannot exclude that three strategies independently did not inhibit all UPR pathways, the fact that each treatment produced the same type of results on insulin signaling, strongly suggests that ER stress is probably not a major player in palmitate-induced insulin resistance in muscle cells. In addition, this is also supported by our *in vivo* data demonstrating that increased expression of ER stress markers in muscle of HPD fed mice is not associated with altered insulin-stimulated PKB phosphorylation.

Insulin receptor substrates (IRS), which are normally phosphorylated on tyrosine by insulin, are phosphorylated on serine in lipotoxic states, and this in turn abrogates the insulin signal. Among the IRS-modifying enzymes, stress activated kinases such as c-Jun N-terminal kinase (JNK) [18], inhibitor of kappa B kinase (IKK) [19], PKC [5] and double stranded RNA-dependent protein kinase (PKR) [20] have been reported to inhibit insulin action by serine phosphorylation of IRS-1. In both liver and adipose tissue, ER stress dampened insulin signaling by increasing JNK activity, and chemical chaperones improved insulin sensitivity by blocking ER stress-mediated JNK activation [8,11]. Why the same chaperone treatments were not effective on insulin signaling in myotubes is unclear. We can hypothesize that palmitate could induce muscle IR independently of JNK activation, by acting directly on IRS-1 through the activation of other stress-related kinases. Alternatively, palmitate could alter insulin action at the level of PKB, independently of an effect on IRS1 serine phosphorylation. In agreement, it was reported that palmitate-induced reduction of insulin-stimulated PKB phosphorylation was not due to a reduction of the upstream insulin pathways, but was attributable to an increased synthesis of ceramides and the subsequent activation of PKCzeta [21]. In addition, palmitate was also reported to alter mitochondria [22] and to induce oxidative stress [14,23] in muscle. Because mitochondrial dysfunction has been associated with IR [6,14] palmitate-induced alterations of mitochondria may explain why reducing ER stress is not efficient to improve insulin signaling in myotubes. In agreement, palmitate-induced oxidative stress contributed to the loss in insulin-stimulated phosphorylation of PKB [23]. Lastly, palmitate can be incorporated in ER phospholipids membranes leading to modifications of ER morphology [13], and such modifications may participate to altered insulin sensitivity [24]. One could thus not exclude that alleviating UPR signaling pathways with chemical chaperones as done in the present work did not counteract a potential effect on ER membrane fluidity during palmitate treatment.

Our results do not exclude also a role of the UPR pathway in the alterations of muscle insulin signaling in other circumstances. Indeed, glucosamine-induced ER stress caused IR in myotubes, and pre-treatment of myotubes with TUDCA or 4-PBA completely preserved these effects [25]. In addition, treatment with TUDCA and 4-PBA increased glucose uptake in muscle and adipose tissue of ob/ob mice [11], whereas TUDCA treatment of obese and insulin-resistant humans increased hepatic and muscle insulin sensitivity [26]. Consequently, all these studies indicate that targeting UPR could modulate skeletal muscle insulin sensitivity. However, our data clearly demonstrate that dampening ER stress using chemical chaperones or grp78 overexpression did not protect myotubes from palmitate-induced alterations of insulin signaling, suggesting that either ER stress is not a major actor of muscle IR or that palmitate have more complex action on muscle UPR signaling and ER homeostasis in muscle than in adipose or liver cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.135.

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