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CTRP5 ameliorates palmitate-induced apoptosis and insulin resistance through activation of AMPK and fatty acid oxidation

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ABSTRACT

Lipotoxicity resulting from a high concentration of saturated fatty acids is closely linked to development of insulin resistance, as well as apoptosis in skeletal muscle. CTRP5, an adiponectin paralog, is known to activate AMPK and fatty acid oxidation; however, the effects of CTRP5 on palmitate-induced lipotoxicity in myocytes have not been investigated. We found that globular domain of CTRP5 (gCTRP5) prevented palmitate-induced apoptosis and insulin resistance in myocytes by inhibiting the activation of caspase-3, reactive oxygen species accumulation, and IRS-1 reduction. These beneficial effects of gCTRP5 are mainly attributed to an increase in fatty acid oxidation through phosphorylation of AMPK. These results provide a novel function of CTRP5, which may have preventive and therapeutic potential in management of obesity, insulin resistance, and type 2 diabetes mellitus.

1. Introduction

Obesity has become a major public health problem worldwide, and is known as an important risk factor for development of insulin resistance [1]. Insulin resistance, defined as the failure of target tissues to show a normal response to insulin, is considered a major characteristic of type 2 diabetes mellitus (T2DM) and metabolic syndrome [2]. Skeletal muscle accounts for more than 70% of post-prandial whole-body glucose disposal; accumulation of excess saturated fatty acids (SFA) in skeletal muscle has been reported to induce lipotoxicity, such as apoptosis and dysregulation of cellular metabolism, which is inevitably linked to development of insulin resistance, as well as T2DM [2,3]. The mechanism underlying SFA-induced apoptosis and insulin resistance in skeletal muscle largely remains unclear, however, several studies have suggested that a high concentration of palmitate, the most abundant dietary SFA, induces significant accumulation of diacylglycerol (DAG), ceramides, and reactive oxygen species (ROS), resulting in oxidative stress, mitochondrial dysfunction, apoptosis, and insulin resistance in skeletal muscle cells [4,5]. Therefore, it is generally accepted that reduction of SFA in skeletal muscle results in decreased lipotoxicity, increased insulin sensitivity, and reduction of risk factors for T2DM and metabolic diseases.

AMP-activated protein kinase (AMPK) is an important regulator of cellular and whole-body energy homeostasis [6]. Activation of AMPK mainly occurs as the result of a reduced energy state, such as increased intracellular ratio of AMP to ATP, and it switches on ATP-generating catabolic pathways, such as fatty acid oxidation and glucose transport [6,7]. Multiple studies have suggested a close association of activation of AMPK with prevention of apoptosis in various ways in many types of cells [8–10]. Activation of AMPK with aminoimidazole carboxamide ribonucleotide (AICAR), a well-known AMPK activator, led to reduced production of DAG and ceramide in palmitate-treated retinal pericytes, and alleviation of palmitate-induced apoptosis [8]. AMPK activation also suppressed palmitate-induced ROS generation in endothelial cells, and inhibited endothelial apoptosis [9]. In a recent study, A769662, an activator of AMPK, inhibited palmitate-induced apoptosis in neuroblastoma cells through prevention of endoplasmic reticulum (ER)-stress by lipotoxicity [10]. In addition to anti-apoptotic capability, activation of AMPK in skeletal muscle increases fatty acid oxidation by phosphorylation of acetyl-CoA carboxylase (ACC), and could reduce the risk of metabolic diseases, such as insulin resistance, T2DM, and metabolic syndrome [6,7]. Hence, drugs such as metformin, thiazolidinediones, and statins effectively increase phosphorylation of AMPK, and exhibit antidiabetic and lipid-lowering potentials [11]. Thus, activation of AMPK led to reduction of harmful byproducts of SFA, such as ceramides, DAG, or ROS, thereby ameliorating lipotoxicity and insulin resistance in many different kinds of cells [8–11].

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C1q tumor necrosis factor α-related protein isoform 5 (CTRP5), a member of a new family of secreted proteins, was recently identified as a novel, highly conserved family of adiponectin paralog [12], and its biological function is similar to that of adiponectin in myocytes [13]. Adiponectin, an abundant adipokine mainly secreted from adipose tissue, participates in regulation of energy metabolism, such as fatty acid and glucose oxidation [14]. We previously reported that mitochondrial dysfunction resulting in insulin resistance leads to increased expression and secretion of CTRP5 in myocytes [13]. Similar to adiponectin, the globular domain of CTRP5 (gCTRP5) induces phosphorylation of AMPK, which subsequently leads to enhanced fatty acid oxidation and insulin sensitivity in myocytes [13]. However, the beneficial effects of CTRP5 on SFA-induced lipotoxicity, such as apoptosis and insulin resistance, in myocytes have not been elucidated. In this study, we attempted to determine whether CTRP5 can inhibit palmitate-induced apoptosis and insulin resistance in L6 GLUT4myc myocytes. In addition, the mechanism underlying inhibition of palmitate-induced lipotoxicity by CTRP5 was investigated.

2. Materials and methods

2.1. Cell culture and palmitate treatment

L6 GLUT4myc cells derived from rat skeletal muscle were provided by Dr. Amira Klip (the Hospital for Sick Children, Toronto, Ontario, Canada). The cells were cultured in MEM alpha with 10% FBS and 1% penicillin-streptomycin, as described previously [15]. For the palmitate treatment, the cells were incubated for 6–18 h with BSA or BSA conjugated-palmitate (0.1–0.5 mM) in culture media. If necessary, recombinant gCTRP5 (5 μg/ml) and/or adenine 9-D-arabinofuranoside (AraA, 2 mM), an AMPK inhibitor, were used with or without palmitate treatment. For insulin stimulation, the cells were serum-starved for the last 4 h of the experiment, followed by stimulation with insulin (100 nM) for the last 30 min.

2.2. Measurement of cytotoxicity and ROS production

Palmitate-induced cytotoxicity was measured by MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Briefly, the cells were incubation in media containing 1.2 mM MTT solution for 4 h. Following incubation, 40% DMSO was added to each well, and the absorbance was measured at 540 nm. Intracellular ROS levels were examined using a DCF-DA ROS detection assay kit (Abcam). Briefly, myocytes were incubated with 20 μl of DCF-DA at 37 °C for 30 min, and the cells were lysed with Triton X-100. Fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

2.3. Determination of cell apoptosis

Apoptosis was assessed by measurement of cellular caspase-3 activity, which is based on the hydrolysis of the peptide substrate of caspase-3, using colorimetric methods according to the manufacturer's instructions (R&D Systems).

2.4. Generation of CTRP5 constructs and production of recombinant protein

pBAD/Thio-TOPO (Invitrogen) vector containing the thioredoxin-fused human CTRP5 [16] was provided by Dr. A.F. Wright (Western General Hospital, Edinburgh, UK). gCTRP5 was cloned into the *NdeI* and *EcoRI* sites of pRSET A vector (Invitrogen). gCTRP5 was expressed in *Escherichia coli* BL21 and purified with Glutathione Sepharose Fast Flow (Amersham, Piscataway, NJ), according to the manufacturer's instructions. For removal of endotoxin, purified proteins were infiltrated to Detoxi-Gel (Pierce) before storage at −70 °C.

2.5. Fatty acid oxidation

For measurement of fatty acid oxidation rate, $^{14}CO_2$ generation in cells from [^{14}C] palmitate (NEN Life Sciences) was measured, as previously described [17].

2.6. Immunoblot analysis

The cells were lysed using Laemmli solution. The lysates were subjected to SDS–PAGE and immunoblot analysis, as described previously [18]. Proteins were detected using ECL Western Blotting Detection Reagents (GE Healthcare, UK). The immunoblot intensities were quantified by densitometry using an analytical scanning system (Alpha Imager HP, Alpha Innotech).

2.7. Antibodies and reagents

The antibody against IRS-1 was obtained from Upstate (Lake Placid, NY) and the antibody against phospho-IRS-1 was supplied by Invitrogen (Carlsbad, CA). Antibodies for AMPK, phospho-AMPK (Thr172), ACC, phospho-ACC (Ser79), Akt2, and phospho-Akt2 (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). Anti-β-actin was purchased from Sigma. All other unspecified reagents were obtained from Sigma.

2.8. Statistical analysis

Values are expressed as the mean ± SEM from at least four independent experiments. Where applicable, the significance of the differences was determined using a Student's *t* test for the unpaired data.

3. Results

3.1. gCTRP5 induces phosphorylation of AMPK and increases fatty acid oxidation in L6 GLUT4myc myocytes

First, we determined palmitate-induced apoptosis in L6 GLUT4myc myocytes, because palmitate is known to activate apoptotic signaling in skeletal muscle [19]. We found that treatment of L6 GLUT4myc myocytes with higher than 0.3 mM of palmitate for 18 h resulted in significantly increased cleavage of procaspase-3, caspase-3 activity and reductions in cell viability and IRS-1 expression, in a dose-dependent manner (Suppl. 1). According to a recent study, generating full-length CTRP5 is limited in quantity and gCTRP5 is more potent than the full-length protein in adiponectin-like effect [13]. Therefore, to evaluate the effect of CTRP5 on AMPK activation and caspase-3 cleavage, we treated myocytes with gCTRP5 (5 μg/ml) in the presence or absence of palmitate (0.5 mM for 18 h). Treatment with gCTRP5 resulted in drastically increased phosphorylation of AMPK and ACC in both control and palmitate-treated myocytes; however, insulin and palmitate had no effect on phosphorylation of AMPK and ACC (Fig. 1A–C). In addition, treatment with gCTRP5 inhibited the palmitate-induced cleavage of caspase-3 in myocytes (Fig. 1D), implying that it may exert anti-apoptotic potential in palmitate-treated myocytes.

As phosphorylation of AMPK increases phosphorylation of ACC, leading to decreased fatty acid synthesis and a concomitant increase in β-oxidation of fatty acids in skeletal muscle [6,7], we next attempted to determine whether gCTRP5 can increase fatty

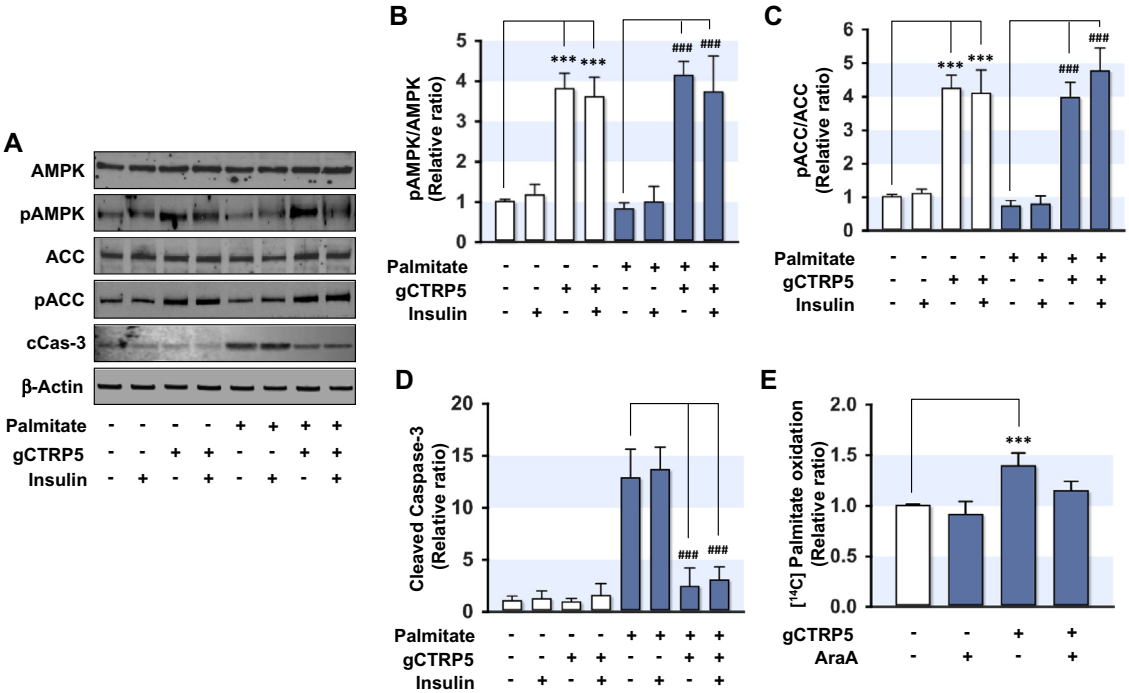

Fig. 1. gCTRP5 activates apoptosis and fatty acid oxidation in myocytes. L6 GLUT4myc myocytes were incubated in the presence or absence of palmitate (0.5 mM) and/or gCTRP5 (5 μg/ml) for 18 h. For insulin stimulation, myocytes were treated with 100 nM of insulin during the last 30 min of incubation. (A) Myocyte lysates (20 μg) were immunoblotted with antibodies specific for AMPK, phospho-AMPK (pAMPK), ACC, phospho-ACC (pACC), cleaved caspase-3 (cCase-3), or β-actin. The immunoblots are representative of five independent experiments. (B–D) The immunoblot intensities for pAMPK/AMPK, pACC/ACC and cCas-3/β-Actin were quantified by densitometry. The values are expressed as the relative ratio, where the intensity of the normal control was set to one. Values are expressed as means ± SEM of five independent experiments. (E) Myocytes were treated with vehicle, gCTRP5 (5 μg/ml) for 2 h with or without a co-treatment with AraA (2 mM). The fatty acid oxidation rate was measured as described in Methods. Values are expressed as means ± SEM from three independent experiments, each with triplicate samples; $^{***}P < 0.001$ vs control; $^{###}P < 0.001$ vs palmitate only.

acid oxidation in L6 GLUT4myc myocytes. Treatment of myocytes with gCTRP5 resulted in significantly increased (approximately 50%) fatty acid oxidation, as assessed by palmitate oxidation, compared to control cells (Fig. 1E). Since the increased palmitate oxidation by gCTRP5 was effectively abolished by an AMPK inhibitor, AraA (Fig. 1E), gCTRP5-induced fatty acid oxidation was mainly due to activation of AMPK in myocytes.

3.2. gCTRP5 inhibits palmitate-induced activation of caspase-3, apoptosis, and ROS accumulation in myocytes

To analyze the effect of CTRP5 on palmitate-induced activation of caspase-3, apoptosis, and ROS accumulation, we co-treated myocytes with gCTRP5 in the presence or absence of palmitate. We found that gCTRP5 significantly inhibited the palmitate-induced activation of caspase-3 and MTT reduction in a dose-dependent manner, whereas gCTRP5 did not affect caspase-3 activity and cell viability in control cells (Fig. 2A and B). AraA almost completely blocked this effect of gCTRP5, indicating that the anti-apoptotic effect of gCTRP5 in palmitate-treated cells is dependent on AMPK activation.

Palmitate increases the accumulation of ROS in the cells, which is known to be a possible cause of apoptosis [19,20]. We treated myocytes with palmitate (0.5 mM for 18 h) in the presence or absence of gCTRP5, and cellular ROS level was measured as described in the Methods. As shown in Fig. 2C, treatment with palmitate caused a significant increase in cellular ROS level in myocytes, while gCTRP5 inhibited ROS accumulation by palmitate, which was also abolished by co-treatment with AraA. Thus, gCTRP5 successfully prevented myocytes from palmitate-induced accumulation of ROS, and the effect was attributed to AMPK activation.

3.3. gCTRP5 improved insulin sensitivity in palmitate-treated myocytes

Because gCTRP5 effectively inhibited apoptosis and ROS accumulation induced by palmitate in myocytes, we next investigated whether gCTRP5 can also inhibit reduction of IRS-1 resulting from the palmitate treatment, thereby preventing insulin resistance. Palmitate caused a drastic reduction in IRS-1 expression, leading to impaired insulin-stimulated tyrosine phosphorylation of IRS-1 and its down-stream target, Akt2 and GSK3β (Fig. 3A–D). Thus, palmitate is causally linked to development of insulin resistance through a substantial reduction in IRS-1 expression in myocytes. However, gCTRP5 almost completely inhibited reduction of IRS-1 by palmitate treatment, which consequently normalized the insulin-stimulated phosphorylation of IRS-1, Akt2 and GSK3β in myocytes (Fig. 3A–D). It should be emphasized that the reduction of IRS-1 induced by palmitate was prevented by gCTRP5 treatment and this effect of gCTRP5 was abolished by the co-treatment of AraA (Fig. 3E). Hence, these results clearly indicate that gCTRP5 normalized insulin sensitivity in palmitate-treated myocytes through activation of AMPK.

3.4. Overexpression of CTRP5 improved insulin sensitivity in palmitate-treated myocytes

As gCTRP5 exhibited improved fatty acid oxidation and insulin sensitivity in myocytes, we attempted to determine whether full-length human CTRP5 has a similar effect on palmitate-induced insulin resistance. As shown in Fig. 4A and B, treatment with palmitate decreased the total protein level of IRS-1, whereas overexpression of CTRP5 restored the IRS-1 level in myocytes. Next, we

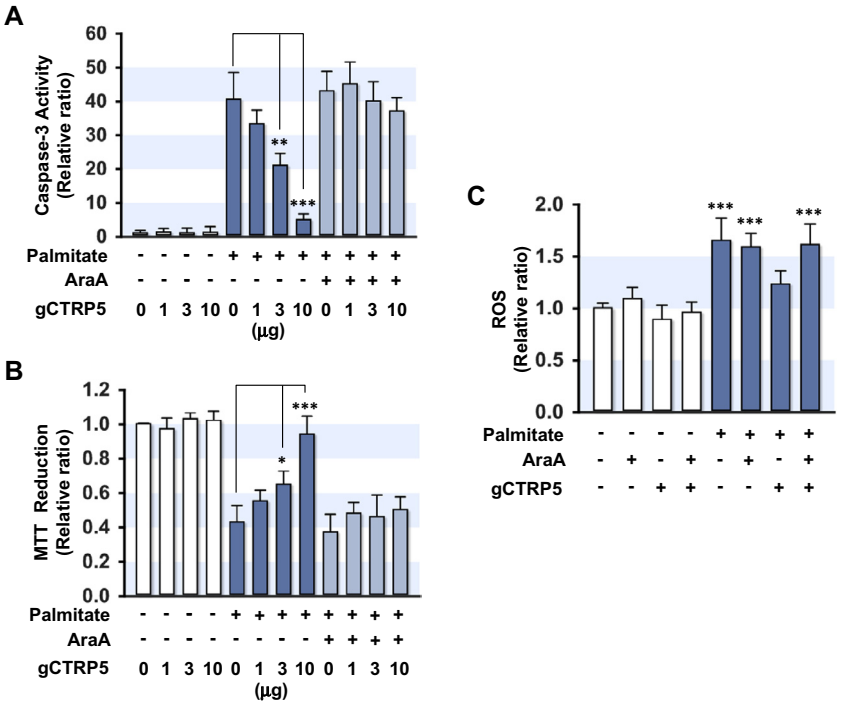

Fig. 2. gCTRP5 prevents myocytes from palmitate-induced activation of caspase-3, apoptosis and ROS accumulation. (A–B) L6 GLUT4myc myocytes were incubated in the presence or absence of palmitate (0.5 mM) and/or gCTRP5 (1–10 μg/ml) for 18 h. For inhibition of AMPK, 2 mM of AraA was co-treated during the incubation. Palmitate-induced apoptosis was analyzed by measuring the cellular caspase-3 activity and MTT reduction. (C) Intracellular ROS levels in myocytes were measured using a DCF-DA ROS detection assay; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

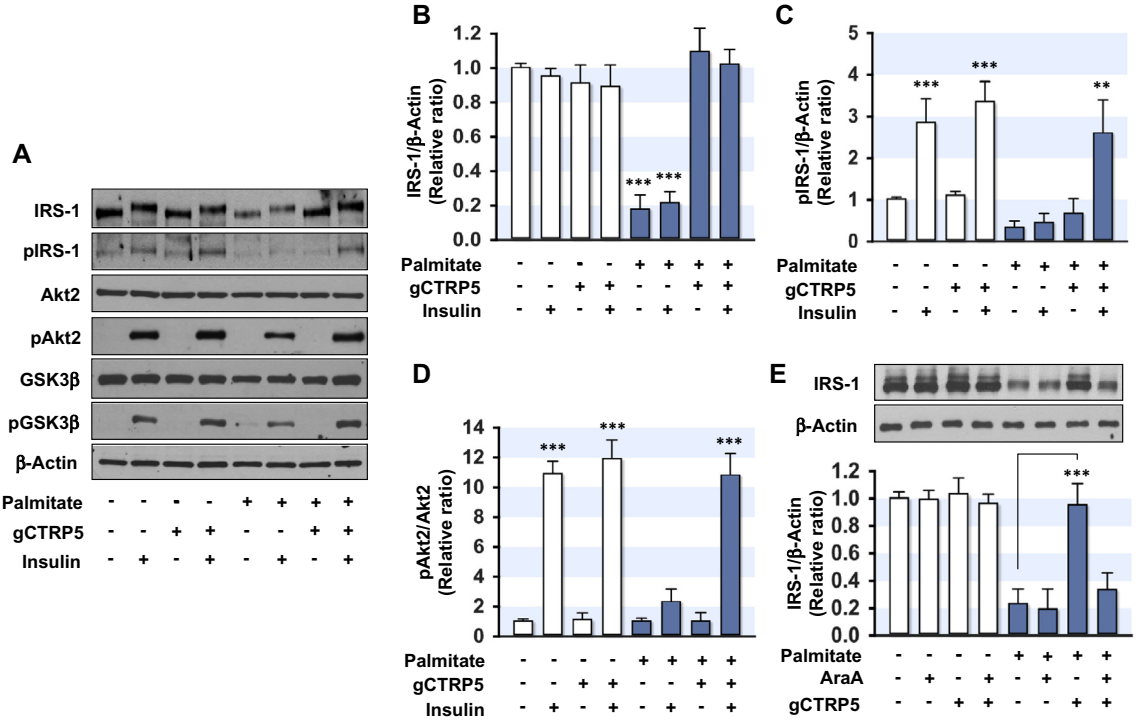

Fig. 3. gCTRP5 ameliorates palmitate-induced IRS-1 reduction and insulin resistance in myocytes. L6 GLUT4myc myocytes were incubated in the presence or absence of palmitate (0.5 mM for 18 h) and/or globular domain of human CTRP5 (gCTRP5, 5 μg/ml). For insulin stimulation, myocytes were treated with insulin (100 nM) during the last 30 min of incubation. For AMPK inhibition, 2 mM of AraA was co-treated. (A) Myocyte lysates (20 μg) were resolved by SDS–PAGE and immunoblotted with specific antibodies. (B–E) The immunoblot intensities were quantified by densitometry. The values are expressed as the relative ratio, where the intensity of the untreated control was set to one. Values are expressed as means ± SEM of five independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$.

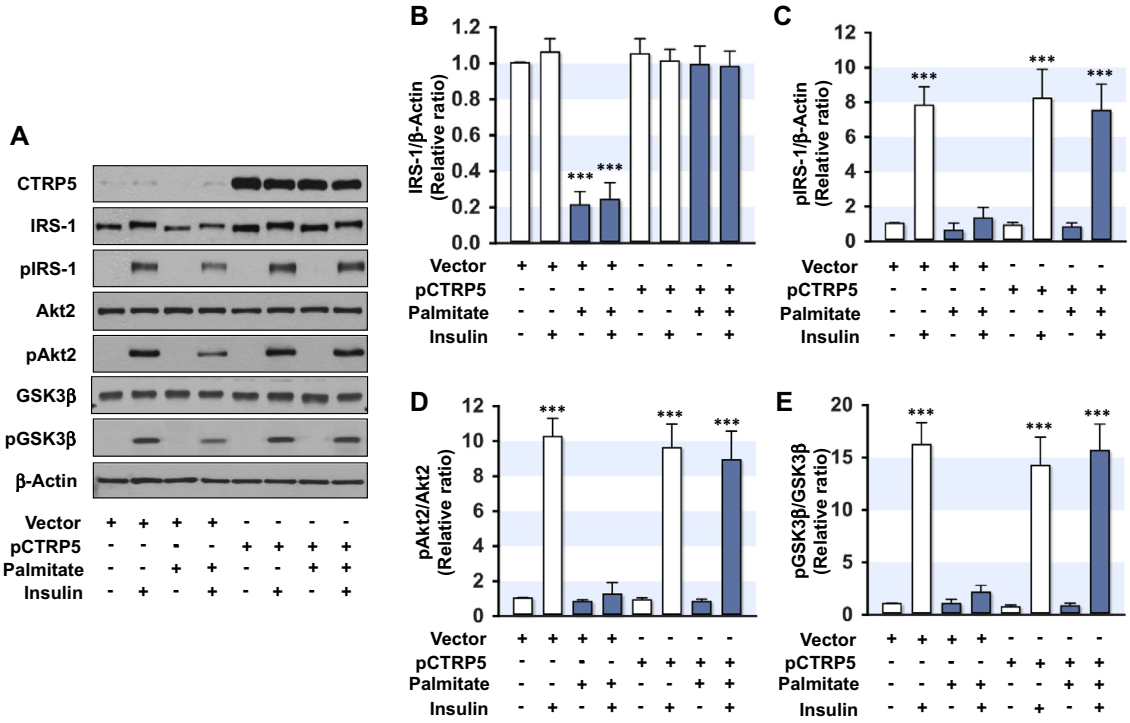

Fig. 4. Overexpression of CTRP5 inhibits palmitate-induced IRS-1 reduction and insulin resistance in myocytes. L6 GLUT4myc myocytes were transfected with empty pcDNA3.1 vector (Vector, 2 μg/ml) or pcDNA3.1 containing full-length human C1QTNF5 (pCTRP5; 2 μg/ml) and incubated for 24 h. Then, the cells were incubated in the presence or absence of palmitate (0.5 mM) for 18 h. For insulin stimulation, myocytes were treated with insulin (100 nM) during the last 30 min of incubation. (A) Myocyte lysates (20 μg) were resolved by SDS-PAGE and immunoblotted with specific antibodies. (B–E) The immunoblot intensities were quantified by densitometry. The values are expressed as the relative ratio, where the intensity of the untreated control was set to one. Values are expressed as means ± SEM of five independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$.

evaluated the insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 4A and C) and showed that overexpression of CTRP5 led to restoration of insulin-stimulated phosphorylation of IRS-1 in L6 GLUT4myc myocytes, which was decreased by palmitate. We also compared tyrosine phosphorylation of Akt2 in myocytes from CTRP5- or vector-transfected myocytes in the presence or absence of palmitate. As shown in Fig. 4D and E, transfection of CTRP5 to myocytes prevented palmitate-induced inhibition of insulin-stimulated phosphorylation of Akt2 and GSK3β. Therefore, overexpression of full-length CTRP5 normalized insulin-sensitivity, as determined by the phosphorylation of IRS-1, Akt2 and GSK3β in palmitate-treated myocytes.

4. Discussion

CTRP5, an adiponectin paralog, is known to be a putative biomarker of mitochondrial dysfunction, and modulates fatty acid metabolism through activation of AMPK in myocytes [13]. The main findings of this study were that CTRP5 ameliorates palmitate-induced apoptosis and insulin resistance in myocytes. These effects of CTRP5 were mainly due to activation of AMPK in myocytes, because inhibitor of AMPK abolished the effect of CTRP5 against palmitate-induced apoptosis and insulin resistance in myocytes. This study unveils a novel mechanism whereby CTRP5 plays an important role in modulation of cell survival and insulin sensitivity, and highlights the significant potential of CTRP5 in prevention and treatment of T2DM.

Apoptosis is mediated by a highly regulated sequential activation of caspases, and among those, activation of caspase-3 acts as a central effector, which cleaves several essential substrates, leading to cell death [21]. Several studies have shown that palmitate causes significant increases of lipotoxic intermediates, consequently leading to activation of caspase-3, ER stress, and apoptosis [2,4,5]. In this study, palmitate induced apoptosis in L6 GLUT4myc myocytes through activation of caspase-3, whereas co-treatment with gCTRP5 prevented the cleavage and activation of caspase-3 induced by palmitate. Although gCTRP5 abolished the activation of caspase-3 in palmitate-treated myocytes, it did not inhibit caspase-3 enzyme activity in a cell-free system (data not shown). Therefore, this preventive effect of gCTRP5 against palmitate-induced apoptosis is thought to occur before activation of caspase-3. In this regard, it is interesting that gCTRP5 attenuated inhibition of Akt phosphorylation by palmitate, because the activation of Akt inhibits apoptosis directly and indirectly by promoting binding of the anti-apoptotic Bcl-2 to the pro-apoptotic Bax, which prevents release of mitochondrial cytochrome c and activation of caspase-3 [21].

Accumulation of ROS has been implicated in the pathogenesis of insulin resistance and T2DM via the inhibition of insulin signaling, as well as cell survival [20]. Several previous studies have shown that palmitate increases generation of ROS in skeletal muscle cells through activation of NADPH oxidase and it alters mitochondrial integrity [19,22]. Oxidative stress derived from palmitate induces mitochondrial DNA (mtDNA) damage, mitochondrial dysfunction, and ER stress, which cause apoptosis and insulin resistance [19,20,22,23]. Indeed, ROS and oxidative stress are modestly increased under dysmetabolic conditions, such as obesity, insulin resistance, and T2DM [24,25]. In this study, gCTRP5 also suppressed ROS accumulation induced by palmitate in myocytes. This beneficial effect was completely abolished by treatment with AraA, an inhibitor of AMPK. Since CTRP5 has not been reported to exhibit ROS scavenging or antioxidant function, the results indicate that activation of AMPK by CTRP5 may also contribute to amelioration of apoptosis and insulin resistance by palmitate via a reduction of ROS and oxidative stress in myocytes.

In the current study, palmitate caused reduction of IRS-1 and impaired insulin signaling in L6 GLUT4myc myocytes, whereas CTRP5 prevented palmitate-induced reduction of IRS-1 through activation of AMPK. IRS-1, a well-known intracellular signaling adaptor molecule, plays a pivotal role in insulin signaling from insulin receptor to PI3K and Akt2 [26]. Therefore, the inappropriate modification and reduction of IRS-1 is closely linked to the pathogenesis of insulin resistance and T2DM [26]. In this aspect, expression of IRS-1 is decreased in skeletal muscle of obese-type T2DM animals, such as ob/ob mice [27], obese Zucker rats [28], and T2DM patients [29–31]. Apart from apoptosis, accumulation of palmitate in skeletal muscle has been proposed as a negative regulator in the insulin signaling cascade by the repression and modification of IRS-1. Palmitate increases ER stress and activates certain serine kinases, including protein kinase C theta (PKC θ), JNK, and IKK, which are known to increase the serine phosphorylation of IRS-1 [4,5]. These serine modifications on IRS-1 interrupt downstream insulin signal transduction, and, consequently, reduce IRS-1 level by a proteasome-dependent degradation mechanism [32]. gCTRP5 also normalized Akt phosphorylation, which is known to be involved in prevention of apoptosis, by inhibiting palmitate-induced reduction of IRS-1. Therefore, prevention of IRS-1 reduction by gCTRP5 in palmitate-treated myocytes can promote both skeletal muscle cell survival and insulin sensitivity.

The activation of AMPK was suggested to decrease the lipotoxicity of excess SFA by preventing the accumulation of harmful byproducts, such as ROS, DAG and ceramide, in various cell types [8–11]. Treatment of myocytes with gCTRP5 resulted in increased phosphorylation of AMPK and ACC, leading to activation of fatty acid oxidation, whereas co-treatment of gCTRP5 with an inhibitor of AMPK failed to prevent palmitate-induced activation of caspase-3, ROS accumulation, and IRS-1 reduction. The mechanism by which CTRP5 can ameliorate the deleterious lipotoxicity of SFA is mainly attributed to an increase of fatty acid oxidation, thereby reducing ROS accumulation and caspase-3 activation. Therefore, activation of AMPK has been regarded as the most important mechanism through which CTRP5 prevents palmitate-induced apoptosis and insulin resistance in myocytes. Taken together, our results demonstrated that palmitate induces apoptosis and impaired insulin signaling in L6 GLUT4myc myocytes, and that gCTRP5 intervenes to ameliorate palmitate-induced apoptosis and insulin resistance in myocytes. The beneficial effect of CTRP5 is mainly due to an increase in fatty acid oxidation through the phosphorylation of AMPK. Thus, this study not only suggests the mechanisms of gCTRP5-mediated anti-apoptosis in myocytes, but also provides a novel function of gCTRP5 as a useful therapeutic agent in management of obesity, insulin resistance, and T2DM.

Acknowledgments

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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.2014.08.145.

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